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Review

Troponin: Regulatory function and disorders

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Abstract

Study of the molecular biology of the calcium regulation of muscle contraction was initiated by Professor Ebashi's discovery of a protein factor that sensitized actomyosin to calcium ions. This protein factor was separated into two proteins: tropomyosin and a novel protein named troponin. Troponin is a Ca^{2+}-receptive protein for the Ca^{2+}-regulation of muscle contraction and, in association with tropomyosin, sensitizes actomyosin to Ca^{2+}. Troponin forms an ordered regulatory complex with tropomyosin in the thin filament. Several regulatory properties of troponin, which is composed of three different components, troponins C, I, and T, are discussed in this article. Genetic studies have revealed that many mutations of genes for troponin components, especially troponins T and I, are involved in the three types of inherited cardiomyopathy. Results of functional analyses indicate that changes in the Ca^{2+}-sensitivity caused by troponin mutations are the critical functional consequences leading to these disorders. Recent results of this pathophysiological aspect of troponin are also discussed.

Keywords: Calcium ion; Muscle contraction; Troponin; Troponin T; Troponin I; Troponin C; Hypertrophic cardiomyopathy; Dilated cardiomyopathy; Restrictive cardiomyopathy

Study of the molecular biology of the calcium regulation of muscle contraction was initiated by Professor Ebashi's discovery that a contractile interaction of actomyosin is sensitized to calcium ions in the presence of a protein factor other than myosin or actin [1,2]. The protein factor was called native tropomyosin, because of its similarity in amino acid composition to tropomyosin, which had already been reported [3,4]. Tropomyosin was isolated from native tropomyosin but did not confer any Ca^{2+}-sensitivity to actomyosin. In 1965, an unknown protein was found to remain in the native tropomyosin solution even after isoelectric precipitation of tropomyosin. This protein, in association with tropomyosin, sensitized actomyosin to Ca^{2+} and was named "troponin" [5,6]. Troponin was demonstrated to be a Ca^{2+}-receptive protein of the Ca^{2+}-regulation of muscle contraction [6]. The contractile interaction between the two contractile proteins, myosin and actin, was thus established to be regulated by Ca^{2+} through two specific Ca^{2+}-regulatory proteins, troponin and tropomyosin. At the same time, electron microscopy revealed that troponin is distributed with regular intervals of 38 nm along the thin filament [7]. This finding led to the construction of a structural model of the thin filament, in which two head-to-tail filaments of tropomyosin run in the grooves of a double-stranded actin filament and troponin binds to a specific region of each tropomyosin molecule [8]. The action of Ca^{2+} on troponin is transmitted to the double-stranded actin molecules through tropomyosin.

Troponin is not a single peptide but consists of three different components, troponins C, I, and T [9–12]. Troponin C is a Ca^{2+}-binding component, troponin I is an inhibitory component of contractile interaction, and troponin T is a tropomyosin-binding component. Two essential mechanisms in the regulation by troponin components are the inhibition of the contractile interaction of myosin–actin–tropomyosin by the inhibitory action of troponin I and the release of the inhibition by troponin I through the bind-

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ing of Ca^{2+} to troponin C. Troponin T functionally acts as an integrating component necessary for the Ca^{2+}-regulatory action of troponin [13].

In the last two decades, results of genetic studies of inherited disorders of the myocardium have developed a new area in troponin research. Genetic studies have revealed that three major types of inherited cardiomyopathy are associated with mutations of genes of sarcomeric and cytoskeletal proteins, including troponin [14,15]. More than 70 mutations of cardiac troponin components have been reported to be associated with the cardiomyopathies. Functional analyses indicate that an increase or decrease in the Ca^{2+}-sensitivity of contraction caused by troponin mutations is the critical functional consequence leading to the three types of cardiomyopathy [16].

In this article, several regulatory properties of troponin and functional studies of the troponin mutations associated with inherited cardiomyopathy are discussed.

Arrangement of troponin in the thin filament

During the initial studies of troponin, localization of this protein in the myofibrillar structure was examined by means of immunoelectron microscopy. An anti-troponin antibody was found to form 24 regular narrow transverse striations along the entire length of the thin filament region in chicken skeletal myofibrils [7]. This finding indicated that troponin is distributed with regular intervals of 38 nm along the thin filament and that troponin molecules in neighboring filaments are aligned in register within the myofibrillar structure. In view of the finding that tropomyosin is a 40-nm long fibrous molecule that forms long filaments with head-to-tail interactions [17,18], the intermittent distribution of troponin was deduced to be based on the underlying head-to-tail filaments of tropomyosin molecules. A model of the thin filament was thus constructed as an ordered assembly of three kinds of proteins, i.e., troponin, tropomyosin, and actin [8]. In this structure, two head-to-tail filaments of tropomyosin lie in the grooves of a double stranded actin filament and each tropomyosin molecule is attached by troponin at a specific region (Fig. 1A).

The detailed observation of isolated thin filaments revealed that the top of thin filament is separated from the terminal location of troponin by about two thirds of the 38-nm period length [19]. Consistent with this finding, an analysis of the paracrystalline structure of troponin–tropomyosin showed that troponin is bound to the region approximately two-thirds of the molecular length from one end of the tropomyosin molecule. The formation of fine transverse striations with the anti-troponin antibody along the thin filament bundle also indicated that two troponin–tropomyosin filaments run almost in register along the actin double strands. However, at the same time, the two troponin–tropomyosin filaments should form the same specific interactions with double-stranded actin molecules. Therefore, the two troponin–tropomyosin filaments should be shifted from each other by half actin size, according to the helical symmetry of actin double strands [19]. Based on these considerations, a refined model of the thin filament was constructed, showing an axial polarity for the arrangement of troponin and tropomyosin [20] (Fig. 1B). In the absence of Ca^{2+}, the contractile interaction of actin with myosin is inhibited by troponin–tropomyosin. The binding of Ca^{2+} to troponin releases the inhibition by troponin–tropomyosin and, thus, the contractile interaction is allowed.

Troponin components

Troponin is composed of three different components, troponins C, I, and T. In 1968, troponin was found to be separated into an activating factor and an inhibitory factor [9]. The activating factor is now known as the Ca^{2+}-binding component of troponin (troponin C), and the essential component of the inhibitory factor was shown to be the

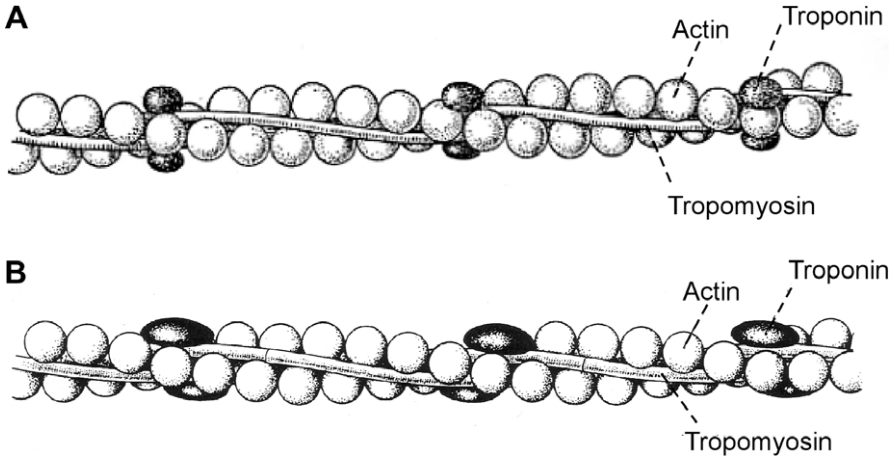

Fig. 1. Molecular arrangement of troponin, tropomyosin, and actin in the thin filament. (A) From Ebashi, Endo, and Ohtsuki [8]. (B) From Ebashi [20]. In this model of thin filament, the filament-top and the Z-line are located on the left and the right, respectively.

inhibitory component of troponin (troponin I) [10]. In addition, a third component, called troponin T, was found [11,12]. Troponin T connects the entire troponin complex to tropomyosin.

The essential regulatory processes of the troponin components are as follows. Troponin I alone inhibits or suppresses the contractile interaction between myosin and actin in the presence of tropomyosin. This action represents the inhibition or suppression of the contractile interaction by troponin–tropomyosin. The inhibition or suppression by troponin I is released Ca^{2+}-independently by the neutralizing action of troponin C in the absence of troponin T but is released Ca^{2+}-dependently in the presence of troponin T. In this respect, troponin T integrates the regulatory function of the troponin complex. It should be noticed that troponin is not a mere assembly of three protein units, but its regulatory structure and function are integrated by the networks of interactions between its three different components accompanied by their conformational changes [13].

The first indication of the molecular arrangement of the troponin components in the thin filament was obtained through immunoelectron microscopy [21]. As expected from the findings of the localization of the entire troponin complex, each of the three components of troponin is distributed with a 38-nm periodicity along the entire width of the bundle of thin filaments extending from the Z-band. However, although antibodies against troponins C and I form narrow striations in each 38-nm period, an antibody against troponin T forms a wider band in each 38-nm period. Detailed examination showed that the wide band formed by the anti-troponin T antibody is composed of a pair of striations that are separated by 13 nm.

Attempts were then made to separate the two domains of troponin T and, after several trials of proteolytic digestion, troponin T was cleaved into two subfragments with chymotrypsin [22]. The subfragment with a larger molecular weight was called troponin T_1 (TNT_1 or TnT1), and the subfragment with a smaller molecular weight was called troponin T_2 (TNT_2 or TnT2). Antibodies against troponins T_1 and T_2 from chicken skeletal muscle were raised in rabbits, and the localization of these subfragmental regions in the thin filament was examined with electron microscopy. Antibodies against troponin T_1 and troponin T_2 were found to form the striations on Z-line side and the filament top side, respectively, of the antibody striations within the wide band formed by the anti-troponin T, clearly indicating that the troponin T molecule and, consequently, troponin C·I·T-complex molecules are axially oriented along the thin filament (Fig. 2).

Troponin T from rabbit skeletal muscle is a single peptide of 259 residues, of which a high percentage are charged residues; the N-terminal region is rich in acidic residues and the C-terminal region is rich in basic residues, although charged residues are distributed along the entire sequence of the molecule [23]. Troponin T is insoluble under physiological conditions of low ionic strength and is soluble only in the presence of KCl or NaCl at concentrations higher than 0.4 M. Although the insolubility of troponin T complicated the study of its properties of interaction under physiological conditions, this problem was largely solved by the finding that troponin T can be split into two soluble subfragments, troponin T_1 and troponin T_2, by mild chymotryptic digestion [13,22]. Troponin T_1 is an N-terminal subfragment of 158 residues, whereas troponin T_2 is a C-terminal subfragment of 101 residues. Affinity chromatography has shown that troponin T_1 binds solely but strongly to tropomyosin, whereas troponin T_2 interacts with tropomyosin, troponin I, and troponin C [24,25]. The tropomyosin-binding region of troponin T_1 is located in the helix-rich region of residues 71–151 [26]. The Ca^{2+}-regulating ability of troponin T resides in the smaller C-terminal subfragment, troponin T_2. Both the Ca^{2+}-regulating and the tropomyosin-binding abilities of the troponin T_2 subfragment are greatly reduced by removing C-terminal residues, indicating that the Ca^{2+}-regulating action of troponin T_2 is closely correlated with the tropomyosin-binding ability through the C-terminal region. A fragment missing the 45 N-terminal residues of troponin T (26K fragment) is also produced by endogenous proteases [27,28]. This fragment has the same Ca^{2+}-regulating ability as intact troponin T, indicating that the N-terminal residues are not essential for the Ca^{2+}-regulating ability of this molecule [29].

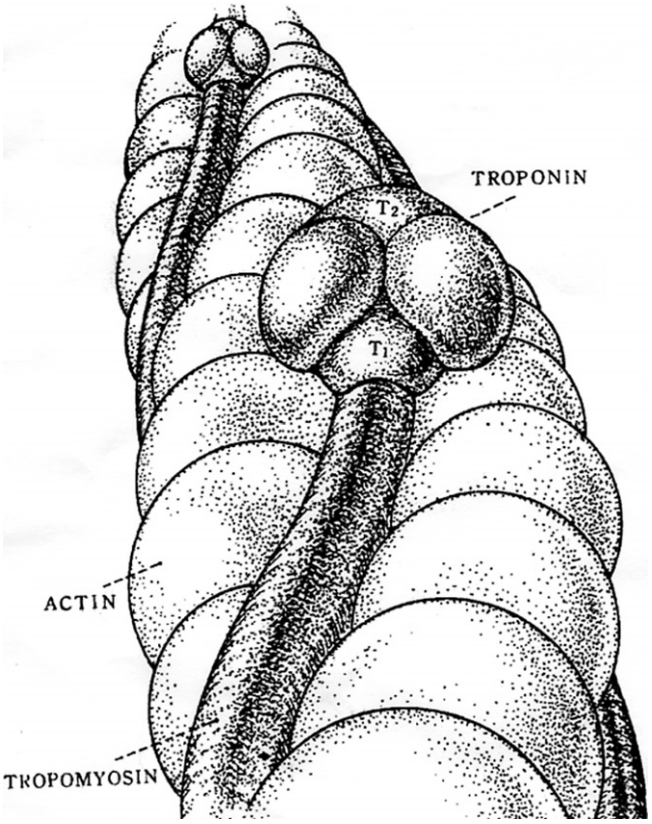

Fig. 2. Arrangement of the T_1- and T_2-regions of troponin T along the thin filament. In the figure, T_1- and T_2-regions of troponin T molecule are located at the near side (Z-line side) and far side (filament-top side), respectively. From Ohtsuki [72].

Crystallographic analyses of the core domain structure of the troponin C·I·T_2-complex demonstrated that troponin T_2 (or TnT2) forms a stable coiled-coil with troponin I and binds to the C-lobe of troponin C within the IT-arm region of the core domain [30,31]. The IT-arm region is stably fixed to tropomyosin through the two tropomyosin-binding regions of troponin T: the helix-rich region of troponin T_1 and the C-terminal region of troponin T_2. The coiled-coil in the IT-arm region might also be involved in the interaction with tropomyosin. In the absence of Ca^{2+}, the inhibitory region and its C-terminal segment of troponin I would suppress actin–tropomyosin, but only when an appropriate steric relation with actin–tropomyosin has been established through the binding of troponin T_2 region to tropomyosin. The binding of the troponin T_1 region to tropomyosin would stabilize the steric relation of the troponin complex to actin–tropomyosin, together with the binding of troponin T_2 to tropomyosin.

The inhibitory activity of troponin I represents the suppression of the contractile interaction of myosin–actin–tropomyosin by troponin in the absence of Ca^{2+}. The position of troponin I in the thin filament is generally considered to be determined through the binding of troponin T to tropomyosin. However, without troponins C and T, the inhibitory action of troponin I on actomyosin is at full strength in the presence of tropomyosin but is extremely weak in the absence of tropomyosin [13,32]. These findings raise the possibility that troponin I alone has a structural relation with tropomyosin along the actin double strands. An immunoelectron microscopic investigation has demonstrated that troponin I or troponin C·I-complex binds to tropomyosin–actin filaments with a 38-nm periodicity, even in the absence of troponin T [33], indicating that troponin I is located at a specific region of each tropomyosin molecule along the tropomyosin–actin filament. Tropomyosin, through its interaction with actin filament, would enhance the affinity of the specific actin, among the seven actins in each 38-nm period, to troponin I, although the possibility that tropomyosin directly interacts with troponin I cannot be ruled out.

Troponin is freely soluble in water immediately after being isolated from minced muscle. An aqueous solution of fresh troponin is transparent and liquid but becomes gradually viscous and occasionally turbid during storage. The Ca^{2+}-regulating ability of troponin also gradually declines during storage. Changes in the shape of troponin molecules during storage have been examined with the rotary shadowing technique [34]. Fresh troponin contains mainly globular particles with about 13×9 nm in size. Aged troponin preparations, however, contain mostly particles with smaller heads attached by thin tail (head–tail-type particle). The axial size of the head region was approximately 8 nm and the tail length was 17 nm. These findings indicate that the thin tail of the head–tail-type particle is folded along the head region in fresh troponin to form the globular troponin particle. Troponin T has been shown to be a rod-shaped particle about 17 nm long, indicating that the tail region of the particle is composed of troponin T and the head region is formed by troponins C and I. A part of troponin T_2 sequence was later shown to form a coiled-coil structure with troponin I within the core domain in troponin C·I·T_2 crystal structure [30,31], suggesting that the troponin T_2 region is partially included in the head region of the head–tail-type troponin particle. The construction of a quaternary structure prediction model of globular troponin was attempted [35]. Elucidation of the detailed structural arrangement of troponin components in the native thin filaments is important for understanding the regulatory mechanisms of troponin under physiological conditions.

Early *in vitro* studies established that the essential processes in the Ca^{2+}-regulation by troponin components are the suppression of contractile interaction between myosin and actin–tropomyosin through the inhibitory action of troponin I and the removal of this suppression by the neutralizing action of troponin C, which becomes sensitive to Ca^{2+} only in the presence of troponin T. However, subsequent studies showed that the maximum contraction level is slightly higher in the presence of the troponin C·I·T complex than in its absence. The presence of these essential processes under physiological conditions was also suggested by examining effects of the removal and reconstitution of troponin components on the Ca^{2+}-activated force generation of glycerinated muscle fibers [36]. The isometric force of the troponin C·I-depleted fibers stays at the level about 30% lower than the maximum force of the intact fibers regardless of Ca^{2+} concentrations and is suppressed by troponin I. The original Ca^{2+}-sensitive contraction recovers with subsequent addition of troponin C.

The activating action of troponin, i.e., the elevation of maximum contraction level by the troponin complex, is a major Ca^{2+}-regulatory mechanism by invertebrate scallop troponin as examined by actomyosin or myofibrillar ATPase activity [37,38] but is only a minor mechanism in vertebrates. The effects of troponin I fragments from Akazara scallop striated muscle and rabbit skeletal muscle were investigated on the Ca^{2+}-activated actomyosin ATPase activity; the fragments contained the inhibitory region and its N-terminal sequence or the inhibitory region and its C-terminal sequence [39]. The fragment of the inhibitory region with the N-terminal sequence from scallop muscle has the same activity as the entire troponin I molecule in terms of the Ca^{2+}-regulating ability of the ternary troponin complex, i.e., the Ca^{2+}-dependent activating action, whereas the fragment with the C-terminal residues does not show any Ca^{2+}-regulating ability. On the other hand, the fragment with the N-terminal sequence from rabbit skeletal muscle shows an activating effect in troponin complex, while the fragment with the C-terminal sequence has the Ca^{2+}-regulating ability of the suppression-de-suppression type in the troponin complex. These findings strongly suggest that the N-terminal region of troponin I, which forms a complex with troponin T_2 and troponin C within the core domain structure [30,31], plays an essential role in the activating action of troponin.

Troponin and genetic disorders

Genetic analyses in the last two decades have revealed that inherited cardiomyopathies, i.e., hypertrophic (HCM), dilated (DCM), and restrictive (RCM) cardiomyopathies, are associated with mutations of genes for sarcomeric (myofibrillar) and cytoskeletal proteins, including cardiac troponin components [14,15]. HCM is an autosomal dominant disorder of the heart that has characteristic symptoms of an asymmetric left ventricular hypertrophy and impaired diastolic function and is associated with a high incidence of sudden death and heart failure. Genes for β-cardiac myosin heavy chain, cardiac myosin binding protein C, and cardiac troponins T and I are the most common sites of the mutations [15]. Mutations have also been found in α-cardiac myosin heavy chain, regulatory and essential myosin light chains, cardiac actin, α-tropomyosin, cardiac troponin C, and titin/connectin. To date, 27 mutations of troponin T, 26 mutations of troponin I, and 1 mutation of troponin C have been associated with HCM.

DCM is characterized by ventricular dilation and depressed systolic function and is associated with frequent sudden death and heart failure. Initial genetic analyses demonstrated that genes for cytoskeletal proteins are involved in DCM, but subsequent studies revealed that mutations of cardiac sarcomeric proteins, such as troponins T, I, and C, actin, myosin binding protein C, α-tropomyosin, and β-myosin heavy chain, are also involved in DCM. Six mutations in troponin T, 1 mutation in troponin I, and 1 mutation in troponin C have been reported.

RCM is the least common by inherited cardiomyopathy and is characterized by impaired diastolic filling of the left ventricle, normal or near normal systolic function, and normal ventricular wall thickness. Six mutations of human cardiac troponin I have been found to be associated with RCM.

Evidence obtained by functional studies indicates that alterations of the Ca^{2+}-sensitivity of force generation of cardiac muscle fibers are the critical functional consequences of the troponin mutations leading to these three types of inherited cardiomyopathy.

Troponin T mutations in HCM and DCM

The mutations of troponin T in HCM, reported in 1994, were the first troponin mutations to be identified in inherited cardiomyopathies. Two missense mutations (Ile79Asn, Arg92Gln) and a splice donor site (Intron16G_1→A) mutation were linked to HCM [40]. Our group at Kyushu University then started investigating the functional consequences of these mutations. For our studies we used a procedure developed a few years earlier to selectively replace all three troponin components in skinned fibers [36]. The principles of the procedure are as follows. Troponin T, when added to the incubating solution in an excessive amount, is incorporated into skinned fibers, in exchange for the intrinsic troponin C·I·T-complex. This exchange results in the removal of troponins C and I from the skinned fibers, and, thus, the development of force becomes insensitive to Ca^{2+}, remaining at about 70% of the maximum level. Force development is then suppressed by the reconstitution of troponin I and the original Ca^{2+}-sensitive contraction is restored by the subsequent reconstitution of troponin C. This procedure has allowed the properties of various troponin isoforms and their recombinant proteins to be examined in skinned muscle fibers under physiological conditions.

First, mutants of two missense mutations, Ile79Asn and Arg92Gln, of human cardiac troponin T were expressed, purified, and incorporated into rabbit cardiac skinned fibers by means of the troponin exchange procedure. Both mutations were found to increase the Ca^{2+} sensitivity of isometric force generation but not to affect the maximum force or cooperativity of Ca^{2+}-activation [41]. The mutation of the splice donor site produced two truncated mutants; one mutant lacks the 14 C-terminal residues ($TnT_{\Delta 14}$) and the other lacks the 28 C-terminal residues but includes 7 novel residues ($TnT_{\Delta 28+7}$). Both truncated mutants, when incorporated into the skinned fibers, produced greater Ca^{2+}-sensitizing effects on the force generation than did the two missense mutations but decreased cooperativity slightly; the maximum force was also decreased somewhat by the $TnT_{\Delta 28+7}$ mutation but not by the $TnT_{\Delta 14}$ mutation [42]. Most mutations, such as Arg92Leu, Arg92Trp, Arg94Leu, Ala104Val, Arg130Cys, ΔGlu160, Glu163Arg, Ser179Phe, Glu244Asp, Lys273Glu, and Arg278Cys, examined thereafter in several laboratories, had a Ca^{2+}-sensitizing effect on the skinned fiber force generation but did not significantly affect the maximum force level [43–49].

In 2000, a deletion mutation (ΔLys210) of cardiac troponin T became the first mutation of sarcomeric proteins causing DCM to be reported [50]. Functional analysis revealed that the force generation by the skinned cardiac muscle, into which the ΔLys210 troponin T mutant had been incorporated, showed lower Ca^{2+}-sensitivity than that of the fibers with the wild-type troponin T, whereas other variables, such as maximum force and cooperativity, were not affected by the mutation [46]. Incorporating the mutant into isolated cardiac myofibrils also caused a decrease in the Ca^{2+}-sensitivity of myofibrillar ATPase activity. Of the 6 DCM-causing mutations of troponin T reported to date, 3 mutations (Arg141Trp, Arg205Leu, and ΔLys210) have demonstrated a Ca^{2+}-desensitizing effect on skinned fiber force development, and 5 mutations (Arg131Trp, Arg141Trp, Arg205Leu, ΔLys210, and Asp270Asn) have decreased the Ca^{2+}-sensitivity of myofibrillar or actomyosin ATPase activity [46,47,51].

The Ca^{2+}-desensitizing effects of the DCM-causing mutations contrast sharply with the Ca^{2+}-sensitizing effects of HCM-causing mutations. In this respect, both systolic and diastolic functions of the heart are affected significantly by the changes in Ca^{2+}-sensitivity, because the force level achieved by the increase in Ca^{2+}-concentration during

myocardial twitch contraction is submaximal, not exceeding half the maximum force. The increased Ca^{2+}-sensitivity would enhance systolic function and impair diastolic function, whereas the decreased Ca^{2+}-sensitivity would impair systolic function and enhance diastolic function. These changes are consistent with the difference in characteristic phenotypes between HCM and DCM: HCM is characterized by impaired diastolic function, whereas DCM is characterized by reduced systolic function. The functional studies on troponin T mutations thus indicate the importance of the difference in the changes, increase and decrease, in the Ca^{2+}-sensitivity of contraction as pathogenic causes triggering the two types of cardiomyopathy, HCM and DCM, respectively.

Human cardiac troponin T is a peptide of 288 amino acid residues, along which are distributed several interacting regions (Fig. 3). The C-terminal region, troponin T_2, in which the Ca^{2+}-regulating activity of troponin T resides, forms a stable coiled-coil structure with troponin I in IT-arm region of the core domain of the troponin C·I·T_2 crystal [30]. The IT-arm region of the core domain is fixed to tropomyosin through the two regions of troponin T: the tropomyosin-binding region in troponin T_1 and the C-terminal region of troponin T_2 [13]. Only a few HCM- and DCM-causing mutations of troponin T are located within the core domain structure of troponin C·I·T_2 The troponin T mutations causing HCM would mainly impair the interactions of troponin T with tropomyosin–actin to increase Ca^{2+}-sensitivity. Concerning the mechanisms of the Ca^{2+}-desensitizing action of the DCM-causing mutations, the affinity of troponin T to tropomyosin was shown to be enhanced by the Arg141Trp mutation but not by the ΔLys210 mutation [47]. The Arg141 residue is located in the helical tropomyosin-binding region within the troponin T_1. A possible consequence of binding of troponin T to tropomyosin is to make fully effective the inhibitory interaction of troponin I with actin–tropomyosin, in competition with troponin C in the absence of Ca^{2+} [13]. The increased affinity of troponin T for tropomyosin due to the Arg141Trp mutation would increase the inhibitory action of troponin I and, consequently, would decrease Ca^{2+}-sensitivity. On the other hand, changes in the affinity of troponin T for tropomyosin are not caused by the ΔLys210 mutation located in the helical H1(T2) segment of the N-terminal region of troponin T_2 of the core domain. Another mutation causing DCM, Arg205Leu, is also located in the H1(T2) helix, but no mutations causing HCM have been found in this helical segment. The phosphorylation of the Thr206 residue of mouse cardiac troponin T, which corresponds to the Thr203 residue of human cardiac troponin T located at the N-terminal end of the H1(T2), has been reported to decrease the Ca^{2+}-sensitivity and the maximum activation of actomyosin ATPase [52]. These mutations and the phosphorylation in the H1(T2) region might affect the interactions of this region with actin–tropomyosin, because this region is exposed on the marginal surface of the core domain structure and includes a sequence of actin binding residues of skeletal troponin T [53].

Troponin I mutations in HCM and RCM

Twenty-six mutations of human cardiac troponin I in the HCM and 6 mutations in the RCM have been found [15]. Most of these mutations are located in the inhibitory region and the C-terminal region of the troponin I. One mutation (Ala2Val) that causes DCM was reported in the N-terminal region of troponin I.

In 1997, six mutations associated with HCM were reported for cardiac troponin I (Arg145Gly, Arg145Gln, Arg162Trp, ΔLys183, Gly203Ser, and Lys206Gln) [54] (Fig. 4). Functional studies of these mutations, examining the skinned cardiac muscle force generation and myofibrillar ATPase activity, have been performed with the troponin-exchange technique [55]. These mutations have the Ca^{2+}-sensitizing actions, consistent with the results of analyses of the troponin T mutations causing HCM. Two mutations, Arg145Gly and Arg145Gln, in the inhibitory region, show almost the same Ca^{2+}-sensitizing effects with the increased minimum force but no significant changes in

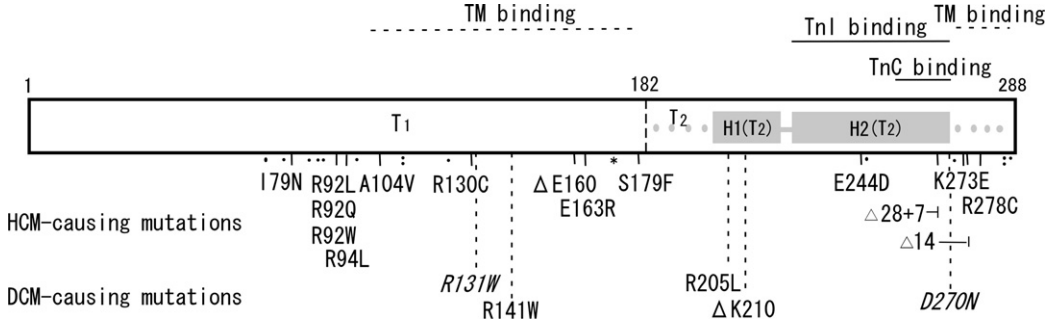

Fig. 3. Distribution of HCM- and DCM-causing mutations along the amino acid sequence of human cardiac troponin T. In this figure, 15 HCM-causing mutations and 5 DCM-causing mutations, whose effects were examined in skinned fiber force generation and myofibrillar or actomyosin ATPase activity, are indicated in the figure. Two DCM-causing mutations (*R131W, D270N in italic letters*) were analyzed only by means of actomyosin ATPase assay. The positions of HCM-causing mutations (●) and DCM-causing mutations (∗), whose functional effects have not been reported, are also indicated in the figure. Two helical regions, H1(T2) (residues 203–222) and H2(T2) (residues 225–271) of troponin T_2 in the IT-arm region of the core domain structure, are indicated [30]. The H2(T2) forms a coiled-coil with H2(I) of troponin I. *Abbreviations:* TM, tropomyosin; TnI, troponin I; TnC, troponin C.

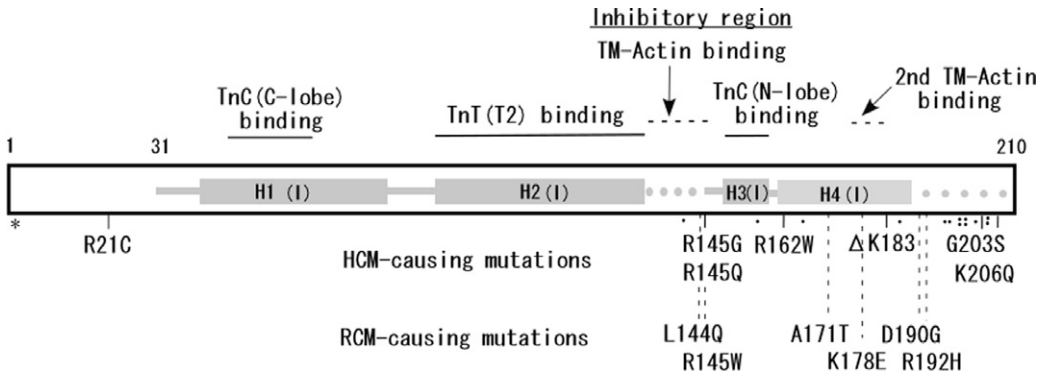

Fig. 4. Distribution of HCM- and RCM-causing mutations along the amino acid sequence of human cardiac troponin I. Seven HCM-causing mutations and 6 RCM-causing mutations, whose effects were examined in skinned fiber force generation and assays of myofibrillar ATPase activity, are indicated in the lower part of the figure. The positions of HCM (●)- and DCM (*)-mutations, whose functional effects have not been reported, are also indicated in the figure. Four helical regions (H1(I), residues 42–80: H2(I), residues 90–136: H3(I), residues150–159: H4(I), and residues 161–189) are indicated based on the core domain structure of troponin CIT_2 [30]. The H2(I) forms a coiled-coil with the H2(T2) of troponin T. The H3(I) is homologous to the part of the second troponin C binding region (signal segment) of skeletal troponin I, and the H4(I) includes the region of residues 176–184 that is homologous to the second actin–tropomyosin binding region of skeletal troponin I [73]. *Abbreviations:* TM, tropomyosin; TnC, troponin C; TnT, troponin T.

the maximum force. Similar effects are produced by the Arg162Trp mutation at the N-terminal end of the H4(I) helix. The ΔLys183 deletion mutation in the H4(I) helix has the Ca^{2+}-sensitizing effect but does not change either the maximum or minimum force. Two mutations, Gly203-Ser and Lys206Gln, in the C-terminal end region of the molecule have small Ca^{2+}-sensitizing effects on the skinned fiber force generation. In addition, an Arg21Cys mutation that causes HCM leads to Ca^{2+}-sensitization of skinned fiber force development without significantly affecting the maximum activation [56].

In 2003, mutations of human cardiac troponin I were found to be associated with RCM, which is the least common type of inherited cardiomyopathy [57]. These mutations cause Ca^{2+}-sensitizing effects on the skinned fiber force generation and actomyosin ATPase activity [58] (Fig. 4). The relative strengths of Ca^{2+}-sensitization by RCM-causing mutations are as follows: Lys178Glu > Arg192His = Arg145Trp > Leu144Gln = Ala171Thr > Asp190Gly. The Ca^{2+}-sensitizing effects by the mutations causing RCM are greater than those by the troponin I mutations causing HCM. The minimum force at low Ca^{2+}-concentrations is slightly but clearly elevated by all 6 RCM-causing mutations. The maximum level of force generation is decreased by four RCM-causing mutations (Leu144Gln, Arg145Trp, Ala171Thr, and Arg192His). The Ca^{2+}-activation profiles are, therefore, affected to a greater extent by the RCM-causing mutations than by the HCM-causing mutations. Similar Ca^{2+}-sensitizing effects of five RCM-causing mutations of troponin I (except Asp190Gly) have also been reported [59]. Examinations of troponin I fragments ($TnI_{129–210}$) have shown that the Lys178Glu mutation decreases the inhibitory activity on actin–tropomyosin without changing the maximum inhibition level and decreases the EC_{50} of the neutralizing action of troponin C [58]. The Asp190Gly mutation, which shows the smallest Ca^{2+}-sensitizing effect among the six RCM-causing mutations, is reported to cause both RCM and HCM in a single family [57], suggesting that the RCM and the HCM are caused by similar mechanisms. The Ca^{2+}-affinity of reconstituted thin filaments is increased by both HCM-causing mutation (Arg145Gly) and RCM-causing mutations (Arg145Trp, Asp190Gly, and Arg192His) [60].

Troponin C mutations in HCM and DCM

Only two mutations of troponin C associated with cardiomyopathy are reported. One mutation is located in the non-functional site I of four EF-hand motifs of the molecule and is associated with HCM (Leu29Gln) [61]. A second reported mutation (Gly159Asp) causing DCM does not significantly affect the Ca^{2+}-sensitivity of the skinned fiber force generation [62] but decreases the Ca^{2+} sensitivity of acto-S1 ATPase activity [51]. This mutation slows the rate of force development [62] and blunts the Ca^{2+}-desensitizing effect of the phosphorylation of troponin I by A-kinase [63,64].

Knock-in mouse model of DCM generated by troponin T mutation

The development of animal models is the critically important step to explore the pathogenesis of inherited cardiomyopathies caused by troponin mutations. Recently, we created knock-in mice in which three base-pairs coding for the amino acid residue Lys210 in cardiac troponin T were deleted from their endogenous cardiac troponin T gene (*TNNT2*) using gene-targeting technology [65]. The knock-in mice developed enlarged hearts and heart failure, and showed a high incidence of premature sudden death, closely recapitulating the phenotypes of human DCM [50,66,67]. Consistent with the previous study showing that the mutation of troponin T has a Ca^{2+}-desensitizing effect on the myofilament *in vitro* [46], skinned cardiac muscle

fibers prepared from mutant mice showed a decrease in Ca^{2+}-sensitivity of force generation, being larger in homozygotes (*TNNT2*$^{\Delta K210/\Delta K210}$) than in heterozygotes (*TNNT2*$^{+/\Delta K210}$). Although the decreased Ca^{2+}-sensitivity of cardiac myofilament would be expected to lead to some reduction in the maximum force-generating capability of cardiac muscle, intact cardiac muscle fibers from mutant mice showed no significant decrease in maximum isometric force per cross-sectional area. Analyses using a Ca^{2+}-indicator, fura-2, revealed a significant increase in the peak amplitude of Ca^{2+}-transient in cardiomyocytes of mutant mice, suggesting that Ca^{2+}-transient is augmented to compensate for decreased myofilament Ca^{2+} sensitivity. Significant increases in the phosphorylation of ryanodine receptor-2 and phospholamben catalyzed by cAMP-dependent protein kinase suggest that an increase in the intracellular cAMP level might be responsible for the augmented Ca^{2+}-transient in mutant mice.

Despite preserved maximum isometric force-generating capability in intact cardiac muscle, echocardiography demonstrated that left ventricular ejection fraction was significantly reduced in mutant mice, with *TNNT2*$^{\Delta K210/\Delta K210}$ being more severely affected than *TNNT2*$^{+/\Delta K210}$. *Ex vivo* analyses of isolated work-performing heart preparations also showed that cardiac pump function was impaired in mutant mice. Fractional sarcomere shortening and peak velocity of sarcomere shortening of isolated cardiomyocytes were also significantly decreased in *TNNT2*$^{\Delta K210/\Delta K210}$ mice, indicating that a defect of dynamic contractile performance was still present in cardiomyocytes of these mutant mice. Consistent with the reduced dynamic contractile performance of cardiomyocytes, the expression of β-cardiac myosin heavy chain isoform in ventricular myocardium, known to produce much lower power output due to its much slower velocity of actin filament sliding and lower actin-activated ATPase activity compared with α-cardiac myosin heavy chain isoform [68,69], was found to be markedly increased in the mutant mice. Our hypothesis is that reduced power output of myocardium due to decreased myofilament Ca^{2+}-sensitivity leads to an augmentation of Ca^{2+}-transient and cardiac enlargement as compensatory responses, but an increase in intracellular Ca^{2+} will increase energy consumption through activating Ca^{2+}-dependent metabolic processes such as the Ca^{2+}-uptake of sarcoplasmic reticulum and cellular hypertrophic response, which in turn would induce the expression of "low power" β-cardiac myosin heavy chain isoform to save energy consumption. Therefore, the myocardium should repeat these compensatory responses involving the augmentation of Ca^{2+}-transient and cardiac enlargement to offset the reduced power output of myocardium until it decompensates and leads to heart failure and sudden cardiac death.

Mutant mice showed high mortality in a mutant-gene dosage-dependent manner. *TNNT2*$^{\Delta K210/\Delta K210}$ mice showed a particularly high incidence of sudden death in their growth periods from 1 to 3 months old. Instantaneous death was frequently observed by mild chest compression, suggesting that their hearts are very susceptible to fatal arrhythmias due to mechanical stimuli. Surface electrocardiography (ECG) showed that *TNNT2*$^{\Delta K210/\Delta K210}$ mice commonly had electrophysiological abnormality in the heart with long QT, which might be involved in their frequent sudden death. Telemetric ECG recordings showed that mice die by abruptly developing repetitive Torsade de Pointes from several hours before death, which ultimately degenerated into ventricular fibrillation. Preliminary experiments suggest that cardiomyocytes of mutant mice have a longer action potential duration and greater tendency to develop early afterdepolarization than wild-type mice, which might be responsible for their frequent sudden death. Further studies are required to clarify the molecular mechanisms of sudden death, probably involving electrophysiological abnormality of the heart.

Administration of pimobendan, a Ca^{2+}-sensitizer, known to increase the Ca^{2+}-sensitivity of myofilament, was found to reduce the heart size of mutant mice and markedly improve the life span of *TNNT2*$^{\Delta K210/\Delta K210}$ mice, suggesting that Ca^{2+}-sensitizers, such as pimobendan, might be beneficial for the treatment of DCM patients affected by mutations of sarcomeric regulatory proteins, troponin, and α-tropomyosin, which have been demonstrated to have a common functional consequence of decreasing myofilament Ca^{2+}-sensitivity [46,48,51,70].

Concluding remarks

Troponin consists of three different components: troponins C, I, and T. Each component has a specific function, such as the Ca^{2+}-binding of troponin C, the inhibitory activity of troponin I, and the tropomyosin-binding of troponin T. In this review article, several aspects of Ca^{2+}-regulatory mechanisms involving troponin were discussed with particular reference to the regulatory and structural roles of troponin T [13]. Troponin T, which has the greatest molecular weight of the three components, is a highly charged molecule that is split by chymotrypsin into two subfragments, troponin T_1 (N-terminal subfragment) and troponin T_2 (C-terminal subfragment). The Ca^{2+}-regulating action of troponin T resides in the C-terminal troponin T_2 region that interacts with tropomyosin, troponin I, and troponin C, whereas the N-terminal troponin T_1 region binds strongly to tropomyosin. The two regions of troponin T are axially oriented along the thin filament: T_1 and T_2 regions are on the Z-line and the filament-top sides, respectively. Troponin T (or the troponin complex) forms a complex with tropomyosin by highly ionic interactions and, hence, is dissociated from tropomyosin as the ionic strength of the solution increases.

Within the myofibrillar lattice, troponin T forms a stable complex with troponins I and C and binds to a specific region of tropomyosin in the thin filament. When excess troponin T is added to the solution bathing the skinned muscle fibers, it binds to tropomyosin in the thin filament

of myofibrils of skinned fibers by expelling intrinsic troponin C·I·T-complex according to the law of mass action [36]. As a result, the contraction of skinned fibers becomes desensitized to Ca^{2+} through the removal of troponins C and I. The contraction of troponin C·I-depleted fibers is suppressed by the addition of troponin I and the original Ca^{2+}-sensitive contraction is regained by the subsequent addition of troponin C. When the ternary complex of troponin, instead of troponin T, is added to the solution, the added troponin complex also replaces the intrinsic troponin complex within the myofibrillar matrix [71]. This exchangeable property of the binding of troponin T to tropomyosin has been used to introduce troponin isoforms and recombinant mutants into skinned fibers and to examine their properties under physiological conditions.

Through the troponin exchange procedure, remarkable progress has been made in understanding the functional consequences of troponin mutations associated with inherited cardiomyopathies. Functional analyses have revealed that two distinct changes in the Ca^{2+}-activation profiles of skinned fiber contraction are caused by the troponin mutations associated with HCM, DCM, and RCM. The Ca^{2+}-sensitivity of contraction is increased by the mutations of troponins T and I causing HCM and is increased to an even greater extent by the mutations of troponin I causing RCM. In contrast, the Ca^{2+}-sensitivity is decreased by the mutations of troponin T causing DCM. These findings strongly suggest that the modulation of Ca^{2+}-sensitivity of contraction is the crucial functional consequence leading to these cardiomyopathies.

Recently, the critical importance of the decrease in Ca^{2+}-sensitivity of contraction by troponin mutations was indicated in a knock-in mouse model of DCM caused by a deletion mutation of troponin T (ΔLys210-mutation) [65]. Mutant mice show the characteristic phenotype of human DCM such as cardiac enlargement, bi-ventricular dilation, systolic dysfunction, heart failure, and frequent sudden cardiac death. These phenotypes in mutant mice can be interpreted as the compensatory responses to the decreased Ca^{2+}-sensitivity of the contractile system caused by the ΔLys210-mutation of troponin T. The development of DCM-phenotype in mutant mice can be virtually prevented by a Ca^{2+}-sensitizing agent. This preventive effect emphasizes the importance of the decreased Ca^{2+}-sensitivity of contraction as a primary cause of the DCM due to the troponin T mutation.

Fig. 5 shows a schematic representation of the structure of human cardiac troponin based on the crystallographic

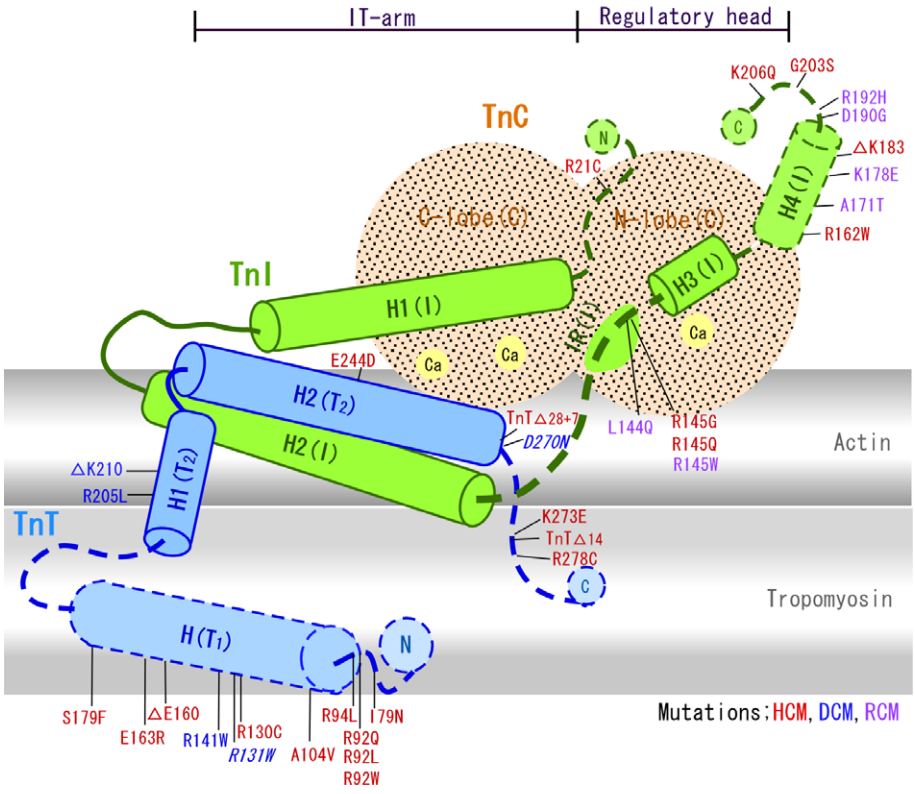

Fig. 5. Schematic representation of the distribution of mutations causing HCM, DCM, and RCM in troponins T and I of human cardiac troponin. The distribution of the mutations of troponins T and I shown in Figs. 3 and 4 are illustrated in the structure of the Ca^{2+}-bound ternary troponin complex based on the core domain structure of the human cardiac troponin C·I·T_2 complex [30]. The core domain is composed of two regions: the IT-arm and the regulatory head. The IT-arm region is the structural core of troponin and is composed of the C-domains of troponin C, troponin I, and troponin T_2. The regulatory head consists of the N-domain of Ca^{2+}-bound troponin C and the H3(I) helical region of troponin I. The regions of troponin components outside the core domain, which were not determined with crystal structure analysis, are indicated by broken lines. See the legends to Figs. 3 and 4 for other details.

analysis of the core domain formed by troponin C·I·T_2 [30]. The core domain of the troponin C·I·T_2 complex consists of an IT-arm region and a regulatory-head region. The IT-arm region is the structural core of the troponin complex and is fixed to tropomyosin through the two regions of troponin T: tropomyosin-binding region in troponin T_1 and C-terminal region of troponin T_2. The regulatory head consisting of the Ca^{2+}-bound N-lobe of troponin C and H3(I) helix of troponin I contains the Ca^{2+}-receptive site for regulating contraction. The inhibitory region and its C-terminal side region of troponin I are involved in the Ca^{2+}-regulatory mechanism through interacting with actin–tropomyosin in the absence of Ca^{2+}. Mutations of troponin T causing HCM or DCM are distributed in the troponin T_1 and T_2 regions, whereas troponin I mutations causing HCM or RCM are concentrated in the inhibitory region and its C-terminal side sequence. Most mutations of troponins T and I are located outside the core domain, and, hence, would cause changes in the Ca^{2+}-sensitivity of contraction through affecting the interactions with other thin filament proteins, tropomyosin and actin, rather than affecting the interactions within the troponin complex. Intricate processes, with which a large number of mutations converge, should exist to exert the Ca^{2+}-sensitizing or Ca^{2+}-desensitizing effects. Further detailed investigations will contribute to a more complete understanding of the physiologically important mechanisms of the regulation of muscle contraction underlying inherited cardiomyopathies.

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