Communication

Exchange of β- for α-Tropomyosin in Hearts of Transgenic Mice Induces Changes in Thin Filament Response to Ca2+, Strong Cross-bridge Binding, and Protein Phosphorylation*

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Despite its potential as a key determinant of the functional state of striated muscle, the impact of tropomyosin (Tm) isoform switching on mammalian myofilament activation and regulation in the intact lattice remains unclear. Using a transgenic approach to specifically exchange β-Tm for the native α-Tm in mouse hearts, we have been able to uncover novel functions of Tm isoform switching in the heart. The myofilaments containing β-Tm demonstrated an increase in the activation of the thin filament by strongly bound cross-bridges, an increase in Ca2+-sensitivity of steady state force, and a decrease in the rightward shift of the Ca2+-force relation induced by cAMP-dependent phosphorylation. Our results are the first to demonstrate the specific effects of Tm isoform switching on mammalian thin filament activation in the intact lattice and suggest an important role for Tm in modulation of myofilament activity by phosphorylation of troponin.

The ability of myosin heads to react with actin in heart muscle occurs with a transition of the thin filament from an "off" to an "on" state that depends on complex alterations involving the tropomyosin (Tm) molecule (for reviews see Refs. 1 and 2). These alterations include possible steric effects associated with changes in the position of Tm on the thin filament, as well as allosteric and cooperative effects associated with Tm-induced changes in actin structure and reactivity with myosin (3–5). The steric and allosteric/cooperative alterations involving Tm are triggered by Ca2+-binding to TnC, but also depend on myosin head binding (6). Ca2+-binding to TnC promotes interactions with other Tn components, Tni (2), an inhibitory protein that binds to actin, as well as TnI and Tm-binding protein. The steric model of activation (3) hypothesizes that Ca2+-TnC-induced movement of Tm or possibly Tnl (2) reverses the off state by releasing actin sites for reaction with myosin. The allosteric model (4, 5) proposes that Ca2+-TnC itself cannot activate the thin filament, but acts as a co-factor shifting the equilibrium between off and on states of Tm such that strongly bound cross-bridges more easily activate the thin filament. Although recent considerations indicate that activation may involve both processes (2), the relative role of the steric and allosteric/cooperative mechanisms in turning on the activity of striated muscle remains unclear.

Our perception of the role of Tm in the regulation of striated muscle, as well as its structure/function relations, has come from a variety of approaches. These include x-ray diffraction of muscle preparations (7) and crystals (8, 9), reconstructions from electron micrographs (9, 10), and reconstitution studies of soluble systems with Tm (11–13), Tm peptides (14), and mutants of Tm (15). In some cases, differences regarding structure/function relations have been made from comparisons of muscle fibers containing isoforms of Tm (16). However, interpretation of these studies is difficult in that there are multiple changes in myofilament proteins that occur along with the natural variations in Tm. A clearer understanding of the structure/function relations of Tm has been hampered by an apparent lack of methods for reversibly extracting Tm from the myofilament lattice in a force-generating system, as has proved so successful in the case of Tn components such as TnC and Tnl (17). Thus, issues such as the role of Tm domains, covalent modifications, and the functional significance of isoform switching of Tm in the intact force-generating lattice of vertebrate-sriated muscle have remained poorly understood. Delineating the functional differences between Tm isoforms has taken on new significance with the identification of missense mutations in the Tm gene causally linked to familial hypertrophic cardiomyopathy (18–20).

In the present experiments, we used a transgenic approach to overcome the difficulties of exchanging Tm isoforms in the intact myofilament lattice. Transgenic mice, which overexpress β-Tm in the heart, were generated as described previously (21). This has permitted us to test explicitly the effects of alterations in Tm isoforms on myofilament activation. Our results provide the first unambiguous evidence that myofilament activation by Ca2+ and strong cross-bridges is affected by the population of Tm isoforms present in the heart. Our results also indicate a role for Tm in the modulation of myofilament activation by phosphorylation of Tn. Some of our results have been published in abstract form (22).

EXPERIMENTAL PROCEDURES

Materials—ATP (from equine muscle), creatine phosphate, creatine phosphokinase, cAMP, EGTA, MOPS, saponin, and the protease inhibitors pepstatin A, leupeptin, and phenylmethylsulfonyl fluoride were...


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purchased from Sigma. Calycin A and Triton X-100 were purchased from Calbiochem-Novabiochem Corp. Alkaline phosphatase-conjugated goat-anti-mouse IgG (Fc), nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Promega Corp. \(^{[\text{32P}]ATP} (3000 \text{ Ci/mmol})\) was obtained from DuPont NEN products. Kodak X-Omat scientific imaging film (Eastman Kodak Co.) was used for autoradiography and was purchased from Fisher.

Transgenic Animals—Transgenic mice (FVB strain) were generated as described previously by Muthuchamy et al. (21). Expression of the transgene was driven by the murine \( \alpha \)-myosin heavy chain promoter which, as demonstrated previously (23), restricts expression to the transgenic mice. These mice showed no evidence of neonatal mortality, and no histological evidence of abnormalities, no evidence of neonatal mortality, and no histological evidence of abnormalities or hypertrophy.

Gel Electrophoresis and Western Blotting—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (24). Myofibrils were isolated as described previously (25), separated on a 12.5% SDS gel, and transferred to nitrocellulose paper according to the method of Towbin et al. (26) for Western blot analysis. Primary antibody incubations were performed with a 1:10,000 dilution of chI antibody which recognizes both \( \alpha \)-Tm isoforms. Secondary antibody incubations were performed with alkaline phosphatase-conjugated goat-anti-mouse IgG. Color development occurred over a 5-min period using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate system.


determinations—Left ventricular trabeculae were excised from the heart and dissected into fiber bundles approximately 100–150 \( \mu \)m in width and 2–3.5 mm in length. Fibers were dissected and stored in a low relaxing solution containing 0.1 mM EGTA in standard buffer (20 mM MOPS at pH 7.0, 1 mM free Mg\(^{2+}\), 2 mM MgATP, 12 mM creatine phosphate) at pH 9.5, pH 7.0 at 4°C. Low relaxing solution also contained the protease inhibitors, pepstatin A (2.5 \( \mu \)g/ml), leupeptin (1 \( \mu \)g/ml), and phenylmethylsulfonyl fluoride (25 \( \mu \)M).

For Measurements—Fiber bundles were mounted between a micro-manipulator and a force transducer (27) with cellulose-acetate glue. Fibers were then extracted for 30 min at 22°C in a 250 \( \mu \)g saponin/ml high relaxing solution (10 mM EGTA in standard buffer). After a maximal contraction at pCa 4.5, the fiber bundle was relaxed in high relaxing solution. The fiber bundles were then immersed in successive solutions of varying pCa or pMgATP. Following relaxation in high relaxing solution, a second pCa 4.5 contraction was performed to assess fiber viability. The ionic compositions of the solutions were computed by solving multiequilibria equations with the use of a computer program (28).

Conditions for \( \text{cAMP} \)-Dependent Phosphorylation—\( \text{cAMP} \)-dependent phosphorylation was induced by incubating detergent-extracted fiber bundles in a phosphorylation buffer containing 10 mM EGTA, 80 \( \mu \)g/ml \( \text{cAMP} \), and 100 \( \mu \)M \( \text{cAMP} \). The protocol used to phosphorylate myofilaments in detergent-extracted preparations was modified after that described by Herzig et al. (29), who demonstrated full phosphorylation of cardiac skinned fiber bundles by endogenous PKA and 100 \( \mu \)M \( \text{cAMP} \) after 10–20 min of incubation. Even so, to ensure full phosphorylation of \( \alpha \)-TnI in both NTG and TG-\( \beta \)-Tm fiber bundles, the preparations were incubated for 30 min with \( \text{cAMP} \) in the presence of calycin A (100 ng/ml), a phosphatase inhibitor. In some experiments, exogenous PKA (30 \( \mu \)g/ml) was also added to the incubation solution, giving the same results as in the absence of added PKA.

To remove excess \(^{[\text{32P}]ATP} \) and to stop the reaction, myofilaments were washed three times with ice-cold phosphorylation buffer. The fibers were then solubilized in 130 mM Tris-HCl, pH 6.8, and total protein was determined by the method of Lowry et al. (30). Equivalent amounts of protein were loaded onto SDS gels. Gels were exposed to Kodak X-Omat scientific imaging film for 24 h at \( -80^\circ \)C and then developed.

Calculations and Statistics—Individual pmGmATP- or pCa-force determinations were normalized to a maximum generated specifically for each measurement by Graphpad Inplot curve fitting software, Graphpad Software Inc. The normalized values where then grouped and averaged and used for calculation of the final curves as illustrated in Figs. 2 and 3. Statistical calculations were made using SigmaStat software, and Excel Corp. Values were considered significantly different if \( p < 0.05 \) as determined by two-way ANOVA.

RESULTS

Fig. 1A illustrates the SDS-PAGE protein profiles of detergent-treated myofilament preparations from NTG and TG-\( \beta \)-Tm mouse hearts (lanes 1 and 2, respectively). \( \alpha \)-Tm was identified in the two preparations by Western blot analysis as illustrated in Fig. 1B (NTG, lane 1, and TG-\( \beta \)-Tm, lane 2). This analysis demonstrated that NTG hearts express only \( \alpha \)-Tm in the myofilaments, whereas \( \beta \)-Tm is abundantly expressed in myofilaments from TG-\( \beta \)-Tm hearts. We found no changes in the expression of other myofilament proteins. The results illustrated in Fig. 1 show that our transgenic approach has made myofilaments containing \( \beta \)-Tm preparations contain an abundance of \( \beta \)-Tm.

Myofilament activation via \( \text{Ca}^{2+} \) binding to TnC was also measured in skinned fiber preparations. Results presented in Fig. 3A indicate that the force developed by TG-\( \beta \)-Tm myofilaments was significantly (\( p < 0.05 \) two-way ANOVA) more sensitive to \( \text{Ca}^{2+} \) than NTG myofilaments. The pCa\(_{50} \) was 5.72 ± 0.01 for TG-\( \beta \)-Tm preparations and 5.57 ± 0.04 for NTG preparations.

A unique property of cardiac TnI that may be important in its reaction with Tm is that it is phosphorylated by PKA (31). In experiments reported in Fig. 3, we compared the pCa-force relations for NTG and TG-\( \beta \)-Tm fiber bundles before and after phosphorylation by cAMP/PKA as described under “Experimental Procedures.” Our results show that the difference in \( \text{Ca}^{2+} \) sensitivity between NTG and TG-\( \beta \)-Tm myofilaments was even more pronounced when the myofilaments were phospho-
Fig. 2. The relation between pMgATP and force of NTG and TG-β-Tm fiber preparations. Detergent-extracted fiber preparations were sequentially exposed to solutions of decreasing MgATP concentration at pCa 9.0. In both cases, the peak force obtained at pMgATP 5.4 in the NTG fibers and pMgATP 5.0 in the TG-β-Tm fibers was only approximately half that obtained under maximal Ca2+-activated conditions at pCa 4.5 (data not shown). Values are expressed as the mean ± S.E. of n = 5 from 3 different hearts. *, p < 0.05 as determined by Student Newman Keul’s post hoc t-test. ○, NTG; ▲, TG-β-Tm.

Fig. 3. The effect of cAMP-dependent phosphorylation on the pCa and force relation in NTG and TG-β-Tm fiber preparations. A, pCa-force relation of detergent-extracted NTG (○, ●) and TG-β-Tm (▲, △) fiber preparations under control conditions (A, ○, ▲) and under phosphorylating conditions (B, ●, △). Inset, protein phosphorylation profile of detergent-extracted NTG and TG-β-Tm myofilament preparations performed as described under “Experimental Procedures.” All phosphorylation experiments represent at least 3 individual determinations. Values are expressed as the mean ± S.E. In the case of the NTG preparations, n = 3 from 3 different hearts. In the case of the TG preparations, n = 5 from 3 different hearts. Each individual determination involved sequentially immersing a fiber in two sets of solutions of varying pCa values. The first set of Ca2+-solutions to which a fiber was exposed (under control conditions) had no added cAMP. After obtaining the initial measurements, the fiber bundle was then incubated under phosphorylating conditions (see text and “Experimental Procedures”) and then reimmersed in solutions of varying pCa in the presence of 100 μM cAMP.

DISCUSSION

Our data provide the first clear evidence that exchange of β-Tm for α-Tm alters thin filament activation by cross-bridge binding. It is known that strong binding of cross-bridges is able to “turn-on” many actins through a cooperative process that requires Tm (6). Moreover, when overlap between adjacent Tm molecules is removed, cooperative cross-bridge-actin binding is significantly reduced (33). In the mouse, isoform switching from α- to β-Tm involves 39 amino acid substitutions. Twenty five of these substitutions occur in the C-terminal half of the molecule,2 a region important for Tm head-to-tail interactions. Previous studies (21) have demonstrated the preferential formation of αβ-Tm heterodimers in these transgenic cardiac myofilaments. This does not, however, preclude the formation of ββ-Tm homodimers which were undoubtedly present due to the abundance of β-Tm expressed. Importantly, Thomas and Smillie (13) have reported that ββ-Tm has a greater propensity to form end-to-end interactions than either αβ- or αα-Tm, which may significantly alter the cooperative potential of the myofilaments containing β-Tm. Additionally, amino acid substitutions Ser229 → Glu and His276 → Asn result in β-Tm having a (−2)

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charge change relative to α-Tm.2 These changes could affect cooperative activation of thin filaments: (i) by altering the interaction of Tm with the seven actins under its control or (ii) by affecting end-to-end interactions linking near neighbor functional units consisting of actin-Tm-Tn in a 7:1:1 ratio.

Based on previous studies, it is not surprising that isoform switching from α-Tm to β-Tm alters thin filament activation by Ca2+. Ca2+ binding activates the thin filament by altering the interactions of TnC with TnT (34) and TnI (35, 36). These changes induce changes in the binding of Tm to actin (10) and promote cross-bridge binding to actin. Importantly, TnT has been shown to bind more weakly to β-Tm than to α-Tm (37). Thus, it would be expected that activation may occur at a lower level of free Ca2+ in myofilaments containing β-Tm.

Our results also implicate a role for Tm isoform switching in the modulation of thin filament activation by phosphorylation of TnI. Although the inhibitory activity of TnI is potentiated by...
Tm (6), how phosphorylation of TnI might alter its interaction with Tm has not been appreciated. In vitro studies of Al-Hillawi et al. (38) demonstrated that cooperative binding of TnI to actin-Tm is abolished when TnI is phosphorylated. Our hypothesis is that isoform switching from α-Tm to β-Tm itself results in a weaker cooperative interaction between TnI, actin, and Tm. Thus, the effect of TnI phosphorylation may be minimized or lost in myofilaments containing β-Tm.

An increased Ca\(^2+\) sensitivity and a reduced effect of TnI phosphorylation on myofilaments containing β-Tm serves to explain results of studies on isolated working hearts from NTG and TG-β-Tm mice. Muthuchamy et al. (21) showed that, compared to controls, TG-β-Tm working heart preparations demonstrated an increase in the time for half-maximal relaxation of ventricular pressure. This fits with our findings of increased Ca\(^2+\) sensitivity in myofilaments containing β-Tm. Moreover, the difference in half-maximal relaxation time was increased with low level β-adrenergic stimulation. This is what we would expect given that β-adrenergic stimulation, which results in TnI phosphorylation, has been shown to reduce myofilament Ca\(^2+\) sensitivity, thereby enhancing relaxation (39). Our results on skinned fiber bundles indicate that this relaxant effect, associated with TnI phosphorylation, would be much reduced or absent in the TG heart.

Our results also have important implications regarding the etiology of FHC, which is genetically linked to sarcomeric mutations (for review, see Ref. 40). Two of the three point mutations found in the α-Tm gene of patients with FHC involve charge changes in the putative Ca\(^2+\)-dependent TnI binding domain (18–20). Our data show that isoform switching involving charge changes near this domain enhance myofilament Ca\(^2+\) sensitivity, an effect expected to slow relaxation as demonstrated in working heart preparations (21). Interestingly, point mutations in myosin heavy chain linked to FHC also slow contraction dynamics (41, 42). Thus, the mechanism for the slowing need not involve myosin, but could occur through changes in myofilament response to Ca\(^2+\) exacerbated during exercise. The ability to test these ideas in transgenic animals brings us closer to understanding the functional differences between Tm isoform populations in physiological and pathological settings.

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