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Hypertrophic cardiomyopathy-causing Asp175asn and Glu180gly *Tpm1* mutations shift tropomyosin strands further towards the open position during the ATPase cycle

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ABSTRACT

To understand the molecular mechanism by which the hypertrophic cardiomyopathy-causing Asp175Asn and Glu180Gly mutations in α-tropomyosin alter contractile regulation, we labeled recombinant wild type and mutant α-tropomyosins with 5-iodoacetamide-fluorescein and incorporated them into the ghost muscle fibers. The orientation and mobility of the probe were studied by polarized fluorimetry at different stages of the ATPase cycle. Multistep alterations in the position and mobility of wild type tropomyosin on the thin filaments during the ATP cycle were observed. Both mutations were found to shift tropomyosin strands further towards the open position and to change the affinity of tropomyosin for actin, with the effect of the Glu180Gly mutation being greater than Asp175Asn, showing an increase in the binding strong cross-bridges to actin during the ATPase cycle. These structural changes to the thin filament are likely to underlie the observed increased Ca^{2+}-sensitivity caused by these mutations which initiates the disease remodeling.

1. Introduction

Hypertrophic cardiomyopathy (HCM) is one of the most frequently occurring inherited cardiac disorders. HCM produces ventricular wall thickening with decreases in ventricular chamber volumes; systolic function is increased, while diastolic function is impaired and this is thought to be responsible for symptoms of heart failure and premature sudden cardiac death of HCM patients [1]. Eleven missense mutations in the *TPM1* gene which encodes α-tropomyosin have been shown to cause HCM (Cardiogenomics: http://cardiogenomics.med.harvard.edu/). Biochemical work aimed at investigating the effects on contractile regulation has mainly focused on the Asp175Asn and Glu180Gly mutations and has shown that these mutations increase myofilament Ca^{2+} sensitivity and elevate thin filament Ca^{2+} affinity [2,3], as well as causing changes to TM structure [4]. Mouse model of these mutations show decreases in the rates of cardiac contraction and relaxation, along with increases in cardiac myofilament Ca^{2+} sensitivity [5]. Asp175 and Glu180 are located in the region that interacts with troponin [6]. It has been postulated that these mutations affect the TM–TnT interaction [7] and induce alterations in the signaling of TnC and TnI [8] that could be responsible for TM movement allowing strong cross-bridge binding [9].

Here we present the first study to examine the effect of HCM *TPM1* mutations on the position of TM at different points of the ATPase cycle using polarized fluorimetry, a technique we have previously used to study DCM *TPM1* mutations [10]. We demonstrate that in reconstituted actin-tropomyosin, the Asp175Asn and Glu180Gly mutations intensify the myosin-induced shift of the TM strands towards the open position and make the binding of the region of Cys-190 to actin stronger during the ATPase cycle; these changes, fundamentally different from those we detected in DCM *TPM1* mutants [10], are likely to provide the structural basis for the altered switching in HCM.

2. Materials and methods

2.1. Preparation and labeling of proteins

Recombinant wild-type and HCM mutant human α-tropomyosin were overexpressed in BL21(DE3)pLysS *Escherichia coli* and subsequently purified as previously described [11]. Each recombinant α-tropomyosin was produced with a Met-Ala-Ser N-terminal tag, which is post-translationally processed to Ala-Ser; this acts as a mimic of native N-terminal acetylation of tropomyosin [12].

Abbreviations: HCM, hypertrophic cardiomyopathy; S1, myosin subfragment 1; 5-IAF, 5-iodoacetamide fluorescein; TM, tropomyosin; TN, troponin.

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Myosin subfragment-1 (S1) was prepared by treatment of skeletal muscle myosin with α-chymotrypsin for 10 min at 25 °C [13]. Purity of the protein preparations, as well as the composition of the fibers after washing out of the unbound proteins, was monitored by SDS-PAGE. Protein concentrations were determined by measuring UV absorbance.

2.2. Preparation and labeling of ghost fibers

Ghost fibers were prepared from single glycerinated fibers of rabbit psoas muscle by extraction of myosin and the regulatory proteins as described previously [13]. The resultant ghost fibers were composed of more than 80% actin. S1 and tropomyosin were incorporated into thin filaments by incubation of the single fibers in a solution containing 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10 mM Tris–HCl, pH 6.8 (buffer A), and 1.0–2.5 mg/ml proteins. Labeling of tropomyosin with 5-iodoacetamide fluorescein (5-IAF) at Cys190 was performed as previously described giving probe to protein ratio 0.8:1 [10]. The molar ratio of tropomyosin to actin was 1:6.5 (±2).

2.3. Fluorescence polarization measurement

Steady-state fluorescence polarization measurements on single ghost muscle fibers were made using a photometer [10]. The polarized fluorescence from 5-IAF-labeled TM was recorded at 500–600 nm after excitation at 437 ± 5 nm. Four components of polarized fluorescence were measured when the fiber was oriented parallel ($_{||}I_{||}$, $_{||}I_{\perp}$) and perpendicular ($_{\perp}I_{\perp}$, $_{\perp}I_{||}$) to the polarization plane of the exciting light. Fluorescence polarization ratios were defined as: $P_{||} = (_{||}I_{||} - _{||}I_{\perp})/(_{||}I_{||} + _{||}I_{\perp})$, $P_{\perp} = (_{\perp}I_{\perp} - _{\perp}I_{||})/(_{\perp}I_{\perp} + _{\perp}I_{||})$. The measurements were carried out in the buffer A in the absence or presence of 2.5 mM ADP, 5 mM AMP–PNP, 5 mM ATPγS or 3 mM ATP [14,15,25]. To estimate the changes in probe orientation we used the helix plus isotropic model [16]. The polarized fluorescence of the fiber was described by the assumptions that in a fiber there were fluorescent probes oriented either disorderly (fraction N) or orderly (fraction $1-N$). The absorption and emission of light is accomplished by linear, completely anisotropic dipoles of absorption (A) and emission (E). The axes of dipoles of the ordered probes are arranged in a spiral along the surface of the cone, the axis of which coincides with the long axis of the thin filament. The dipoles of absorption and emission form the angles Φ_A and Φ_E, respectively, at the top of the cone. In terms of this model, the changes in Φ_A, Φ_E, and N are considered as a reflection of the changes in orientation and mobility of the probe [17]. Since in all experiments the values of Φ_A were very close to that of Φ_E, only Φ_E and N values are described. The statistical reliability of the changes was evaluated using Student's t-test.

3. Results and discussion

In order to study the myosin-induced movement of wild-type and HCM mutant α-tropomyosins along thin filaments during the ATPase cycle we used our established model system of thin filaments reconstituted in the ghost fibers from F-actin, S1 and 5-IAF labeled tropomyosin, with steps of ATP hydrolysis mimicked either by the presence of MgADP, MgAMP–PNP, MgATPγS or MgATP or by the absence of the nucleotides [10,17]. In accordance with previous work, the effect of the labeled tropomyosin on the acto-S1 ATPase activity was found to be similar to that of the unlabeled tropomyosin, indicating that the labeling does not greatly affect the structure and function of tropomyosin [17]. Consistent with our previously published data [10,17], the incorporation of 5-IAF-labeled recombinant Ala-Ser-α-tropomyosin into the ghost fibers induced the emergence of anisotropy in polarized fluorescence. The $P_{||}$ values were uniformly lower than $P_{\perp}$, thus showing that the dipoles of 5-IAF were predominantly oriented perpendicular to the fiber axis (Table 1). The Asp175Asn and Glu180Gly mutations decreased the difference between $P_{||}$ and $P_{\perp}$ (Table 1), indicating that the conformational states of the wild type and the mutant TMs are different. The angle of emission dipole of the probe Φ_E was found to be close to 54° (Fig. 1A), and the proportion of disorderly oriented probes, N, did not exceed 0.15 (Fig. 1B). This indicates a well-ordered orientation of 5-IAF-labeled TM on the thin filament and rigid binding of the probe to tropomyosin in the ghost fibers [10,17].

Tropomyosin is a coiled–coil protein that assembles end-to-end to form continuous strands which run along the entire length of F-actin and have the ability to shift azimuthally around actin filaments [18]. The bound probe will also undergo azimuthal movements in concert with the protein [17]. The movement of the probe towards the center of the thin filament (an increase of Φ_E) can be interpreted as a consequence of the shift of TM to the open position [18]. Similarly, the movement of the probe towards the periphery of the thin filament (a decrease in the angle Φ_E) (Fig. 1A) reflects the shift of TM strands towards blocked position [10,17]. According to Fig. 1A, in the absence of S1, the Asp175Asn and Glu180Gly mutations evoke a decrease in Φ_E by 0.3° and 0.8°, respectively, compared to the wild type ($p < 0.05$). Thus both mutations shift TM strands towards the periphery of the thin filament (to the blocked position).

According to Fig. 1, the shift to the blocked position was accompanied by changes in the mobility of the C-terminus of TMs located close to the region of Cys190. For both TM mutations, the value of N decreased on average by 20% (Fig. 1B). Since N is a measure of the proportion of disorderly arranged probes bound to Cys-190, a decrease in this value indicates an increase in the affinity of the Cys-190 region of TM to actin [10,17]. It is known that the Asp175Asn and Glu180Gly mutations are charged-to-neutral amino acid substitutions in the TnT–TM binding region [6], destabilize

Table 1
The effect of the HCM mutations on the polarization ratios of 5-IAF-labeled tropomyosin in the absence and in the presence of nucleotides and S1.

S1	Nucleotide	Wild-type			Asp175Asn			Glu180Gly		
		n	$P_{||}$ ± SEM	$P_{\perp}$ ± SEM	n	$P_{||}$ ± SEM	$P_{\perp}$ ± SEM	n	$P_{||}$ ± SEM	$P_{\perp}$ ± SEM
–	–	9	0.074 ± 0.001	0.182 ± 0.002	10	0.080 ± 0.001	0.149 ± 0.002	11	0.093 ± 0.001	0.133 ± 0.002
+	–	7	0.064 ± 0.002	0.174 ± 0.002	10	0.050 ± 0.002	0.156 ± 0.002	11	0.053 ± 0.002	0.157 ± 0.002
+	ADP	7	0.075 ± 0.003	0.175 ± 0.001	9	0.054 ± 0.003	0.147 ± 0.001	10	0.060 ± 0.003	0.145 ± 0.001
+	AMP–PNP	7	0.075 ± 0.003	0.158 ± 0.002	9	0.063 ± 0.003	0.142 ± 0.001	10	0.119 ± 0.003	0.163 ± 0.001
+	ATPγS	6	0.092 ± 0.003	0.158 ± 0.002	9	0.091 ± 0.003	0.137 ± 0.002	9	0.124 ± 0.003	0.145 ± 0.002
+	ATP	6	0.143 ± 0.003	0.183 ± 0.002	7	0.128 ± 0.003	0.158 ± 0.002	7	0.158 ± 0.003	0.143 ± 0.002

$P_{||}$ and $P_{\perp}$ were calculated as described in Section 2. n is the number of fibers used in the experiments. S1 and the nucleotides have a pronounced effect on the values of $P_{||}$ and $P_{\perp}$, indicating the changes in the conformational state of TM in the ghost fibers ($p < 0.05$).

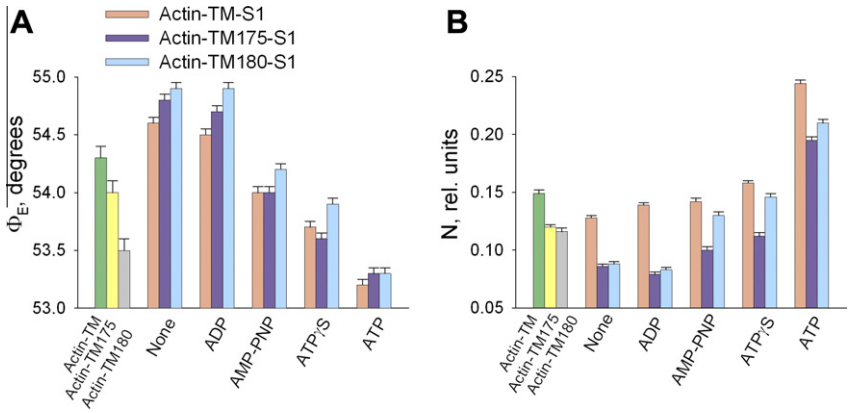

Fig. 1. The values of the angle Φ_E (A) and N (B) of the polarized fluorescence from 5-IAF bound to Cys190 of the wild-type, Asp175Asn and Glu180Gly mutant TMs revealed in ghost fibers at mimicking various stages of the ATPase cycle. Φ_E is the angle between the emission dipole of the probe and the thin filament axis; N is the number of disorderly oriented probes. Calculations of the values of Φ_E and N, the preparation of the fibers, their composition, and the conditions of the experiments are described in Section 2. The bars in each group represent (from the left to the right) the data for ghost fibers containing the wild-type, Asp175Asn and Glu180Gly mutant TMs, respectively. Φ_E and N values are significantly altered by the nucleotides ($p < 0.05$). Error bars indicate ± SEM.

the coiled–coil structure and increase TM flexibility in the regions surrounding the mutations [4]. As the increase in TM flexibility allows surface residues to assume an optimal conformation for binding to F-actin [19], the decrease in the values of N indicate that the mutations make the binding of the region of Cys-190 to actin stronger. As the strong-binding TM to actin can induce a positive allosteric effect of TM on actin [20], it is possible that this underlies the less effective inhibition of the ATPase activity by the Glu180Gly mutant TM [21]. A positive allosteric effect of HCM mutant TMs on actin was confirmed by experiments with S1 (Fig. 1).

The binding of S1 to F-actin-TM increased the value of Φ_E by 0.3° ($p < 0.05$) for the wild type TM (Fig. 1A), showing that S1 shifts the wild type TM strands towards the open position. This conclusion agrees well with the data on the shift of native [18] and the wild type TMs [17] towards the open position initiated by S1. For the Asp175Asn and Glu180Gly mutant TMs the value of Φ_E rises by 0.8° and 1.4° ($p < 0.05$), respectively, indicating that both mutations enhanced the myosin-induced movements of TM strands towards the open position, which could allow for an increase in the strong cross-bridge binding.

The value of N for the wild type TM decreases in the presence of S1 from 0.149 to 0.128 ($p < 0.05$) by 15% (Fig. 1B). Because the cross-bridge binding to actin makes the TM binding to actin stronger [22], it is possible that a decrease in the values of N shows the increased affinity of the region of Cys-190 to actin for the wild type TM [17]. Both mutations enhance this effect. The values of N decrease on average by 30% (Fig. 1B). As the values of N are smaller for both mutant TMs in comparison with wild type TM, it is possible to suggest that the Asp175Asn and Glu180Gly mutations strengthen the binding of the region of Cys-190 to actin, resulting from an increase in the stereospecific matching between actin and myosin molecules [17]. Thus, HCM mutations in TM most likely increase the stereospecific and hydrophobic interaction between actin and myosin molecules and thus enhance a positive allosteric effect of TM on actin.

To study the myosin-induced movement of TMs during the ATPase cycle we mimicked the steps of the ATP hydrolysis cycle in the absence of nucleotides and in the presence of MgATP, MgANP–PNP, MgATPγS or MgADP. In the absence of nucleotides and in the presence of MgADP and MgANP–PNP, the strong-binding states of the actomyosin complex AM, AM^·ADP and AM*·ADP, respectively, are mimicked. MgATPγS and MgATP were used for mimicking the weak-binding states, AM*·ATP and AM**·ADP·Pi [14,15]. Our data indicate that S1 shifts the position of the wild type and the Asp175Asn and Glu180Gly mutant TM strands on the surface of the thin filaments and changes the affinity of the region near Cys190 for actin in a nucleotide-dependent manner. According to Fig. 1, the transformation from AM**·ADP·Pi to AM state produced a multistep increase in Φ_E and a decrease in the values of N, showing the progressive shifting of TM strands towards the open position [18] and made the binding of the region near the Cys-190 region to actin stronger when S1 varied from weak to progressively stronger binding states.

At the transition from the AM**·ADP·Pi to the AM state the values of Φ_E increase from 53.2° to 54.6° for the wild type, for the Asp175Asn and the Glu180Gly mutant TMs from 53.3° to 54.8° and from 53.3° to 54.9°, respectively (Fig. 1A). As the increase in the Φ_E angle correlates with the movement of TM strands to the centre of the thin filament [17], our data indicate that this transition shifts the wild type and both mutant TMs towards the open position and the Asp175Asn and Glu180Gly mutation enhance this effect. Maximal and minimal effect of both mutant TMs was observed at mimicking AM^·ADP and AM*·ATP states, respectively (Fig. 1A). The changes in the values of Φ_E at transition from the AM**·ADP·Pi to the AM state for the wild type, Asp175Asn and Glu180Gly mutant TMs were 1.4°, 1.5° and 1.6°, respectively, showing small increases in the amplitude of the movement of TM strands by 7% and 14% for the Asp175Asn and Glu180Gly, respectively, which can indicate a small increase in the efficiency of the work of the cross-bridges.

It should be noted that the values of Φ_E for the Glu180Gly TM at mimicking AM^·ADP, AM*·ADP, AM**·ADP·Pi and AM*·ADP states were close to those for the wild type TM in the absence of nucleotides and in the presence of MgADP, MgANP–PNP and MgATPγS, respectively. This suggests that the mutations shift TM to the position typical for stronger binding states at mimicking all intermediate states of the ATPase cycle. A smaller effect was observed for the Asp175Asn (Fig. 1A). When mimicking intermediate states of myosin using nucleotides and ATP analogs, each state is most probably not uniform but consists of several different sub-states (see, for example [23]) and it is possible that TM-induced changes in actomyosin conformational state do not imply the formation of new myosin head sub-states. Instead, the mutant TMs can alter relative changes in the populations of sub-states upon the transition from one mimicked state to another [17]. The maximal increase in the proportion of strong-binding sub-states in the S1 population can be expected at mimicking the AM^·ADP state for the Glu180Gly mutant TM (Fig. 1A). It is interesting to note that even in the presence of MgATPγS the Glu180Gly mutant mutation shifts the

position of TM strands towards the filament center, showing the formation of the stronger binding states. Thus, both HCM mutations can increase the fraction of the strong-binding sub-states in the S1 population at mimicking each of the intermediated states of the ATPase cycle (Fig. 1A). It means that HCM Asp175Asn and Glu180Gly mutations disturb the balance between the strong and weak binding states in the population of actomyosin at different stages of the ATPase cycle, indicating that these mutations in TM can disorganize the work of the cross-bridges at muscle contraction. A similar increase in the strong-binding states of the cross-bridge population in the muscle fibers induced by the Glu180Gly mutation in TM was recently postulated in the presence of TN [24]. It is possible that disorganized work of the cross-bridges is one of the reasons for a decrease in the rates of contraction and relaxation when assessed by in vivo work-performing cardiac analyses, although these parameters may also be affected by secondary changes and subsequent remodeling (for a review, see [25]).

It is known that TM alone increases actomyosin ATPase activity by promoting the myosin-induced movement of TM to the open position [18]; this enhances the stereospecific and hydrophobic interaction between actin and myosin molecules, and in this way increases the ability of actin to activate myosin ATPase (a positive allosteric effect of TM on actin [20]). In the presence TN and at low Ca^{2+}, the positive allosteric effect of TM can enhance actomyosin ATPase activity. Therefore, the increase in the shifting of the mutant TM strands to the open position during the ATPase cycle (Fig. 1A) can be one of the reasons for the increase in the Ca^{2+} sensitivity of ATPase activity that was observed for HCM mutant TMs in the presence of TN [26]. The difference in the movement pattern revealed for two mutant TMs (Fig. 1A) implied that they would also differ in their ability to activate the actin-activated ATPase activity of myosin heads. Indeed, the Ca^{2+} sensitivity of the ATPase activity of S1 was more strongly activated by the Glu180Gly than by the Asp175Asn TM [26].

Our data suggest that the affinity of the region of Cys-190 to actin throughout the ATPase cycle is increased for both HCM mutant TMs compared to the wild type (Fig. 1B). In particular, at the mimicked AM and AM^·ADP states the value of *N* decreases on average by 30–35% for both mutation. In contrast, the changes in this parameter at mimicked AM^{**}·ADP·Pi and AM^{*}·ATP states are smaller for the Glu180Gly mutation than for the Asp175Asn (Fig. 1B). It means that the Asp175Asn and Glu180Gly mutation have different effects on TM-actin interaction during the ATPase cycle. The Asp175Asn mutation in TM had a weaker effect on the strong-binding TM strands to actin than the Glu180Gly mutation in TM (Fig. 1B). Since the strong-binding of TM to actin can be a result of an increase in the region of the stereospecific and hydrophobic interaction between actin and myosin molecules [17], which can increase the ability of actin to activate myosin ATPase, it is possible to suggest that the positive allosteric effect of the Glu180Gly TM on actin activated ATPase activity of S1 is higher than that of the Asp175Asn TM [26].

Hypertrophic cardiomyopathy-causing Asp175Asn and Glu180Gly mutations are charged-to-neutral amino acid substitutions in the TnT Ca-sensitive binding region [27]. These substitutions occur in the g position for Asp175Asn and in the e position for Glu180Gly in the heptad repeat at sites that allow interchain and interhelical electrostatic interactions. Disruption of a salt bridge through amino acid charge reversal caused by mutation is likely to cause a local change in the tropomyosin conformation, which can be a structural basis for the altered switching. TM molecule has 14 pseudo-repeats of 19–20 residues, divided into seven pairs of α- and β-bands. These bands may act as alternate 7-fold sets of sites for specific binding to actin in different conformational states of the regulated thin filament. As the ∽20-residues spacing between α- and β-bands correspond to a ∽90° rotation in the coiled–coil superhelix, a corresponding shifting of TM triggered by the binding of Ca^{2+} and myosin heads to the regulated thin filament has been proposed [27]. The pattern of the binding of the α- and β-bands to F-actin defined by TM shifting could determine not only the position of the TM strands and direction of their movement but also the sequence of the conformational changes in actomyosin during the ATPase cycle. If this assumption is true, a modification of TM structure may alter the pattern of the binding of the α- and β-bands with F-actin in the process of shifting, which could affect the amplitude of TM movement and alter effectiveness of the work of myosin cross-bridges. In the presence of Ca^{2+} TN increases the amplitude of TM motion towards the center of the thin filament and the formation of the strong-binding states [10,17]. A similar increase in the amplitude of TM motion towards the open position was observed for Asp175Asn and Glu180Gly mutant TMs alone (Fig. 1A). In contrast, the DCM-causing Glu40Lys and Glu54Lys mutations in α-tropomyosin inhibit the stereospecific and hydrophobic interaction between actin and myosin molecules and the formation of the strong-binding states by shifting TM strands towards the periphery of the thin filament (a negative allosteric effect of TM on actin); during the ATPase cycle the amplitude of tropomyosin movement was reduced and at some stages of the cycle even reversed [10]. It means that a well-defined sequence of the conformational changes in actomyosin during the ATPase cycle depends on the ability of the TM strands to move towards the center of the thin filament, realizing a definite pattern of interaction with actin.

Our data presented here (Fig. 1) and earlier [10,17] provide a strong evidence in support of the suggestion that the regulation of actomyosin interaction by TM is realized not only via a simple movement of the TM strands from a blocked position to an open one but also via an allosterical mechanism. It is possible that during the ATPase cycle the myosin-dependent shift of the native or wild type TM strands from the periphery to the centre on the thin filament increases the interplay between actin and myosin [17]. The HCM Asp175Asn and Glu180Gly mutations increase this effect by shifting the TM strands further to the centre of the thin filament during the ATPase cycle (Fig. 1A) and make the binding of TM to actin stronger (Fig. 1B), thus enhancing the cross-bridge performance. It is likely that the increase in the myofilament Ca^{2+} sensitivity caused by these HCM mutations is not only due to the effects of the TM–TnT interaction and the signaling of TnC and TnI but also to the shifting of the TM strands further to the open position, which entails the enhancement of the proportion of the strong cross-bridge during the ATPase cycle.

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