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CALCIUM, THIN FILAMENTS, AND THE INTEGRATIVE BIOLOGY OF CARDIAC CONTRACTILITY

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■ **Abstract** Although well known as the location of the mechanism by which the cardiac sarcomere is activated by Ca^{2+} to generate force and shortening, the thin filament is now also recognized as a vital component determining the dynamics of contraction and relaxation. Molecular signaling in the thin filament involves steric, allosteric, and cooperative mechanisms that are modified by protein phosphorylation, sarcomere length and load, the chemical environment, and isoform composition. Approaches employing transgenesis and mutagenesis now permit investigation of these processes at the level of the systems biology of the heart. These studies reveal that the thin filaments are not merely slaves to the levels of Ca^{2+} determined by membrane channels, transporters and exchangers, but are actively involved in beat to beat control of cardiac function by neural and hormonal factors and by the Frank-Starling mechanism.

INTRODUCTION

The objectives of our review are to provide an up-to-date picture of Ca^{2+} and molecular signaling in the thin filaments and to summarize advancements in the translation of this information from the molecular and cellular levels to the level of the integrated biology of the intensity and dynamics of the heart beat. A focal point is the hypothesis that altered thin filament response to Ca^{2+} is an essential element in the control of cardiac dynamics. Publication of the excellent review by Tobacman (1) in *Annual Review of Physiology* on the topic of thin filament regulation was about 10 years ago. Since that time, other excellent and detailed reviews, which track progress in this field, have been published (2–11). However, more recently major new experimental findings have provided a clearer picture of molecular signaling in the thin filament and a better understanding of alterations in this signaling in various physiological and patho-physiological conditions. Moreover, the identification of many new sarcomeric proteins has led to fresh concepts of

what thin filament regulation means. Although space limitations required us to focus here on regions of the thin filament (generally the A-band region) that react with crossbridges, there is an exciting and developing area of research concerned with regions of the thin filament that rarely if ever react with crossbridges and dwell near and within the Z-disk. Recent papers (10, 11) review the significance of direct and indirect interactions of the thin filaments with cytoskeletal and Z-disk proteins in controlling cardiac function, signal transduction, and signal processing.

STRUCTURE AND INTERACTIONS AMONG THIN FILAMENT PROTEINS IN THE A-BAND REGION OF THE SARCOMERE

States of the Thin Filament

Following the original proposal of the steric blocking model of muscle regulation (12–14), structural studies have provided major advances in our perception of the thin filament. Several models for muscle regulation involve either two (on and off) or three (blocked, closed, and open) different states of thin filament (15–17). Our discussions employ the three-state model to describe molecular mechanisms, but other models may also fit the data as discussed in papers by Chalovich (18) and by Squire & Morris (7), which summarize the ambiguities and difficulties in this area of research. Current descriptions of the state of the thin filaments derived from biochemical (16, 19, 20) and structural studies (21, 22) include a blocked state or B-state, reflecting steric block of crossbridges; a closed state (C-state), reflecting weakly bound crossbridges; and an open or strong myosin-binding state (M-state). Figure 1, which is a reconstruction based on electron micrographs, shows the B-, C-, and M-states of the thin filaments as indicated by the position of tropomyosin (Tm). The B-state, which is occupied when the cytoplasmic [Ca^{2+}] is below the threshold for binding to regulatory sites on troponin (Tn), is similar to the original concept that Tm blocks crossbridge reactions with actin by a steric block, although part of the putative myosin-binding site seems to be exposed on actin (21–23). In the B-state, Tm is positioned at the outer domains of actin away from the groove formed by the actin helix. With Ca^{2+} activation, Tm moves closer to the inner domain of actin, nearer the groove. The closed or cocked state reflects a refinement of the original steric hindrance hypothesis that is required to fit biochemical data indicating that some or all crossbridges bind weakly to actin in a non-force-generating but stereo-specific manner. Crossbridges are poised to enter into the force-generating state with the thin filament in the C-state, hence the term cocked (7). Evidence for a Ca^{2+}-dependent movement of Tm on the thin filament has been observed by Förster resonance energy transfer (FRET). Although some studies could not determine Ca^{2+}-induced movement of Tm by FRET measurements (24), others using multi-site lifetime FRET measurements were able to detect Ca^{2+}-dependent Tm movement relative to actin (25). The M-state is associated with the strong binding of force-generating crossbridges

that induce further movement of Tm and/or actin subdomains, which is apparently important in spreading activation laterally along the thin filament. Ca^{2+}-dependent crossbridge-induced movement of Tn relative to actin on the thin filament was also observed by FRET measurements (26–28). The functional importance of these movements of Tn and Tm are not fully understood. The discussion concerning their function is couched in two major theories of activation. One is the so-called rate modulation theory originally proposed by Julian (29) and refined by Brenner (30); the other is the classic recruitment-based theory. In the extreme, the rate theory holds that recruitment of crossbridges following Ca^{2+} activation of the thin filaments is relatively unimportant, that crossbridges are all in the C-state, and that Ca^{2+} acts as an allosteric effector increasing the probability that crossbridges enter into force-generating state(s). As discussed here, it appears possible that in heart, a fraction of crossbridges is in each of these states.

Reconstructions of electron micrographs also indicate that Tn may move on the thin filament in order to release the actin-crossbridge reaction from a steric block. To get around the difficulty of visualizing Tn on the thin filament, Lehman et al. (31) used a deletion mutant of Tm, where internal pseudorepeats 2, 3, and 4 were missing. The deletion mutant Tm forms Tm polymers on F-actin, still binds the Tn complex, and demonstrates normal Ca^{2+}-induced movement (32, 33). These modified thin filaments contain seven-fourths-fold more Tn than native thin filaments, which aids in localizing Tn densities and detecting Ca^{2+}-dependent movement of Tn. The three-dimensional images reported by Lehman et al. (31) indicate that in the absence of Ca^{2+}, Tn approached residues 1–4, 23–27, and 47 of actin. This prediction agrees with NMR data (34) and cross-linking studies (35, 36). A different picture of Ca^{2+}-induced structural changes of the thin filament was reported by Narita et al., who used single-particle analysis of cryo-EM images to reconstitute three-dimensional models (37). One structural unit of actin filament (7actin-1Tm-1Tn) was divided into seven segments, I through VII. In the presence of Ca^{2+}, the globular domain of Tn (the T2-TnC-TnI region) was located over the inner domain of actin at segment V. In the absence of Ca^{2+}, it shifted toward the outer domain of actin by ∼28 Å. The C-terminal one-third of Tm (segments V–VII) shifted toward the outer domain by ∼35 Å, whereas the N-terminal half of Tm shifted less than ∼12 Å. In the presence of Ca^{2+}, Tm was located entirely over the inner domain of actin to allow crossbridge attachment. This model suggests that Tn covers at least part of the myosin-binding site on one or two actin molecules among the seven actin molecules in a structural unit.

The Troponin Complex

A major advance in our understanding of the inhibited and active state of the thin filaments came with the determination of the crystal structures of the Ca^{2+} bound form of the core domain of a ternary cardiac troponin (cTn) complex by Takeda et al. (38) (Figure 2). They crystallized two different cTn complexes. One had a M_r of 46 kDa and consisted of the full-length Ca^{2+} sensor, cTnC (residues

1–161),* a fragment of the inhibitory protein, cTnI (residues 31–163), missing the first 30 residues and the C-terminal 48 residues, and the C-terminal end of the Tm-binding protein, cTnT (residues 183–288; T2). The other Tn complex had a M_r of 52 kDa owing to inclusion of a larger cTnI fragment (residues 31–210) missing only the N-terminal extension that is unique to the cardiac variant. Thus in both cases, the cardiac-specific N-terminal region of cTnI, the C-terminal region of cTnI, the N-terminal half of TnT (T1), and the C-terminal part of TnT, which may interact with tropomyosin (Tm)-actin, are missing. Two Tn molecules present in an asymmetric unit of each crystal resulted in four different structures (identified as Tn46KA, Tn46KB, Tn52KA and Tn52KB). The overall architecture of each of these four structures is similar, except that TnI in Tn52KB has an extra α-helix spanning residues 164–188. The crystal structure of the core-domain of the cTn complex is divided into two subdomains, the regulatory head and the TnI-TnT arm, which are connected by a flexible linker. The regulatory head is composed of the N-terminal regulatory domain of cTnC and the regulatory or triggering site (downstream of the inhibitory region) of cTnI. The IT arm is made up of the C-domain of cTnC, T2, and two long TnI α-helices, one of which forms a coiled coil with TnT. The D/E linker of cTnC and the inhibitory region of cTnI form a flexible linker that is likely involved in the regulatory mechanism by allowing the two domains to rotate and alter their relative orientation.

Troponin C

TnC ($\sim$18 kDa) is a member of the EF-hand family of Ca^{2+}-binding proteins and consists of two globular domains (39–41). As illustrated in Figure 3, each EF-hand Ca^{2+}-binding site consists of an α-helix-loop-α-helix structural motif (42). The TnC molecule has eight α-helices (designated as A-through H-helix) associated with four Ca^{2+}-binding sites and an extra α-helix at the N terminus (N-helix). Each globular domain contains a pair of Ca^{2+}-binding sites designated sites I through IV from the N terminus. Sites I and II in the N-lobe are generally referred to as Ca^{2+}-specific sites, and sites III and IV in the C-lobe as Ca^{2+} and Mg^{2+} sites. Vertebrate fast skeletal muscle TnC (fsTnC) binds two Ca^{2+} at each domain, whereas in the cardiac/slow skeletal muscle isoform of TnC (cTnC), site I does not bind Ca^{2+} in the physiological range because there are several amino acid replacements in the coordinating sites. Analysis of equilibrium Ca^{2+}-binding data of cTnC in detergent-extracted fiber bundles in ATP-free solutions resolved these two distinct classes of binding sites (43). These sites saturated over the physiological range of Ca^{2+} concentrations with the following binding constants: sites III and IV; $K_{Ca} = 7.4 \times 10^7\ M^{-1}$, $K_{Mg} = 0.9 \times 10^3\ M^{-1}$, and site II; $K_{Ca} = 1.2 \times 10^6\ M^{-1}$, $K_{Mg} = 1.1 \times 10^2\ M^{-1}$. Following dissociation of rigor complexes by MgATP, there was a fall in K_{Ca} of site II to $\sim 3 \times 10^5\ M^{-1}$

*The residue numbers for cardiac and slow skeletal muscle are from human including the initial Met, and for fast skeletal muscle from rabbit.

with no changes in the affinities for site III and IV. This result, which agreed with earlier data in soluble reconstituted thin filaments reacting with myosin heads (44), indicates that the strong actin crossbridge reaction influences Ca^{2+} binding to the regulatory Ca^{2+}-binding site of cTnC just as Ca^{2+} binding to cTnC influences the actin-crossbridge reaction. Kinetic analysis of exchange of Ca^{2+} with sites III and IV indicated that these sites exchange Ca^{2+} much too slowly for it to occur within a heart beat (43). In contrast, kinetic analysis and site-directed mutagenesis studies indicated that site II is able to exchange Ca^{2+} in the time course of the heart beat and thus must be responsible for its regulation (45–47). However, the precise rate constants occurring with cTnC in its native environment remain unknown. In early studies (48), the kinetics of Ca^{2+} binding to the single regulatory site of cTnC was estimated using the fluorescent probe, IAANS, covalently attached to Cys-35 and Cys-84. These studies gave a dissociation rate constant of $\sim 20\ s^{-1}$. More recent data indicate that this rate constant may reflect a change in structure more than a change in Ca^{2+} binding. When the IAANS label was placed only at Cys-35 as a reporter of Ca^{2+} binding to cTnC in the cTn complex (49, 50), two phases were observed in Ca^{2+} association steps: a fast phase with $[Ca^{2+}]$ dependency and the slower phase without $[Ca^{2+}]$ dependency. Dong et al. (49, 50) concluded that the fast component corresponds to the Ca^{2+}-binding event, whereas the slower component corresponds to the conformational change triggered by Ca^{2+} binding. The results of Hazard et al. (51) are in general agreement with these data. They measured Ca^{2+}-binding kinetics for cTnC using both IAANS-labeled protein at Cys-84 and unlabeled protein. Ca^{2+} dissociation from the unlabeled cTnC was determined using stopped flow and the spectroscopically sensitive chelators, Quin 2 or BAPTA. The off-rate constant for Ca^{2+} binding to site II of cTnC was $700\ s^{-1}$, whereas the Ca^{2+}-independent off rate determined by IAANS fluorescence was $90\ s^{-1}$. Together with the results of Dong et al. (49, 50), these data indicate that site II rapidly binds and releases Ca^{2+} and induces relatively slow conformational steps. Because Cys 35 is located in the region surrounding the inactive site I in cTn, the data indicate a more important role for this region in regulation than is generally appreciated. The data of Dong et al. (50) also demonstrated that these steps are sensitive to phosphorylation of cTnI (see below).

Regions surrounding the inactive site I and the D/E linker region, which connects the N- and the C-lobes, may also be of special significance with regard to regulation of thin filament activity by cTnC. The D/E linker region is involved in Ca^{2+}-dependent activation of actomyosin ATPase activity; replacement of acidic amino acid residue(s) in this region with neutral Ala resulted in the loss of activation (52, 53). A direct role of TnT in Ca^{2+} activation is consistent with the observation that the interaction between TnC and TnT is involved in the regulatory function of Tn, at least in fast skeletal muscles (54, 55). In the crystal structure, the C-terminal domain of cTnC interacts with TnT, although interaction sites on TnC are different from those determined by NMR (56). The meaning of these variable results from different approaches should be clarified with the emergence of more cTn structural data in the sarcomeric lattice.

Troponin I

TnI, which was originally named because of its ability to inhibit actin-activated myosin ATPase activity, is a basic protein with a $M_r \sim 21$ kDa for fast skeletal TnI (fsTnI) and slow skeletal TnI (ssTnI) and a $M_r \sim 24$ kDa for cardiac TnI (cTnI). TnI has six distinct functional regions: (*a*) an N-terminal cardiac-specific extension that contains PKA-dependent phosphorylation sites (Ser-23 and Ser-24); (*b*) a region that binds to the C-lobe of TnC; (*c*) a region that binds to C-terminal regions of TnT; (*d*) a basic inhibitory region or first actin-binding region; (*e*) a regulatory or triggering region; and (*f*) a second actin-binding region. A critical function of TnI is to interact with actin and thereby inhibit actomyosin ATPase activity at low cytoplasmic [Ca^{2+}]. TnI is able to inhibit ATPase activity in the absence of Tm and other Tn subunits in vitro. Maximum inhibition occurs at an actin:TnI molar ratio of 1:1. In the presence of Tm, maximum inhibition is observed at a 7:1 ratio of actin:TnI. There is a periodic distribution of TnI along the Tm-actin filaments (57), which indicates that Tm not only amplifies the inhibitory action of TnI but also serves to direct TnI to its optimal thin filament location during sarcomere assembly. A critical actin-binding region on TnI is the inhibitory region (58, 59), whose sequence is highly conserved among muscle types, although Pro-110 in fsTnI and ssTnI is replaced by Thr-143 in cTnI. Thr-143 of cTnI is a major phosphorylation site for protein kinase C (PKC), which may affect velocity of shortening and plays an important role in post-translational modifications of cTn associated with hypertrophic signaling and the transition to heart failure (60). Rarick et al. (61) identified two other actin-binding sites on cTnI that are outside the inhibitory region and contain residues 151–188 of cTnI (residues 120–157 of fsTnI) and 188–210 of cTnI (residues 157–179 of fsTnI). In general agreement with this finding, Ramos (62) reported that residues 166–182 of fsTnI are also involved in actin-Tm binding, and Tripet et al. (63) identified a second actin region in fsTnI containing residues 140–148.

Although not defined in the crystal structure, there are several reports addressing the structure of the TnI inhibitory region. Data from circular dichroism spectroscopy and NMR studies using a binary complex of TnI with TnC indicate that the peptide derived from the fsTnI inhibitory region, residues 96–115, has an extended conformation with a possible kink around Gly-104 (64). A refined model based on further studies employing cross-linking and FRET studies indicates the inhibitory region is a flexible β-hairpin (65). Lindhout & Sykes (66) also determined the solution structure of cTnI inhibitory peptide, residues 129–148 (residues 96–115 of fsTnI), by NMR in a binary complex with the C-domain of cTnC, showing that a segment spanning residues Leu-135 to Lys-140 adopts a helical conformation. Using electron paramagnetic resonance, Brown et al. (67) reported that, among residues 129–145 of cTnI, residues 129–137 adopt an α-helical structure in the Tn ternary complex. Note that in the crystal structure (Figure 1), residues only up to Phe-133 of cTnI form an α-helix, H2(I), with C-cap residue Asp-134. In Tn46KA, the segment (residues 137–144) was not visible. The distance between α carbon atoms of Arg-136 and Arg-145 is 24.4 Å. If the peptide bond is fully extended, the

distance between these same atoms of adjacent residues is 32.7 Å. Thus in this case, the conformation of the inhibitory region that was not visible in Tn46KA is not completely extended but rather appears to be a flexible loop. Dong et al. measured the Ca^{2+}-dependent distance change between the inhibitory region and regulatory region of cTnI in a cTn complex, as well as in a cTnI-cTnC complex (68, 69). They observed a large distance increase (~9 Å) between residues 128 and 152 of cTnI upon Ca^{2+} binding to the regulatory site of cTnC, whereas the distance between the residues in the regulatory site (residues 149 and 166 of cTnI) remained unchanged. Dong et al. concluded that as cTnI is released from actin, residues within the cTnI inhibitory region switch from a β-turn/coil to an extended quasi-α-helical conformation. In contrast, the cTnI regulatory region remains α-helical.

Comparison of the molecular mechanisms by which Ca^{2+} binding to the N-lobe of fsTnC and cTnC triggers contraction reveals unique features in heart versus skeletal muscle. In the case of fsTnC, Ca^{2+} binding to sites I and II induces a conformational change in the N-lobe. Helices B and C move away from helix D, exposing a hydrophobic patch that serves as a new protein-interacting site (70–73). The regulatory site of fsTnI binds to this hydrophobic patch (74–76), and the free energy from this interaction accounts for most of the Ca^{2+}-dependent interaction between fsTnC and fsTnI (77). In studies of isolated cTnC, Ca^{2+} binding to the single regulatory site (site II) cannot open the hydrophobic patch in the N-lobe (78, 79). Opening the hydrophobic patch of cTnC requires an interaction with the regulatory site of cTnI (80, 81). Ultimately, the open structures of the N-lobe of cTnC and fsTnC are indistinguishable (Figure 3). This Ca^{2+}-induced interaction between the N-lobe of TnC and the regulatory region of TnI in turn induces a structural rearrangement among thin filament proteins such that the inhibitory region and the C-terminal part of TnI move away from actin, as determined from fluorescence energy transfer measurements (82–86) in the skeletal system. It is highly likely that a similar mechanism occurs in the cardiac thin filament.

Troponin T

TnT is not only the longest and largest component of Tn but also has the most extensive and diverse interactions with its neighbors on the thin filament. TnT binds to TnI and TnC to form the Tn complex and also binds to Tm and actin. TnT is an elongated protein with a C-terminal region forming part of the globular head of Tn and an N-terminal region forming the tail. Mild chymotryptic digestion yields two soluble fragments, T1 (the tail region) and T2. T1, which was not included in the crystal structure, binds to the Tm-Tm overlap region and confers cooperative myosin S1 binding to the thin filament. As can be seen in the crystal structure (Figure 2), T2 binds to TnC and TnI. T2-TnC-TnI acts as the Ca^{2+} sensor of the thin filament. The M_r of TnT is 31–36 kDa, with 250–300 amino acid residues. Cardiac isoforms are longer than skeletal isoforms at and near the N terminus. In cTnI and cTnC, isoforms arise from separate genes, but in addition, cTnT isoforms arise from alternative splicing of TnT transcripts. In human heart development and in heart failure there are four cTnT isoforms ($cTnT_1$–$cTnT_4$) that are expressed in

a variable isoform population (87). The charge differences associated with these shifts in isoform population may contribute to altered myofilament response to Ca^{2+} as reported by Gomes et al. (88).

A role for the tail region of cTnT, T1, in myofilament activation and relaxation is also evident from other studies. The N-terminal region appears to be important in maintaining Tm in the blocked or B-state associated with steric block of the actin-crossbridge reaction. Hinkle et al. (89) reported that $cTnT_{1\text{-}153}$ strongly promoted Tm binding to actin and was able to inhibit crossbridge interactions with the thin filament that promote ATPase activity and filament sliding in the motility assay. This region of the cTnT tail also inhibited myosin-S1-ADP binding to the thin filament. Three-dimensional reconstructions from negatively stained images of actin-Tm-$cTnT_{1\text{-}153}$ support the idea that the B-state of Tm is associated not only with TnI binding to actin but also with interactions of portions of the TnT tail domains with Tm-actin. The complexity of the functions of the N-terminal tail of TnT is indicated by data showing that deletion of the first 76 amino acids of cTnT results in an inhibition of maximum tension and ATPase rate (90). $cTnT_{77\text{-}289}$ bound more tightly to Tm than full-length cTnT. Interestingly, Hinkle et al. (89) reported that whereas deletion of 94 residues from the N terminus of cTnT had no effect on myosin S1 ATPase activity, deletion of 119 residues resulted in a greatly reduced affinity of Tn for actin Tm. Although the fsTnT-T1 region (residues 1–158 of fsTnT) inhibited actomyosin S1 ATPase activity (91), detailed functional mapping of the fsTnT molecule by Oliveira et al. (92) demonstrated that a peptide containing $fsTnT_{77\text{-}191}$ was able to activate actomyosin ATPase activity. These results point to the complexity of data interpretation from investigations aimed at functional identification of the various domains of TnT.

In addition to this special role of cTnT in relaxation, a role for cTnT in thin filament activation separate from cTnI has also been proposed. Potter et al. (54) indicated that Ca^{2+} fsTnC not only releases the thin filament from inhibition by fsTnI but also plays a role in promoting the thin filament–myosin interaction via a direct reaction with fsTnT. This hypothesis is based on data generated from experiments with thin filaments reconstituted with a fsTnI N-terminal deletion mutant (fsTnId57), which does not bind to fsTnT. These preparations could still activate myosin ATPase activity, presumably by a direct interaction with fsTnC. Whether this occurs in the cardiac system remains unclear, but there is evidence in general agreement with the studies in fast skeletal preparations. A cardiac deletion mutant ($cTnI_{80\text{-}211}$), which is analogous to fsTnId57, also lost its ability to bind to cTnT but retained binding to cTnC, albeit weakly (93). In this case, despite the lack of binding between cTnI and cTnT, Ca^{2+} was able to activate ATPase activity in a reconstituted system to 50% of that obtained with full-length cTnI. The influence of cTnT on Ca^{2+} activation is not restricted to regions that interact with cTnC in the regulatory head identified in the crystal structure. Using metal binding and epitope mapping, Jin & Root (94) reported that the alternatively spliced variable T1 region of chicken fsTnT affects the structure of the T2 region. Data demonstrating that the isoform population of developmental splice variants of the T1 region of cTnT

affect the Ca^{2+} sensitivity of tension development of skinned fiber bundles (88) also indicate an effect of the N-terminal tail on the regulatory head of cTn.

Phosphorylation of cTnI and cTnT

Phosphorylation of cTnI and cTnT has been recently reviewed in detail elsewhere (9, 95). Protein kinase A (PKA) specifically phosphorylates Ser-23 and Ser-24 of cTnI. PKA phosphorylates Ser-24 first and, subsequently, much slower phosphorylation occurs at Ser-23 (96, 97). PKC phosphorylates both cTnI and cTnT. Phosphorylation sites on cTnI were previously determined to be Ser-42, Ser-44, and Thr-143 (98). Recently, we found that both beta-PKC and epsilon-PKC phosphorylate Ser-23, Ser-24, and Thr-143 of cTnI in the Tn ternary complex or in the reconstituted thin filament (99). There is also evidence from in vitro studies that Ser 162 of cTnI is a substrate for p21-activated kinase (Pak) (100). However, active Pak in the cellular environment induced dephosphorylation of cTnI via activation of PP2a (101).

Phosphorylation of Ser-23, Ser-24 appears specialized for regulation of sensitivity of the myofilaments to Ca^{2+} and for enhancing crossbridge cycling rate, whereas phosphorylation of the PKC-specific sites appears specialized for depressing crossbridge cycling rate. Data indicate that PKC-dependent phosphorylation of cTnI either enhances (102) or depresses (103) myofilament Ca^{2+} sensitivity. Pak-dependent phosphorylation of Ser-162 increased Ca^{2+} sensitivity of skinned fiber bundles. Residues Thr-194, Ser-198, Thr-203, and Thr-284 are the major PKC-dependent phosphorylation sites in cTnT (104). Among these four phosphorylation sites, Thr-203 appears to be by far the most important for modulation of cTn function (105). Phosphorylation of Thr-203 significantly inhibits tension and Ca^{2+} sensitivity of skinned fiber bundles, indicating the significance of this region of TnT in thin filament function.

Most of the potential phosphorylation sites in cTnI and cTnT were not resolved in the crystal structure. Some were among the segments removed to improve the crystal growth. Others were not seen owing to structural flexibility. In the crystal structure, Ser-42 and Ser-44 of cTnI were visible. Ser-42 is the N-cap residue of a long helix H1(I). Ser-42 forms a side chain backbone hydrogen-bond network with Arg-45, which represents the most common pattern of α-helix capping, i.e., a capping box (Figure 4). Phosphorylation of Ser-42 is likely to affect the capping box formation and thus alter cTnI local structure. Ser-44 interacts with Glu-10 of the N-lobe of cTnC, indicating that phosphorylation of Ser-42/Ser-44 might affect the interaction between cTnI and cTnC and thus Ca^{2+} activation. Pseudophosphorylation (replacement of Ser with Glu) of Ser-42/Ser-44 significantly reduces myofilament response to Ca^{2+} (103). Moreover, pseudophosphorylation of these sites, together with pseudophosphorylation of Thr-143, reduces the affinity of cTnI-cTnC interaction (106). Another potential phosphorylation site visible in the crystal structure is Thr-203 of cTnT (Figure 4). This residue also acts as the N-cap of the H1(T2) helix. Modeling of the structural change induced by

phosphorylation of Thr-203 predicts an extension of the helix. Compared with controls, skinned fiber bundles containing cTnT-Thr-203-Glu demonstrate a significantly reduced maximum tension and ATPase rate, as well as a reduced Ca^{2+} sensitivity (105).

The domains surrounding Ser-23, Ser-24, and Thr-143 of cTnI were not determined in the crystal structure. Results of NMR structural studies (97, 107) and cross-linking studies (108) support a mechanism in which the dephosphorylated cTnI N-terminal extension interacts with the N-lobe of cTnC. Upon phosphorylation of Ser-23/Ser-24, the N-terminal region dissociates from cTnC, inducing a reduction in Ca^{2+} affinity of the cTn complex and a decrease in the Ca^{2+} sensitivity of myofilament activity. Although the structure of the cardiac-specific region seems flexible, Keane et al. (97) were able to determine the solution structure of the peptide (residues 17–30 of cTnI) derived from this region with phosphorylation at Ser-23 and Ser-24. According to their model structure, this segment adopted a looped conformation with interactions between the side chains of Arg-21 and Arg-22 and phosphate groups on Ser-23 and Ser-24. Thr-143 is located in the middle of the minimum inhibitory region of cTnI. Phosphorylation of this site reduces the affinity for TnC (109) and depresses filament sliding velocity in the motility assay (103).

Tropomyosin

Tm, which consists of 284 amino acids, is a highly extended parallel coiled-coil protein. Structure-function properties of Tm have been reviewed by Perry (4) and by Wolska & Wieczorek (110). The two chains are about 400 Å long and overlap to form continuous strands winding around the actin filaments (Figure 1). The sequence contains a hepta-peptide repeat of hydrophobic amino acid residues, which are essential for coiled-coil formation. The sequence also contains a less characteristic sevenfold periodic repeat that corresponds to seven actin interaction sites. Each periodic repeat has a different functionality (32, 111, 112). Whereas $\alpha\alpha$Tm from cardiac muscle was found in the blocked-state position along the actin filament in the absence of Tn, $\beta\beta$Tm and $\alpha\alpha/\alpha\beta$Tm from skeletal muscle were found in the closed-state position (113). The two isoforms differ by a relatively small number of amino acids, suggesting a low energy barrier between blocked and closed states of the thin filaments. A high-resolution crystal structure of the N-terminal 81 residues of αTm revealed that the coiled coil is staggered at the segment with a high content of Ala, a structural specialization resulting in the bending of the coiled coil (114). Many such "Ala-staggering" segments along the Tm sequence appear to allow Tm to wind on the actin filament. Changing an Ala cluster to a canonical coiled-coil interface with Leu greatly stabilized Tm, but reduced its affinity for actin more than tenfold (115). The structure of the C-terminal 31–34 residues of Tm was also determined by X-ray crystallography (116) and NMR (117). In the structure generated from a crystal formed from a chimera of the C-terminal 31 residues of Tm with GCN-4, the last 22 residues

splay and expose a large surface area (116). In the solution structure of the C-terminal 34 residues, residues 253–269 formed a coiled coil, whereas residues 270–279 formed parallel linear helices. This C-terminal uncoiled-coil region may be important for TnT-recognition. Greenfield et al. (118) found that when fragments with residues 251–284 of Tm were titrated with residues 1–33 of Tm, NMR signals from residues 274–284 were perturbed. Further addition of TnT perturbed most of the signals. When the unnatural amino acid hydroxy-Trp was introduced into Tm by Farah & Reinach (119), they observed that Ca^{2+} binding to Tn changed the fluorescence of hydroxy-Trp located in the region outside the Tn-binding site of Tm. Thus Ca^{2+} binding to Tn showed a long-range effect on the Tm molecule.

Variations in the flexibility of Tm may be important for its function in relaxation and activation mechanisms within the thin filament (8, 117, 120–122). Increasing evidence indicates that cooperative activation of the thin filament requires Tm to function as a continuous strand along the thin filament. A model presented by Smith & Geeves (121) considers Tm flexibility in the context of a continuous chain of molecules that includes Tn. They hypothesize that TnI in the relaxed state binds to actin, immobilizes Tm on the thin filament, and impedes the strong actin-crossbridge reaction. Release of TnI from actin by Ca^{2+}-TnC permits movement in Tm that results in crossbridge binding and induces a kink in the Tm-Tn chain. In their concept (121), therefore, the crossbridge and TnI compete for binding sites on actin and induce oppositely directed chain kinks. It is also likely that the tail of TnT may immobilize Tm, as discussed above (89). Smith et al. (122) extended this concept of activation to a model describing the influence of crossbridge binding to cooperatively promote the binding of neighbors on the thin filament.

An alternative model was proposed by Tobacman & Butters (17) on the basis of data from many investigators showing that myosin and Tm strengthen each other's binding to actin. There is a ~10,000-fold strengthening of Tm attachment to actin when crossbridges bind to the thin filament. Implicit in the Smith et al. model is the notion that crossbridge binding to the thin filament should loosen the actin-Tm interaction. A continuous chain of Tm molecules is critical to both models, but the models differ greatly: The Tobacman model invokes specific, tight Tm-actin interactions; the Smith et al. model hypothesizes nonspecific actin-Tm interactions. To account for the observed effects of myosin and Tm on each other, Tobacman proposed that strong myosin-actin attachments alter the actin inner domain so that Tm binds to it tightly (the open or M-state position). Similarly, tropomyosin binding to the actin inner domain alters actin so that myosin binds more tightly on the actin outer domain. These changes in actin permit Tm to shift position on the thin filament cooperatively, removing the steric block to the actin-crossbridge reaction, and positioning Tm at the inner domain of actin, a requirement of strong binding of crossbridges. In the Tobacman model, activation of one longer region of the thin filament is 100 times favored over activation of two shorter regions. In both models the Tm shifts position on actin cooperatively. However, they differ in what mechanism, other than its own rigidity, holds the Tm stiffly in place at the same position along multiple successive actins.

INTEGRATION OF THIN FILAMENT MOLECULAR SIGNALING AND CONTROL OF CARDIAC FUNCTION

Pressure, Volume, and Time as Descriptors of Cardiac Function

The dynamics of pressure and volume changes occurring during the heart beat and the relation between volume and pressure during the heart beat (123) provide a framework in which to discuss functional correlates of the molecular interactions at the level of the sarcomere (Figure 5). In the following sections, we discuss molecular reactions involving thin filament proteins in the context of the system's behavior. The two left panels of Figure 5*A* depict left ventricular pressure (LVP) and volume (V) changes during a beat of a mouse heart under basal conditions and during stimulation with a beta-adrenergic agonist, isoproterenol (ISO). The

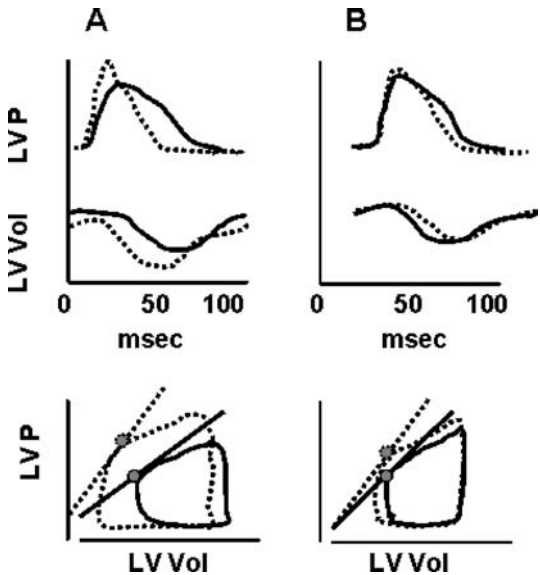

Figure 5 Pressure, volume, and time relations during a beat of the isolated, ejecting mouse heart in control conditions (*solid lines*) and in response to beta-adrenergic stimulation by isoproterenol (*dashed lines*). (*A*) Nontransgenic hearts. (*B*) Transgenic hearts expressing slow skeletal TnI in place of cardiac TnI. The upper panels show the time course of left ventricular pressure (LVP) and volume (LV Vol) and the lower panels show the relation between LV Vol and LVP. Points on the P-Vol loops indicate the end systolic pressure, and lines drawn through these points indicate contractility. Note that the presence of ssTnI signficantly attenuates the enhanced relaxation and contractility associated with adrenergic stimulation. See text for further description.

data are from isolated ejecting mouse hearts beating under conditions of constant venous return and pressure head (afterload) and at constant heart rate (124). The bottom panel of Figure 5*A* illustrates the P-Vol loop, which is a plot of the relation between LV volume and LVP in a beat. The end systolic pressure (ESP) in Figure 5*A* is indicated as the level of LVP where ejection from the ventricle stops. Consideration of sarcomeric function in various phases of the P-Vol loop provides a useful framework for relating molecular properties of the thin filaments to cardiac function. Although the relationships between cellular function and ventricular function are complex, the correlate of pressure is sarcomeric tension and the correlate of volume is sarcomere length. The ESP is thus a point at which the sarcomeres are neither shortening nor lengthening. The line drawn through the ESP points in Figure 5 reflects the sarcomere length tension relation in the heart. Points on the line can be generated by systematically altering the venous return (see 123 for further explanation). The increase in ESP with increases in ventricular volume is the essence of Starling's law of the heart.

The response to ISO reported in Figure 5*A* (dashed lines) identifies important parameters that define the contractile or inotropic state (ability to contract; contractility) and lusitropic state (ability to relax) of the heart. ISO induces an increase in the amplitude of LVP and the rate of rise of pressure. It also induces a decrease in the end systolic volume (ESV) and end diastolic volume (EDV) and an increase in the rate of decay of LVP. Thus there is contractile reserve that can be called upon to meet hemodynamic demands by increasing pressure and the extent of shortening of the sarcomeres during ejection of blood. There is also a relaxation reserve in which enhanced relaxation is called into play. This is especially important in the physiological context where heart rate increases, as during exercise, and thus cycle time of filling and ejection must be reduced. This rotation of the ESP-Vol relation defines a change in contractility; ISO stimulation induced an increase in developed pressure (tension) at a given ventricular volume (∼sarcomere length).

Thin Filaments in Diastole

Sarcomeres in the diastolic state operate along the trajectory of the points connecting the ESV to EDV in the P-Vol loops in Figure 5*A*. It is important that the thin and thick filaments slide past one another during filling in order to maintain the low ventricular pressures that are optimal for filling in light of the low upstream pressures in the atria and large veins of the body and lungs. The nature of the thin filaments in the diastolic state remains unclear. One issue is whether the crossbridges are all sterically blocked during diastole or whether the crossbridges are all weakly bound. Alternatively, it has been proposed that a fraction of crossbridges are blocked (B-state) and a fraction are in the weak binding C-state. Estimates based on kinetics of myosin-S1 binding and fitting to the three state models described above indicate that 50% of the crossbridges are in the B-state (20).

Another issue related to the thin filament in diastole concerns the position of Tm and Tn and the state of actin. Evidence from X-ray structural studies on intact muscle fibers, which formed the basis of the steric blocking model, indicated early on that accessibility of crossbridge-binding sites on actin resulted from a movement of Tm induced by Ca^{2+} binding to Tn (12–14). However, analysis of the X-ray studies focused only on Tm and was interpreted as a movement of Tm over the actin surface in such a way that subdomains 1 and 2 of actin became exposed. Re-evaluation of the data indicates that interpretation of the X-ray diffraction is ambiguous and may, in part, be accounted for by a movement of subdomains of actin and Tn itself (7). This raises questions as to whether the diastolic state requires a particular structure of the actin domain, rather than a static structure waiting for the crossbridge to bind, and whether Tn itself engages in the steric block. Evidence summarized by Squire & Morris (7) supports the concept that both a particular position of the actin subdomains 1 and 2 and the position of Tn may be important determinants of the relaxed state of striated muscle. However, movements of actin subdomains are difficult to resolve in the X-ray data, and data both support (125) and contradict (126) the idea that actin subdomain motion is involved in Tn-Tm regulation of the filaments. Structural studies indicate that, in addition to tethering or latching Tm in a position that inhibits the ATPase rate and force generation, Tn may occupy a position on actin that interferes with both weak and strong binding of crossbridges. The three-dimensional reconstructions from electron micrographs fitted with the atomic model of actin (31) indicate the involvement of residues 1–4, 23–27, and 47 of actin in the interaction with Tn in the relaxed state, as mentioned above. Acidic residues in the N terminus form a likely interface for ionic interactions with basic residues in TnI. However, extensive alterations in charged residues in the N terminus of actin had no effects on the Ca^{2+} sensitivity of thin filaments as determined in the motility assay, or on the inhibitory activity of TnI (127). Tn binding could, however, induce a Ca^{2+}-insensitive change in the structure of the N terminus of actin. This was theorized by Wong et al. (127) to be of possible significance in the effects of Tn-Tm in improving S-1 binding to the thin filament and in the increase in force developed by myosin reacting with regulated thin filaments, as compared with unregulated actin filaments (128). We (129) tested the significance of modifications in actin subdomains 1 and 2 by comparing tension and ATPase rate in heart myofilaments from nontransgenic (NTG) and transgenic (TG-actin) mice in which enteric γ-actin replaced >95% of the cardiac α-actin. The difference between cardiac α-actin and enteric γ-actin is the introduction of an Asp at the N terminus together with Asp9Glu and Asn360Pro substitutions in subdomain 1, and Thr17Cys and Thr89Ser substitutions in actin subdomain 2. Maximum tension and rate of tension redevelopment (k_{tr}) were the same for myofilaments from NTG and TG-actin mice. However, TG-actin myofilaments were less sensitive to Ca^{2+} than the NTG controls, and economy of tension development (unit tension /unit ATP hydrolyzed) was significantly increased. These data indicate that minor amino acid differences in the actin domains reacting with myosin can induce large differences in function

when measured in the lattice of myofilament proteins. Studies at the level of the intact heart demonstrated a hypodynamic phenotype in the TG-actin myocardium (130).

In summary, detailed understanding of the interactions among regions of the thin filament proteins that are critical in maintenance of the diastolic state remain to be determined, especially in the lattice of the sarcomere. However, some general conclusions seem reasonable. We think that the diastolic state involves both steric and allosteric effects on regions of actin that react with crossbridges. Relaxation requires an interaction of cTnI with actin and most likely an interaction of TnT with actin-Tm, both of which immobilize Tm, and possibly Tn, at the outer domains of actin. The result is that most of the crossbridges are blocked from reacting with actin, but a substantial number of crossbridges are cocked, in the terminology of Squire & Morris (7), in that they appear to bind weakly with the thin filament. This makes sense for heart muscle contraction in which basal function involves a small fraction of strong crossbridges reacting with the thin filament and in which there is no spatial summation of motor units. Increases in contractility, as indicated by the change in slope of the ESP-V relation, involve recruitment of crossbridges. It is possible that the B-state of the thin filament holds a portion of this pool in reserve and moves to the C-state only with increases in Ca^{2+} delivered to the myofilaments or alterations of the thin filament (discussed below).

Thin Filaments and the Triggering and Sustaining of Systole

At the end of diastole, there is an abrupt transition to systole triggered by Ca^{2+} binding to the thin filament receptor, cTnC. Flow of blood into the left ventricle ceases as pressure rises in the ventricle and the atrio-ventricular valves close. The LV volume does not change until the pressure exceeds that in the aorta, so for a time, pressure rises isovolumically (Figure 5) or, from the perspective of the myocytes, there is relatively little change in cell length. The function of the thin filaments in this transition from the diastolic state early in systole is reflected reasonably well either by studies using release of caged Ca^{2+} to activate sarcomeres from a relaxed state or by studies using a rapid release/restretch protocol to determine the rate constant for crossbridge entry into the force-generating state. As pointed out by Wolff et al. (131), it is not unreasonable to think that k_{tr} may limit the rate and extent of ventricular pressure development during the early isovolumic phase inasmuch as the duration of Ca^{2+} transients is brief compared with tension development. It is noteworthy that submaximal k_{tr} may increase with the increases in sarcomere length associated with increased end diastolic volume (132). This is of some relevance to Starling's law of the heart (see below).

As discussed in detail by Gordon et al. (8), substantial evidence indicates that the mechanism by which Ca^{2+} regulates the transition of crossbridges from weak- to strong-binding states is by affecting the kinetics of thin filament activation. There is little support for an alternative mechanism involving a direct effect of Ca^{2+} on crossbridge kinetics, for example, by Ca^{2+} binding to myosin light chain 2.

However, whether the dynamics of thin filament–related processes can be rate limiting in the transition from weak to strong crossbridge binding remains to be thoroughly tested in heart muscle preparations. Studies in fast skeletal muscle preparations by Brenner & Chalovich (133) indicate that thin filament processes following Ca^{2+} binding to thin filament are in rapid equilibrium and are not rate limiting. They tracked thin filament structural changes employing fluorescent probes attached to TnI in fast skeletal psoas fibers. Changes in TnI fluorescence were not rate limiting in their measurements of force redevelopment after the rapid release/restretch protocol. The data were well-fit assuming a rapid equilibrium among thin filament states and only two (on and off) states of the thin filament. A role for strongly binding crossbridges was minimized by Brenner & Chalovich (133), who argued that the rigor state is short lived in the intact muscle. They also argued that a time-resolved X-ray diffraction study of the position of Tm on the thin filament (134) demonstrated movement well before tension generation and little movement after tension generation. However, as mentioned above, Squire & Morris (7) have brought the interpretation of these early X-ray data into question, especially with regard to motions of actin and Tn. Moreover, when Ca^{2+}-binding properties of fsTnC were modified by mutations or treatment with small molecules that bind with high affinity, k_{tr} at submaximally activating levels of Ca^{2+} is altered. For example, binding of of calmidazolium (CDZ) alters kinetics of Ca^{2+} binding to TnC with no direct effects on crossbridge cycling and also influences the rate of k_{tr} in skinned rabbit psoas muscle fibers (135). These data indicate that modulation of the thin filament Ca^{2+} signaling, which is apparently most well developed in the heart (60), may modulate k_{tr}. Treatment of skinned fiber bundles with PKA induces an increase in rate of force development as determined by measurements of k_{tr} (136) even though the sensitivity of tension and k_{tr} to Ca^{2+} was reduced. A plot of relative tension versus relative k_{tr} revealed the effect of PKA-dependent phosphorylation on k_{tr}. Patel et al. (136) also reported a similar increase in k_{tr} with a switch of Tm isoforms.

Thin Filaments, Relaxation, and the Return to Diastole

In the context of P-Vol loops (Figure 5), left ventricular relaxation is a complex process that begins somewhere around the time of aortic valve closure, which occurs when pressure in the ventricular chamber falls as blood flows into the arterial tree and as activation declines. Relaxation involves Ca^{2+} removal from cTnC, deactivation of the thin filament, dissociation of crossbridges from the thin filament with the transition from strong to weak and blocked states, and, with the loss of strong crossbridge binding, a loss of cooperative activation of the thin filament. It has also been proposed, on the basis of studies with isolated myofibrils (137) relaxed by a rapid drop in Ca^{2+}, that interactions between sarcomeres induce strain-dependent rebinding of inorganic phosphate to myosin, which is a significant factor in rapidly turning off the actin-crossbridge reaction. The rate of relaxation is also dependent on the afterload, i.e., the pressure against which the ventricle is ejecting blood. With increases in afterload, relaxation slows. Although many investigators focus solely on the kinetics of Ca^{2+} removal from the cytoplasm by

the sarcoplasmic reticulum as the rate-limiting step in relaxation, there is abundant evidence that this is not the case and that processes in the sarcomere are significant factors in relaxation. Alterations in relaxation kinetics that occur with alterations in the isoform population of myosin heavy chains, which alter crossbridge cycling rates, provide perhaps the best evidence (138). An important point with regard to the thin filaments in relaxation is the demonstration (139, 140) that enhanced relaxation is more closely correlated with enhanced crossbridge cycling than enhanced release of Ca^{2+} from TnC. Therefore, it is important to consider potential ways that altered thin filament function could modify crossbridge cycling. For example, altered thin filament function appears to alter crossbridge cycling by phosphorylation of cTnI at the PKA sites. Direct evidence that cTnI-P affects crossbridge cycling comes from measurements of tension as a function of imposed sinusoidal length oscillations over a broad range of frequencies. The frequency at the minimum tension (dip frequency) is an indirect measure of the forward and back transitions between weakly bound and strong force-generating crossbridges. Studies in both intact (141) and skinned fiber bundles (139) with specific changes in phosphorylation of cTnI at the PKA sites concluded that phosphorylation induces an increase in crossbridge cycling.

Testing the Hypothesis that Altered Thin Filament Response to Ca^{2+} is an Essential Element in the Control of Cardiac Dynamics

The generation of transgenic mouse models has been critical in testing the hypothesis that specific alterations at the level of the thin filament affect the intensity and dynamics of the myocardium. There are many examples of studies involving introduction of mutant thin filament proteins into the cardiac sarcomeres of transgenic mice. In most cases, the mutations mimic those linked to hypertrophic (HCM) and dilated cardiomyopathies (DCM), but in some the mutations involve deletion of phosphorylation sites (102, 142–145) thought to be critical in modulating thin filament response to Ca^{2+}. Compared with controls, hearts of these transgenic mice demonstrate altered dynamics and response to inotropic interventions. However, as expected from the linkage to cardiomyopathies, cardiac-directed expression of familial hypertrophic cardiomyopathy (FHC) or DCM-related mutant thin filament proteins results in other abnormalities such as fibrosis and cellular remodeling, which makes it difficult to interpret the data with respect to the connection between altered thin filament function and altered cardiac function. In the case of mice harboring transgenes that express thin filament proteins lacking phosphorylation sites, it is not clear that the Ala/Ser or Ala/Thr mutations are benign. For example, we have found, both in vitro (146) and in vivo (147), that the mutant cTnI (S42A, S44A) apparently alters the specificity of sites at Ser-22 and Ser-23 of cTnI as substrates for PKA such that they become excellent substrates for PKC. The Ala substitutions also induce a depression in maximum tension (144). Transgenic models in which there is incorporation of naturally occurring isoforms into the thin filament proteins may hold more promise for testing the hypothesis that

thin filament response to Ca^{2+} is an essential element in the control of cardiac dynamics. We have investigated two mouse models of this sort. In one we (148) altered the isoform population of Tm, which is predominantly the α-isoform in the adult, by introducing a transgene expressing β-Tm that resulted in a Tm isoform population consisting of about 60% β-Tm. In another model (149, 150), we used transgenesis to completely switch the isoform population of TnI from 100% cTnI to 100% slow skeletal TnI (ssTnI). In both models there was an increase in myofilament response to Ca^{2+} and a depression in the rate of cardiac relaxation in the basal state, which was exacerbated during stimulation with ISO.

A particularly useful model has been one in which the adult form, cTnI, was replaced with ssTnI, which is the isoform expressed in the embryonic and neonatal heart in all species studied to date. Data from investigations of changes in cardiac function with a drop in pH support the hypothesis. Inasmuch as the peak amplitude of the Ca^{2+} transient increases with the fall in tension (151), the most straightforward interpretation of the mechanism for this depression in cardiac activity is a desensitization of the myofilaments to Ca^{2+}. The observation that myofilaments from neonatal dog hearts demonstrate a greatly reduced deactivation by acidic pH indicates a developmental change in the thin filament. With the identification of ssTnI as the embryonic and neonatal isoform in the heart, together with data indicating that isoform switching of TnI may be the most important difference between adult and immature hearts with respect to response to acidosis, it was natural to generate a transgenic (TG) mouse in which ssTnI replaced cTnI. Compared with NTG controls, the Ca^{2+} sensitivity is enhanced in myofilaments from ssTnI-TG mouse hearts, and deactivation by acidic pH is significantly blunted (150). Moreover, papillary muscles from ssTnI-TG hearts generate the same tension when superfused with buffer at physiological pH and buffer at a reduced pH induced by hypercapnia. Under these same acidotic conditions there was the expected significant reduction in force developed by NTG papillary muscle. In view of evidence that expression of ssTnI in the TG mouse heart has very minor effects on cellular Ca^{2+} flux, these results support the hypothesis that altered response to Ca^{2+} may dominate regulation of cardiac activity and dynamics under certain conditions.

The dependence of ventricular systolic pressure on ventricular volume, more commonly known as the Frank-Starling relation, represents another mode of regulation apparently dominated by a length-dependent myofilament response to Ca^{2+}. The relation between ESP and ventricular volume is a measure of Starling's law, which is believed to be rooted in a dependence of cellular tension on sarcomere length (SL). In addition to the increase in maximum tension developed by the myofilaments with increases in SL, there is also an increase in myofilament sensitivity to Ca^{2+}. The mechanism for these changes has been attributed to a decrease in interfilament spacing with increasing SL, but explicit measurements indicate no correlation between lattice spacing and length-dependent activation. More complicated mechanisms involving titin interactions with the thick filament or thin filament may account for length-dependent activation (11). Whatever the case, myofilaments from ssTnI-TG hearts show a significant blunting of length-dependent activation that is not correlated with a change in interfilament spacing

(152). The reduced length dependence of activation predicts a reduction in the steepness of the ESP-ventricular volume relation. This reduction in slope has, in fact, been demonstrated in preliminary studies with ssTG hearts beating in situ during stimulation with ISO. It has also been reported that PKA-dependent phosphorylation of the myofilaments enhances length-dependent activation. Thus under beta-adrenergic stimulation, differences between ssTnI-TG hearts, in which there is no PKA-dependent phosphorylation of TnI, and NTG hearts would be expected to be amplified. These results provide additional and compelling evidence that regulation by alterations in myofilament response to Ca^{2+} is a fundamentally important regulatory device in the heart.

Three recent studies (124, 153, 154) provide strong evidence for a significant role of thin filament proteins as a determinant of contraction and relaxation reserve. These studies also illustrate the importance of evaluating the role of alterations in Ca^{2+} control of thin filament function with the sarcomeres operating under physiological conditions. Investigations by Pena & Wolska (153) of hearts beating in situ (therefore innervated and perfused with blood under physiological loading conditions) support a pivotal role of cTnI phosphorylation as a determinant of relaxation. This study investigated phospholamban knockout (PLBKO) mice expressing either cTnI or ssTnI. PLBKO/cTnI and PLBKO/ssTnI hearts demonstrated similar $-dP/dt$ in the basal state, but during perfusion with ISO, $-dP/dt$ was significantly slower in PLBKO/ssTnI than in PLBKO/cTnI hearts. In another study, Takimoto et al. (154) investigated TG mice expressing cTnI(D22/D23) in which Asp residues replaced Ser in the N-terminal extension unique to cTnI. In vitro studies demonstrated that this substitution produces a pseudophosphorylated cTnI, which mimicked effects of cTnI phosphorylated at the Ser-22 and Ser-23 PKA sites. In the basal state, diastolic and systolic function in hearts of control and cTnI(D22/D23) mice were similar. However, the responses to increases in frequency and afterload were significantly different in these hearts. Rate-dependent increases in contraction and relaxation were enhanced in the cTnI(D22/D23) hearts compared with those in controls. Moreover, prolongation of the time course of relaxation associated with increases in afterload was greatest in the controls. Following beta-adrenergic stimulation, these differences in cardiac function between cTnI(D22/D23) and NTG mice were not evident.

Layland et al. (124) investigated isolated, perfused working hearts from TG mice expressing slow skeletal TnI (ssTnI) in place of cTnI, which lacks the N-terminal extension containing Ser-23, Ser-24 present in cTnI. Replacement of cTnI with ssTnI renders the myofilaments insensitive to effects (reduced sensitivity to Ca^{2+}) of phosphorylation by PKA (155). Perfusion with ISO reduced relaxation time and ESV in control hearts, as illustrated in Figure 5, but not in hearts of ssTnI mice. Contractility, as reflected in the ESV-pressure relation, was also increased by ISO to a greater extent in the controls than in the ssTnI hearts. Isovolumic hearts beating at constant pressure did not demonstrate such significant differences as in the auxotonically loaded, ejecting heart. Similarly, the difference noted above between hearts expressing cTnI(D22/D23) was not present in isometric papillary muscle with increased frequency of stimulation or beta-adrenergic stimulation. Arelatively

small effect of myofilament phosphorylation had been previously reported in studies comparing tension in isometric papillary muscles or ventricular myocytes isolated from hearts of control mice and mice lacking phospholamban (149, 156). Thus functional effects of phosphorylation of cTnI are fully expressed only when the myofilaments are shortening against a load. This indicates that strain-dependent effects on crossbridge cycling rate may be modified by thin filament proteins.

SUMMARY AND FUTURE DIRECTIONS

Investigation of the control of thin filament function in the heart is at an exciting juncture. The recent data on the role of thin filament alterations in the beating heart have brought fresh understanding to the complexity of control mechanisms at the level of the sarcomere. It is certain that new and exciting lines of investigation will emerge, based on the structural information derived from the crystal structure of the Tn complex. It should not be too long before additional structures emerge showing more detail and different states of the thin filament. The confluence of this information, together with the biochemistry, biophysics, and systems physiology of the heart, will permit a clearer understanding of the role of the thin filament in cardiac function. This information is highly relevant to the diagnosis and treatment of prevalent cardiac diseases genetically linked to thin filament proteins. It is also relevant to a continuing quest for therapeutic agents that modify the sarcomere. A particularly interesting and exciting idea mentioned at the outset is the modern view of the thin filament as it functions in the A-band region and in the I-Z-I region of the sarcomere. Future studies must incorporate the full thin filament in their approaches in order to understand the multiplex functions of the sarcomere apart from shortening and generating force.

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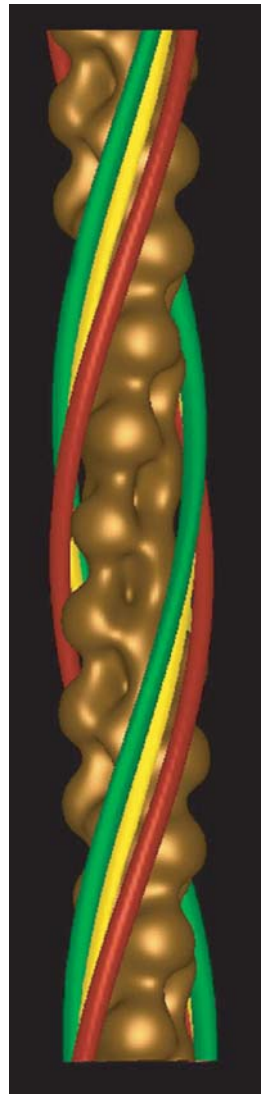

Figure 1 Three-dimensional reconstruction of a surface view of the states of the thin filament under relaxed conditions (Tm, *red*), in the Ca^{2+}-activated condition (Tm, *yellow*), and in rigor (Tm, *green*). Reproduced from Craig & Lehman (22) by permission.

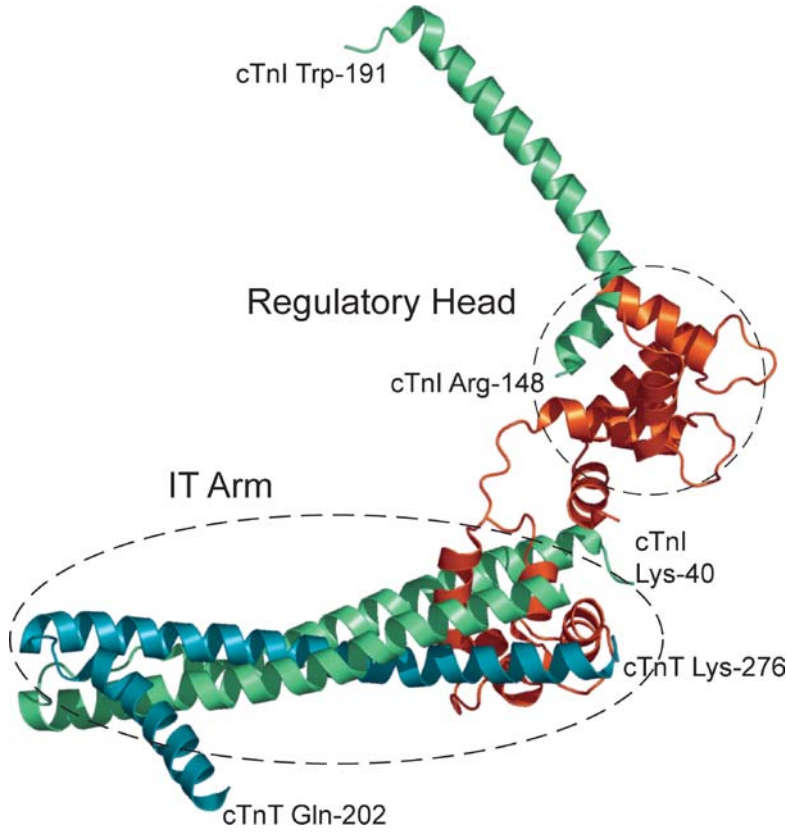

Figure 2 Ribbon model of the crystal structure of the core domain of Tn complex determined by Takeda et al. (38). Shown here are chains D, E, and F of PDB 1J1E. Orange, cTnC; blue, cTnT; green, cTnI.

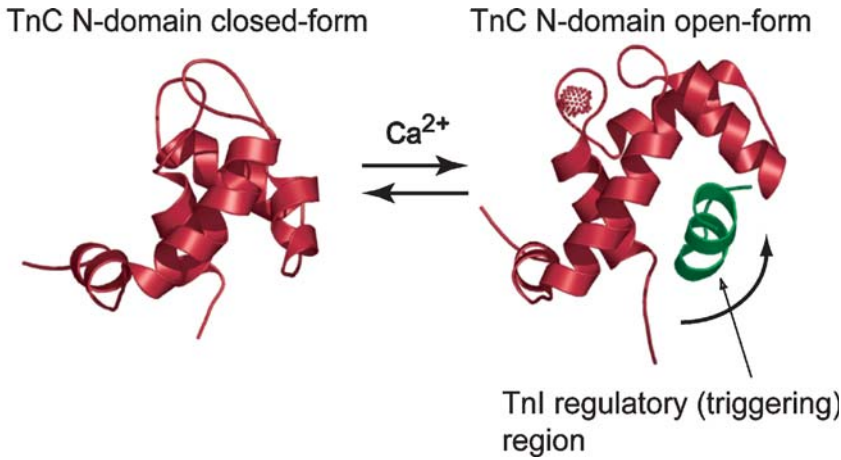

Figure 3 Ca^{2+}-dependent structural change in the N-lobe of TnC. Only the N-terminal domain of TnC and the regulatory (triggering) region of TnI are shown.

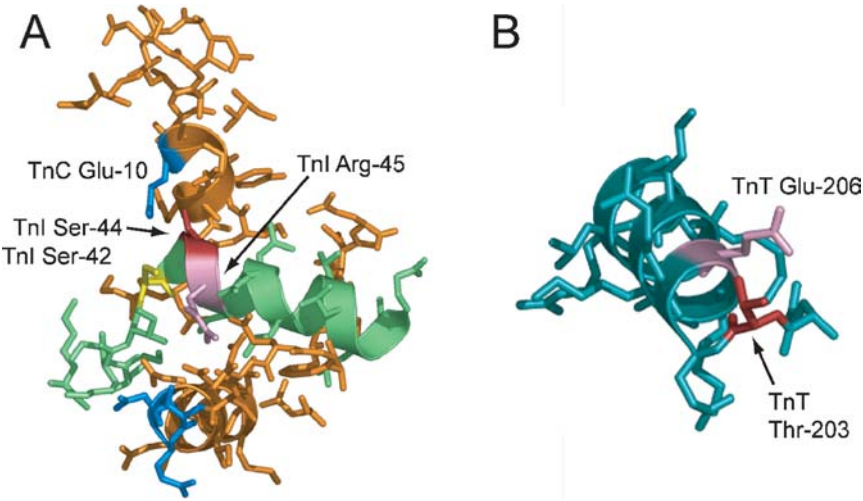

Figure 4 Structural model of phosphorylation sites in cTn. (*A*) cTnI Ser-42 (*yellow*)/Ser-44 (*red*) and nearby residues. Ser-42 forms a capping box with Arg-45 (*magenta*). Whereas Ser-44 interacts with Glu-10 (*blue*) from the N-terminal domain of cTnC in the crystal structure, pseudophosphorylation (Asp-substitution) of Ser-42/Ser-44 perturbs the N-terminal structure of the G-helix (*also shown in blue*) from the C-terminal domain of cTnC (157). (*B*) cTnT Thr-203 (*red*) and nearby residues. Thr-203 forms a capping box with Glu-206 (*pale magenta*) of cTnT.

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