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Original article

Regulatory domain of troponin moves dynamically during activation of cardiac muscle

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ABSTRACT

Heart muscle is activated by Ca^{2+} to generate force and shortening, and the signaling pathway involves allosteric mechanisms in the thin filament. Knowledge about the structure-function relationship among proteins in the thin filament is critical in understanding the physiology and pathology of the cardiac function, but remains obscure. We investigate the conformation of the cardiac troponin (Tn) on the thin filament and its response to Ca^{2+} activation and propose a molecular mechanism for the regulation of cardiac muscle contraction by Tn based uniquely on information from *in situ* protein domain orientation. Polarized fluorescence from bifunctional rhodamine is used to determine the orientation of the major component of Tn core domain on the thin filaments of cardiac muscle. We show that the C-terminal lobe of TnC (CTnC) does not move during activation, suggesting that CTnC, together with the coiled coil formed by the TnI and TnT chains (IT arm), acts as a scaffold that holds N-terminal lobe of TnC (NTnC) and the actin binding regions of troponin I. The NTnC, on the other hand, exhibits multiple orientations during both diastole and systole. By combining the *in situ* orientation data with published *in vitro* measurements of intermolecular distances, we construct a model for the *in situ* structure of the thin filament. The conformational dynamics of NTnC plays an important role in the regulation of cardiac muscle contraction by moving the C-terminal region of TnI from its actin-binding inhibitory location and enhancing the movement of tropomyosin away from its inhibitory position.

1. Introduction

Contraction of cardiac and skeletal muscle is triggered by Ca^{2+} binding to the regulatory protein Tn in the actin-containing thin filaments, leading to an azimuthal movement of the tropomyosin around the filament that uncovers myosin binding sites and allows myosin motors to interact with actin and generate force [1–3]. Tn consists of three subunits, troponin I (TnI), troponin T (TnT) and troponin C (TnC). At low [Ca^{2+}], troponin docks on to the thin filament by binding of TnT to tropomyosin [4,5] and the C-terminus of TnI (CTnI) to actin [6,7]. Except for the N-terminus of TnT (NTnT, residues 1-158), the interactions of these troponin components with actin and tropomyosin are sensitive to the Ca^{2+} level (Fig. 1A). The removal of CTnI from its actin binding site by binding of Ca^{2+} to TnC and the consequent shifting of equilibrium position for tropomyosin are key steps in the regulation of muscle contraction. The crystal structures of the troponin core complex [8,9] have provided a platform for building molecular hypotheses about this steric blocking mechanism, but these are limited by uncertainties about the position and conformation of the troponin components in the filament and the structures of some key regulatory regions of Tn outside the core complex. Until recently, studies on determining the organization of the Tn in the thin filament using a variety of techniques produced inconclusive and contradictory results [10–12].

Crystal structures of the troponin core complex [8,9] have focused on the less dynamic components of the complex in the muscle regulatory system. The 'regulatory head' of Tn is the N-terminal lobe of the TnC (NTnC) which contains the regulatory Ca^{2+} site. The 'IT-arm' is a rigid domain and has an arrowhead shape containing a long coiled-coil formed by helices from TnI and TnT, plus the C-terminal lobe of TnC (CTnC) (Fig. 1B). The backbone fold of the IT-arm does not depend on the presence of Ca^{2+} in the regulatory site. Understanding of interactions between these proteins and their structural changes underlying Ca^{2+}-control of heart muscle contraction is critical for the broad strategy and the detailed molecular design of therapeutic interventions in heart disease in which cardiac contractility is altered.

Here, we investigated the conformation of the cardiac Tn on the thin filament and its response to binding of Ca^{2+} to elucidate the molecular mechanism of the regulation of contraction. The conformation of the Tn core domain in the intact cardiac muscle sarcomere was determined by

Abbreviations: BR, bifunctional rhodamine; CTnC, C-terminal lobe of TnC; CTnI, C-terminus of TnI; IT arm, coiled-coil formed by the TnI and TnT chains; ME, maximum entropy; NTnC, N-terminal lobe of TnC; NTnI, N-terminus of TnI; NTnT, N-terminus of TnT; Tn, troponin.

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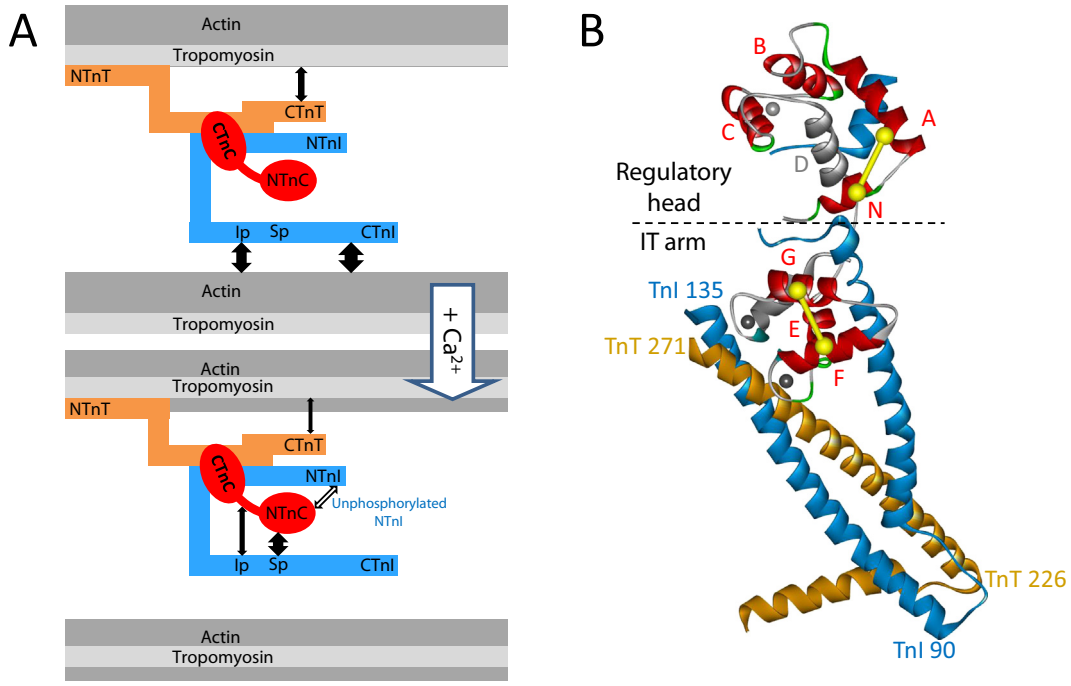

Fig. 1. A schematic representation of the interactions between components of cardiac thin filament (A) and the Ca^{2+}-bound form of the troponin core complex from cardiac muscle (B). A, The Ca^{2+}-dependent interactions between TnI, TnC, TnT and actin are shown in solid double-headed arrows. Ip, TnI inhibitory peptide; Sp, TnI switch peptide. The interaction between cardiac specific NTnI and NTnC in the presence of Ca^{2+} occurs when Ser23/Ser24 of NTnI are unphosphorylated. B, structure of the cardiac Tn core complex contains TnC (red, grey) and parts of TnI (blue) and TnT (gold). Bifunctional rhodamine (BR) probes cross-linked cysteines along either the N, A, B, C, E, F or G helix of TnC (red). BR probes cross-linking cysteines across either N and A, or F and G helices are shown in yellow dumbbells. D helix which is inaccessible is in grey.

a fluorescence-based approach. We attached bifunctional fluorescent probes (BR) to pairs of surface-accessible cysteines of TnC. The probe attachment sites were chosen using the high resolution structures of isolated Tn components, and engineered by expressing mutants of TnC with cysteines at the chosen sites (i.e., 10–15 Å apart on α-helices with their β-carbons at surface-exposed positions) (Fig. 1B). The bifunctional attachment constrains the orientation of the probe dipoles with respect to the protein backbone, so that the polarization of the fluorescence from a heart muscle cell containing such a labelled TnC gives information about the *in situ* orientation of the probe, and thus the vector joining the cysteine pair. We chose four cysteine pairs for the CTnC in the IT arm of Tn and five cysteine pairs for the NTnC in the regulatory head domain of Tn (Fig. 1B). Data from sets of cysteine pairs were combined to estimate the orientation of troponin domains with respect to the thin filament axis.

By combining polarized fluorescence data from each set of the labeled TnCs with *in vitro* structures of the Tn domains, we estimated the orientation of the IT arm and the regulatory head domain in ventricular trabeculae, and their orientation changes associated with binding of Ca^{2+} and of myosin heads during contraction. Finally, by combining the data on the *in situ* orientation of both the IT arm and the regulatory head domain of Tn and on the proximity of the relevant regions of Tn and actin, we constructed an model of the thin filament that suggests a plausible molecular mechanism for the Ca^{2+} regulation of heart muscle involving the conformational dynamics of the Tn regulatory head domain.

2. Materials and methods

2.1. Preparation of BR labeled TnC

Nine double cysteine mutants of human cardiac TnC (D3C/E10C, E10C/L17C, E15C/A22C, K39C/R46C and E55C/D62C for the NTnC; E95C/R102C, E115C/Q122C, D131C/K138C and K118C/E134C for the CTnC) (Fig. 1B) were produced by site-directed mutagenesis, expressed in *E.coli* and purified as described previously [13,14]. Each pair of introduced cysteines was cross-linked with a bifunctional rhodamine probe (BR).

2.2. Reconstitution of TnC into ventricular trabeculae

Ventricular trabeculae from rat right ventricle were prepared as previously described [14]. Native TnC was partially replaced by incubation of trabeculae in relaxing solution containing 30 μmol/L BR-TnC overnight at 4 °C. The fraction of TnC replaced by BR-TnC was estimated as 80 ± 2% (mean ± SE, n = 8) based on SDS-PAGE and immunoblot analysis (see Fig. S1). Following incubation, the demembranated trabeculae were mounted via aluminium T-clips between a force transducer and a fixed hook in a 60 μl trough containing relaxing solution. The sarcomere length was set to 2.1 μm. The experimental temperature was 20–22 °C.

Each trabecular activation was preceded by a 1-min incubation in pre-activating solution. Isometric force and fluorescence intensities were measured after steady-state force had been established. The maximum Ca^{2+}-activated force above the passive tension was 42.2 ± 2.4 mN mm^{-2} (mean ± SE, n = 50 preparations) in the present study, which was comparable to the maximum force recorded in our previous study before incorporation of labeled TnC into trabeculae (37.9 ± 1.6 mN mm^{-2}, n = 14) [14]. When calculated separately for the nine BR-TnCs, none of the average forces was statistically different from these two values ($P > 0.1$; Table S1). Our previous study also showed that the introduction of double-cysteine and BR probe on the C- or E-helix of TnC reduced the Ca^{2+} affinity of the regulatory site of TnC (by about 0.3 units of pCa) with no significant effect on the steepness of the force-Ca^{2+} relationship [14]. Since the exchange of TnC itself using previous extraction-reconstitution protocol causes the reduced Ca-sensitivity of force-Ca^{2+} relationship [13,14], it is reasonable to assume that the present TnC exchange protocol without the extraction step should produce a milder effect. However, it was impractical to test the trabeculae mechanically before an over-night exchange of TnC to assess its impact.