Structural differences between C-terminal regions of tropomyosin isoforms

Tropomyosins are actin-binding regulatory proteins, which overlap end-to-end along the filament. High resolution structures of the overlap regions were determined for muscle and non-muscle tropomyosins, however conformations of the junction regions in complex with actin are unknown. In this work, orientation of the overlap on actin alone and on actin-myosin complex was evaluated by measuring FRET distances between a donor (AEDANS) attached to tropomyosin and an acceptor (DABMI) bound to actin’s Cys374. Donor was attached to the Cys residue introduced by site-directed mutagenesis near the C-terminal half of the overlap. The recombinant alpha-tropomyosin isoforms used in this study – skeletal muscle skTM, non-muscle TM2 and TM5a, and chimeric TM1b9a had various amino acid sequences of the N- and C-termini involved in the end-to-end overlap. The donor-acceptor distances calculated for each isoform varied between 36.4 Å and 48.1 Å. Rigor binding of myosin S1 increased the apparent FRET distances of skTM and TM2, but decreased the distances separating TM5a and TM1b9a from actin. The results show that isoform-specific sequences of the end-to-end overlaps determine orientations and dynamics of tropomyosin isoforms on actin. This can be important for specificity of tropomyosin in the regulation of actin filament diverse functions.
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Introduction

Tropomyosins are a family of two-chain coiled coil proteins and are regarded as actin “gate keepers”, which control access of numerous actin-binding proteins to actin filaments (Gunning et al. 2008). Tropomyosin (TM) binds cooperatively to actin and due to end-to-end overlap between adjacent molecules it forms long chains along both sides of the filament. Binding of TM to actin involves weak, but specific electrostatic interactions between periodic actin-binding sites on TM’s coiled-coil and residues exposed on actin subunits (Barua et al. 2011; Li et al. 2011). In this closed state, TM inhibits activation of actomyosin ATPase at low myosin concentrations (Lehrer & Morris 1982). Strongly bound myosin heads (S1) cooperatively shift the filament into the open state, which is associated with an azimuthal shift of TM away from the position occupied in the closed state (Lehman & Craig 2008). The S1-induced shift from the closed to the open state is a universal mechanism of actin filament activation executed in the presence of muscle and non-muscle TM isoforms.

TM isoforms are generated by several genes (four in vertebrates), selection of alternative promoters, and alternative splicing of the transcripts. In α-tropomyosins, the products of the TPM1 gene, the N- and C-terminal regions are encoded respectively by two (1a and 1b) and four (9a-d) alternative exons. Selection of the alternative promoter gives rise to high molecular weight (HMW) and low molecular weight (LMW) isoforms of TM. HMW tropomyosins bind along seven actin subunits, whereas LMW isoforms bind along six actin subunits. The main structural difference between these two TM types is the N-terminal sequence, which is encoded by exon 1a or 1b respectively in HMW and LMW isoforms.
Structures of tropomyosin intermolecular junctions were studied with the use of model peptides imitating sequences of the end-to-end overlaps. Peptides with sequences of skeletal TM encoded by exons 1a and 9a (Greenfield et al. 2006; Murakami et al. 2008), non-muscle TM encoded by exons 1b and 9d (Greenfield et al. 2009), and smooth muscle TM encoded by exons 1a and 9d (Frye et al. 2010) were analyzed to obtain NMR and X-ray structures. Although the structures differ in the number of amino acids forming the overlap and in specific interactions between amino acid chains, the three complexes are similar—the two \( \alpha \)-helical chains of the C-terminus spread apart and interlock with the N-terminal coiled coils. The structures revealed, however, a slightly different tilt of the axis of the C- and N-terminal coiled coil (Frye et al. 2010).

For understanding of the molecular mechanisms controlling numerous functions of actin, high-resolution structures of F-actin in complex with different TM isoforms are required. Models of actin complexes with skeletal and cardiac muscle TM s in different activation states are available (Barua et al. 2013; Barua et al. 2011; Barua et al. 2012; Behrmann et al. 2012; Li et al. 2011; Miki et al. 2012). The models show periodic interactions between conserved residues repeating along TM and charged or hydrophobic residues exposed on actin (Barua et al. 2011; Li et al. 2011). When bound to actin alone, the N-terminal residues Lys6 and Gln9, located with the overlap region, make important interactions with Asp25 on actin. On the other hand, the C-terminal half of the overlap does not contribute directly to actin binding. This region could may be important for regulation of myosin interactions with the filament, however but there is no experimental data supporting this hypothesis.

In our earlier work, we used steady-state Förster resonance energy transfer (FRET) to determine apparent distances between donors specifically attached to the N-terminal regions of different TM isoforms. Our data suggested that, in closed and myosin-induced open states the N-
terminal segments of tropomyosin isoforms are differently oriented on F-actin (Sliwinska et al. 2011). However, the results did not provide any insight into the orientation position of C-terminal segments of the studied isoforms. We do not know whether the regions located in the close vicinity to the end-to-end junctions are stiff or rather flexible. Various flexibilities within this region of TM might be an important determinant of differences between TM isoforms in regulation of actin-myosin interactions.

The aim of this study was to analyze the orientation positions of the C-terminal regions, adjacent to the end-to-end overlap, in four tropomyosin isoforms. Steady-state FRET between a donor attached to the Cys residue introduced into the C-terminal segment of each isoform and acceptor bound to actin’s penultimate Cys374 was used. The data shows that orientation localization of the C-terminal region in relation to actin’s C-terminus is unique for each type of the studied isoform. Specific conformational changes associated with activation of the filament by strongly bound myosin heads suggest that depending on the sequence, the end-to-end overlap regions have different flexibilities.

Materials and Methods

Chicken skeletal muscle α-A-actin, chicken skeletal myosin subfragment 1 (S1) and recombinant rat α-tropomyosin isoforms were used. TM2, TM5a and TM1b9a were obtained as described in (Sliwinska et al. 2011). Recombinant skTM was modified by insertion of AlaSer at the N-terminus to compensate for low actin affinity of recombinant skTM due to the lack of N-terminal acetylation obtained as described in (Robaszkiewicz et al. 2012). The Department of Biochemistry and Cell Biology is authorized by the Minister of the
Environment (Poland) for laboratory use of genetically modified organisms (permit nr GMO: 01-112/112).

PCR-based oligonucleotide-directed mutagenesis (Stratagene) was used to create an attachment site for a fluorescent probe at the C-terminal region of TM. First, cDNA encoding all TM isoforms used in this study was mutated to replace the single Cys codon for Ser. The procedure was described in (Sliwinska et al. 2011). Then cDNA from the first stage of mutagenesis was used to create Ala269Cys-skTM, Ser269Cys-TM2, Ala232Cys-TM1b9a and Ser232Cys-TM5a mutants. The oligonucleotides used at this stage were the following:

9a Ala269(232)Cys: 5’-ctgaagtacaagtgctacgcaggagctggaccacg-3’
9d Ser269(232)Cys: 5’-gccaaagaagaaaacctttgcgcaccagatgctggac-3’

All primers were synthesized and HPLC purified by the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Science (Warsaw, Poland).

Tropomyosin mutants were labeled with AEDANS and actin was labeled with DABMI according to the procedure described previously (Sliwinska et al. 2011).

Tropomyosin affinity for actin was measured in a co-sedimentation assay. Increasing amounts of tropomyosin (final concentrations varied between 0 and 10 µM) were mixed with actin (5 µM) in F-buffer: 2 mM HEPES, pH 7.6, 40 mM NaCl, 5 mM MgCl₂ at 22°C and ultracentrifuged. Protein content in supernatants and pellets was analyzed electrophoretically on SDS-PAGE as described in (Skorzewski et al. 2009).

The activity of actomyosin ATPase was measured in F-buffer. Myosin S1 concentration was at 0.8 µM, F-actin was 9.2 µM, and tropomyosin was 1.2 µM. The reaction was started by
addition of MgATP to 5 mM and stopped after 10 min. with 3.3% SDS and 30 mM EDTA. The amount of liberated phosphate was measured colorimetrically according to the method described in White (1982).

Fluorescence anisotropy, FRET experiments, calculation of Förster critical distance \( R_0 \), and donor-acceptor distance \( R \) were conducted according to the methods described in Sliwinska et al. (2011).

**Results**

*Rationale for the labeling—site selection and characterization of the labeled tropomyosin isoforms*

End-to-end overlap sequences present in the studied TM isoforms were formed by two N-terminal and two C-terminal variants of alternative sequences of rat \( \alpha \)TM. A schematic illustration of the overlap regions are shown in Fig. 1. In skeletal muscle \( \alpha \)TM isoform (skTM), non-muscle isoforms (TM2, TM5a), and in chimeric TM1b9a, an isoform which has no natural counterpart, N-termini encoded by exons 1a or 1b formed complexes with C-termini encoded by exons 9a or 9d. In order to create a specific reactive site for attachment of a fluorescent probe to the C-terminal regions of tropomyosin isoforms, residue 269 in HMW isoforms or its counterpart in LMW isoforms (residue 232) were changed for Cys (Table 1). This residue was selected for the following reasons: (a) it is located at the outskirts of the end-to-end junction (Frye et al. 2010; Greenfield et al. 2006; Greenfield et al. 2009); (b) it is not involved in any interactions within the studied end-to-end complexes (Frye et al. 2010); and (c) it is located on the outer surface of the two-chain coiled-coil.
tropomyosin (position c of the coiled-coil heptapeptide repeat), which allows for free motions
of the attached label. (d) it is part of the flexible region, where coiled coil structure turns into
parallel helices (Frye et al. 2010; Greenfield et al. 2003). Thus we expected that the attached
label was sensitive to conformational differences within the end-to-end overlap.

Replacement of Cys for Ser in the central region of TM did not affect the basic
functions such as actin binding and regulation of actomyosin ATPase activity (Sliwinska et al.
2011). In order to check whether these functions were conserved in TMIs with Cys mutations
in the C-terminal region, we measured actin binding regulatory functions of all studied
AEDANS-labeled TM isoforms. Binding constants ($K_{app}$) obtained in the co-sedimentation
assay show that all TM mutants bound to actin with high affinity (Table 2), consistent with
previous work (Moraczewska et al., 1999). The mutations also did not change significantly
change the interactions between actin and myosin heads. In the presence of wild-type and
mutant TMIs the activities of actin-myosin S1 ATPase were similar (Table 2).

To check whether all isoforms of AEDANS-labeled TM bound to DABMI-labeled
actin stoichiometrically, TMIs were titrated with increasing concentrations of actin. The
titration curves shown in Fig. 2 indicate that the fluorescence was maximally quenched at the
ratio of TM to F-actin, which that was close to stoichiometric. For skTM and TM2 the
maximal quenching was reached at 1 TM to 6-7 actins and in the case of TM5a and TM1b9a at
1 TM to 4-5 actins. The results confirm high actin affinity of TM isoforms labeled in the C-
terminal segment. Additionally, the titration curves show that in case of the isoforms with the
9d-encoded C-terminus (TM2 and TM5a), the maximal quenching of AEDANS was reached
at a lower TM/actin molar ratio than in the case of their 9a-encoded counterparts (skTM and
TM1b9a). Because As fluorescence quenching was due to FRET, the observed differences
suggest that the C-terminal sequence encoded by exon 9d facilitates interactions of the C-
terminal regions of TM2 and TM5a with actin.
Distances separating the donor attached to the C-terminal region of tropomyosin from the acceptor bound to actin

Random orientation of the labels is important for FRET distance measurements (Lakowicz 1999). To ensure that the probe was flexibly attached to Cys269/232 in all studied TM isoforms, we measured AEDANS fluorescence anisotropies. As shown in Table 3, the obtained anisotropies were low, and therefore we concluded that the label bound to free TM as well as to TM in complex with actin and with myosin S1 was randomly oriented. Thus, the orientation of the donor did not limit the FRET distance measurements.

Quantum yield of the donor (Q_D) and spectral overlap between emission of the donor and absorption of the acceptor (J) affect donor-acceptor critical distance (R_0) (Lakowicz 1999). Because the local environment surrounding AEDANS bound to Cys269/232 could influence both parameters, Q_D and J were determined for each labeled TM mutant in the presence of unlabeled actin. The results show that the label attached to the C-terminal segment of the studied TM isoforms was exposed to different environments, which caused variations in Q_D, small shifts in J and, in consequence, differences in R_0 (Table 4).

The FRET efficiency (E) was calculated from the fluorescence intensity of AEDANS-labeled TM in the absence and in the presence of acceptor (TM saturated with DABMI-F-actin). The fluorescence of the donor was corrected for the increase caused by binding of unlabeled actin (3-7% depending on TM isoform). The efficiencies obtained for the studied TM isoforms were used for calculations of the apparent distances (R) separating donor and acceptor. All calculated FRET parameters are collected in Table 4. The results suggest isoform-specific localization of the overlap region. The differences between the distances obtained for HMW isoforms (skTM, TM2) were small, which indicates similar position of the
donor in relation to the acceptor. In LMW isoforms (TM5a and TM1b9a) the donor-acceptor
distances were larger. It is worth noting that the distance obtained for TM1b9a was far from
$R_0$, thus the sensitivity of FRET measurements was limited and the result can only be regarded
as a rough estimate.

Changes in tropomyosins `C-termini as an effect of myosin S1 binding to actin

Strong binding of myosin heads (S1) to the filament increases affinity of TM to actin
and induces an azimuthal shift of TM chains. This changes the TM interactions with actin and
activates the filament allowing for actin-myosin cross-bridge cycling (Lehman & Craig 2008;
Moraczewska 2002). Saturation of the filament with myosin S1 caused a significant increase
of the fluorescence of AEDANS bound to C-terminal segments of all studied TM isoforms.
The average increase was about 14% and 18% for skTM and the non-muscle isoforms,
respectively. Since the fluorescence intensity of the probe attached to the N-terminal segment
increased by about 2-9% (Sliwinska et al. 2011), it appeared that the fluorophore bound to the
C-terminal segment of TM was more sensitive to myosin binding. The change of fluorescence
observed in this work suggested that the C-terminal region of TM either directly interacted
with myosin or significantly changed conformation upon myosin binding to actin. To explore
the later possibility, changes in FRET distances in the presence of myosin were analyzed.

Strongly bound S1-shifted the C-terminal regions of all TM isoforms, however but the
direction of the shift observed for HMW and LMW isoforms was different. As compared to
the TM-actin complex, in the presence of S1 the energy transfer efficiency between AEDANS
attached to skTM or TM2 and DABMI-actin decreased. In contrast, when the energy donor
was attached to TM5a or TM1b9a, an increase in transfer efficiency was observed (Table 5).
Binding of unlabeled actin-S1 did not shift the maximum of the fluorescence spectrum, thus
the spectral overlap (J) was unchanged. However, the quantum yield of AEDANS-TM bound to unlabeled actin-S1 increased (Table 5), which called for recalculation of the critical distances (see equation 5 in Sliwinska et al. (2011)). Based on the new values of $R_0$ and transfer efficiencies (E) obtained for AEDANS-TMs saturated with DABMI-actin-S1, donor-acceptor distances were calculated (Table 5).

The degree of the maximal shift induced by S1 binding to actin ($\Delta R_{S1}$) was calculated as the difference between the donor-acceptor distance in the absence and in the presence of S1. The data indicates that the C-terminal region of the HMW isoforms was shifted away from the donor, whereas in LMW it was shifted closer to the donor.

The S1-induced activation of the filament is a very cooperative process, which means that the final change in TM orientation is achieved at S1 concentrations much far below the concentrations required for actin saturation (Eaton 1976; Moraczewska 2002). Fig. 3 shows the effects of increasing S1 concentrations on energy transfer between donor-labeled TM and acceptor-labeled actin. The experimental data was normalized and fit to the Hill equation. The ratios of strongly bound myosin heads to actin required for the half-maximal change in the energy transfer (S1/actin molar ratio) are collected shown in Table 5. The numbers in parentheses show the S1/TM molar ratio, obtained by multiplying S1/actin by the number of actin subunits bound by one TM molecule. The data shows that the cooperativity was very high for each of the isoforms. The differences depended on the type of end-to-end overlap.

**Discussion**

The position tropomyosin assumes on the filament controls interactions of the filament with many actin-binding proteins, thus it is an important determinant of actin filament functions. The present work is a continuation of our previous studies on the structural diversity among
tropomyosin isoforms determined by sequences of the end-to-end junctions (Sliwinska et al. 2011). Here, in this study, orientations of the C-terminal segments of four tropomyosin isoforms relative to the actin C-terminal region were analyzed with the use of steady-state FRET. Distances were measured between a donor specifically attached to the C-terminal segment of TM and an acceptor bound to actin’s penultimate Cys374, which is located in the outer domain of the filament.

The TM-actin distances obtained by steady-state FRET do not reflect the single donor-acceptor distance, because donor bound to a specific site on TM transfers energy to several acceptors attached to actin subunits. However, as discussed in our earlier work (Sliwinska et al. 2011), the apparent distances calculated from steady-state FRET data were comparable to the distances obtained by modeling of lifetime fluorescence data, which estimated transfer efficiency from single donor to the closest single acceptor (Bacchiocchi et al. 2004).

According to the NMR as well as the crystal structures of muscle and non-muscle TM model peptides, the overlap region is flexible. When forming a complex with the N-terminus, the C-terminal helices open up and interlock with the N-terminal coiled-coil crystal structures of C-terminal segments of muscle TMs, the C-terminal helices open upon formation of the end-to-end complex (Greenfield et al. 2006; Greenfield et al., 2009; Frye et al. 2010). Although the high resolution structure of tropomyosin overlap bound to actin is not known, most probably this mode of tropomyosin end-to-end interaction along the actin filament is maintained, as it fits well into atomic models of F-actin-TM (Barua et al., 2011; Li et al., 2011). The results obtained in this work suggest that, when bound to actin, the overlap complex remains flexible revealed that the orientation of C-terminal segments of the studied TM isoforms was not determined by exon 9-encoded sequence, but rather by the sequence of the end to end overlap complex. The donor-acceptor distance obtained for the isoforms with the same C-terminal sequence encoded by exon
9a (skTM and TM1b9a) or 9d (TM2 and TM5a) differed by about 6-10 Å. This suggests that interdigitation of the C-terminal sequences with different types of N-termini changes conformation of the C-terminal helices with various degrees. The position of each C-terminal segment of the studied TM isoforms was not determined by the exon 9-encoded sequence, but rather by the sequence of the end-to-end overlap complex.

Interestingly, in the case of TM1b9a, which shares the same C-terminal sequence with skTM (encoded by exon 9a), the donor was separated from the acceptor actin by a much larger distance than in the three other isoforms indicating. This shows that the non-muscle 1b-encoded N-terminus strongly distorts the structure of the striated muscle-specific 9a-encoded C-terminus, which is specific for skeletal muscle TM and forms complex with the 1a-encoded N-terminus.

In the earlier study, we used three out of the four TMs used in this work were studied: TM2, TM5a, and TM1b9a. The TMs were labeled with AEDANS in the N-terminal segment (residues 23 or 28) to measure the distance separating the donor located near the N-terminal half of the end-to-end junction and the acceptor bound to actin’s (Cys374) (Sliwinska et al. 2011). In TM2, the FRET distances obtained for donors attached to the N-terminal and the C-terminal regions were 40.2 and 36.4 Å, respectively. In the case of TM5a, the respective distances were 39.3 and 42.7 Å. Taking into account the length of the donor and acceptor probes (about 10 Å) and their random distribution, the differences of about 3.5 Å between both distances in the two isoforms were small. Thus, when bound to actin, TM2 and TM5a seem to be slightly bent within the end-to-end junction. In contrast, in TM1b9a the FRET distances measured from the N- and C-terminus were 34.8 and 48.1 Å respectively. The 13.3 Å difference suggests that the overlap of this isoform is bent or even broken. The cartoon shown in Fig. 4 compares the FRET distances obtained in this and the previous work. To localize the ends on the surface of actin monomer, we positioned the N-terminus of TM2, encoded by exon 1a, over a path of actin amino acid residues
D25, K326 and K328, which, according to the models of TM-actin, directly interact with TM’s K6, E16, and D20 (Barua et al. 2013; Barua et al. 2011; Li et al. 2011). Since the C-terminal residues of skeletal TM do not seem to contribute to the TM-actin interface (Barua et al. 2013; Barua et al. 2011; Li et al. 2011), this region was positioned arbitrarily, to illustrate the apparent distance separating Cys269 from actin. Atomic models of actin in complex with LMW TMs are not available, thus our FRET distances were used to position the ends of TM5a and TM1b9a relative to the position of TM2. Differences in FRET distances measured from C- and N termini of TM2 and TM5a were 3.8 Å and 3.4 Å, respectively, therefore it appears that in complex with actin the N- and C-terminal regions of these two isoforms were slightly bent. The difference between donor acceptor distances measured from C- and N termini of TM1b9a was about 13.3 Å, which suggested that the end to end junction of this isoform was either strongly bent or even broken. Significant curvature of the end-to-end overlap was also observed in X-ray and NMR structures of muscle and non-muscle TMs (Frye et al. 2010; Greenfield et al. 2009; Murakami et al. 2008). Together the results suggest that curvature of the overlap region is an inherent attribute of all TM isoforms. As suggested before, bending is important to adopt the helical structure of the actin filament (Holmes & Lehman 2008).

Our recent studies have shown that during the activation of the filament by strongly bound myosin heads (open state), the N-terminal segments of TM isoforms were differently shifted from the positions they occupied on the filament in the absence of strongly bound myosin-heads (Sliwinska et al. 2011). In the present work we have observed that the donor-acceptor distances measured for the C-terminal segments of HMW isoforms (skTM and TM2) increased the C-terminal segments of the isoforms shifted by S1 not only by different distances but also in the opposite directions. Upon binding of myosin heads to actin, whereas in LMW isoforms (TM5a and TM1b9a) the distances decreased. This shows that the extent of the S1-induced shift of the C-terminus is determined
by the type of the N-terminal sequence. However, the direction of the shift is not certain. If FRET measured a single donor-acceptor distance between AEDANS bound to TM and DABMI attached to Cys374 of only one actin subunit, shortening the distance would mean shifting the C-terminal segment towards actin’s subdomain 1, where myosin-binding sites are located. This, however would be in discrepancy with the earlier observations that LMW isoforms are better activators of actomyosin ATPase than HMW isoforms (Schorzewski et al., 2009). However, the single TM-bound donor in the TM-actin complex is surrounded by multiple acceptors attached to actin subunits, which contribute to the energy transfer with various efficiencies (Bacchiocchi et al. 2004). According to 3D reconstructions of the filaments’ electron micrographs, binding of myosin to actin shifts TM azimuthally towards the inner domain of actin filament (Lehman et al. 1994; Lehman et al. 2000; Xu et al. 1999). Such a shift might increase FRET transfer efficiency between donor and acceptors attached to actin subunits, which belong to the second chain of the long-pitch actin helix. To verify this possibility, we used atomic models of actin-TM (Li et al., 2011) and actin-TM-S1 (Behrman et al., 2012) and analyzed changes in distances between Cys190 in the central region of TM and the five closest Cys374 residues. The cysteines were located in three actin subunits, which directly bound TM along the filament (A\(_{-1}\), A\(_{0}\) and A\(_{+1}\)), and in two subunits across the filament (A\(_{2}\) and A\(_{+2}\)). The analysis showed that in the absence of S1 Cys374 in A\(_{0}\), A\(_{+1}\) and A\(_{2}\) were the closest to Cys190. In the presence of S1 Cys190 was shifted away from Cys374 in A\(_{0}\) and A\(_{+1}\), whereas it was moved towards Cys374 in A\(_{+2}\) and A\(_{+2}\). Thus, in the S1-induced open state the acceptors bound to actin subunits across the filament significantly contributed to FRET efficiency. Even though the ends are missing in the actin-TM-S1 model, and in the actin-TM model the overlap is not resolved, we assume that these considerations also hold true for the end-to-end junction. Binding of S1 diminished the FRET distances of LMW.
isoforms, and therefore it appears that, in these isoforms, the end-to-end overlap was more strongly shifted towards actin subunits across the filament than in HMW TM isoforms.

According to the actin-TM-S1 model (Behrman et al., 2012), binding of myosin heads to actin shifts TM by as much as 23 Å. The S1-induced shift which we observed in this and the previous work was much shorter, which could be explained by the multi-acceptor system discussed above as well as by local differences in TM isoforms bending.

C-termini of HMW and LMW isoforms were shifted respectively further or closer to actin’s outer domain. However, at this stage of investigation it is difficult to locate the junction region of the isoforms in the open state. The reconstructions of the filament electronmicrographs consistently show that upon binding of myosin, TM chains shift away from actin’s outer domain towards inner domain (Lehman et al. 1994; Lehman et al. 2000; Xu et al. 1999). Thus, shortening the donor-acceptor distance, observed by steady-state FRET, might indicate that the actual shift is not in the direction of outer domain but towards acceptors attached to the second chain of actin subunits, which become significant contributors to the transfer.

Our data has also shown very high cooperativity of the S1-induced activation of the filament. Depending on the isoform, the maximal shift was completed when about 1-2 myosin heads per one TM molecule was bound. It is worth noting that the C-terminal segment of TM1b9a showed similar cooperativity as the C-termini of the other isoforms. However, it was much larger than the cooperativity of the N-terminal segment of TM1b9a, which required about 0.4 S1/actin for half maximal saturation of the changes in FRET distance (Sliwinska et al. 2011). This result supports our conclusion that both ends of this chimeric TM are not compatible with each other.

Conclusions

The FRET data gives us an insight into the dynamic changes in the positions of various end-to-end junctions. The isoform specific sequences determine differences in FRET distances
measured between C-termini of tropomyosin isoforms and actin in two (closed and open) filament activation states. The degree of TM shift in response to the filament activation by myosin is individually determined by the sequences of both ends for each isoform. The results agree very well with the observation based on crystal structure of the TM end-to-end overlap that the intermolecular junction is flexible (Greenfield et al. 2009; Frye et al. 2010). Because TM isoforms are functionally specific, the present data give a structural explanation of this specificity and help us to understand the steric and cooperative mechanisms of the thin filament regulation.

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Figure 1

Schematic illustration of the types of end-to-end junctions present in TM isoforms used in this work.

C-terminal sequences encoded by exon 9a (green) or 9d (red) overlapped with N-terminal sequences encoded by exons 1a (orange) or 1b (blue). The residues in C-terminal segments, which were mutated to cysteines are shown.
Figure 2

Quenching of AEDANS-TM fluorescence by DABMI-actin.

sk TM (closed circles, solid line), TM2 (open circles, long dash), TM5a (open squares, short dash) and TM1b9a (closed squares, dotted line) at 0.6 µM were titrated with DABMI-actin. The data was normalized using equation: \( \frac{I - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}} \), where \( I_{\text{max}} \) is the minimal intensity of the fluorescence of AEDANS-TM alone and \( I_{\text{min}} \) is the minimal fluorescence intensity obtained in the presence of DABMI-actin. The lines were generated by fitting the experimental points to ligand-binding equation in Sigma Plot. Conditions: 2 mM HEPES, pH 7.6, 40 mM NaCl, 5 mM MgCl\(_2\) at 22 °C. Excitation and emission wavelengths were 340 nm and 495 nm, respectively. The points were averaged from 3 to 5 independent experiments.
Figure 3

Myosin S1-induced changes in fluorescence of AEDANS-TM bound to DABMI-actin.

(A) HMW TM isoforms: skTM (closed circles, solid line), TM2 (open circles, long dash line) (B) LMW TM isoforms: TM5a (open squares, short dash line), TM1b9a (closed squares, dotted line). AEDANS-TM isoforms at 0.6 µM were bound to 4.8 µM DABMI-actin and titrated with myosin S1. The data obtained for HMW isoforms was normalized by using the equation \((I-I_A)/(I_{S1}-I_A)\). For LMW isoforms the equation was: \((I-I_{S1})/(I_A-I_{S1})\), where I was the fluorescence intensity at given titration point; \(I_A\) was the intensity of TM-F-actin complex in the absence of S1; \(I_{S1}\) was the fluorescence intensity of AEDANS-TM at maximal S1 concentration. The points were averaged from 3 to 4 independent experiments. The lines were obtained by fitting the experimental data to the Hill equation. Conditions as described under Table 3.
**Table 1** (on next page)

Localization of cysteine mutation sites in C-termini encoded by exons 9a and 9d

The upper row shows positions of the amino acid residues in the coiled-coil heptapeptide repeat. Ala or Ser residues changed into Cys to create AEDANS attachment sites are in red.
Table 2 (on next page)

Functional properties of wild type and mutant tropomyosin isoforms labeled with AEDANS

Conditions: 2 mM HEPES, pH 7.6, 40 mM NaCl, 5 mM MgCl$_2$ at 22 °C. The numbers are average values ± S.E. taken from 2 to 9 independent experiments.
| TM isoform      | $K_{app} \times 10^7 \text{M}^{-1}$ | Actin-myosin S1 ATPase activity (nmol Pi/mg S1/min.) |
|-----------------|-------------------------------------|--------------------------------------------------|
| skTM            | 1.63 ± 0.57                         | 103.0 ± 8.0                                      |
| skTM/A269C      | 1.80 ± 0.26                         | 103.8 ± 7.0                                      |
| TM2             | 2.25 ± 0.76                         | 91.7 ± 13.0                                      |
| TM2/S269C       | 2.04 ± 0.45                         | 112.0 ± 10.0                                     |
| TM5a            | 1.42 ± 0.36                         | 169.0 ± 16.0                                     |
| TM5a/S232C      | 1.49 ± 0.58                         | 183.8 ± 20.0                                     |
| TM1b9a          | 1.27 ± 0.40                         | 108.2 ± 9.0                                      |
| TM1b9a/A232C    | 1.24 ± 0.44                         | 125.5 ± 9.0                                      |
Table 3 (on next page)

Anisotropy of AEDANS bound to C-terminal cysteine residues of TM isoforms

Conditions: 0.6 µM AEDANS-labeled TM alone and with 4.8 µM actin ± 5 µM S1 in 2 mM HEPES, pH 7.6, 40 mM NaCl, 5 mM MgCl\(_2\) at 22 °C. Excitation and emission wavelength were 340 nm and 495 nm, respectively. Average values ± S.E. were taken from 3 to 8 independent measurements.
| TM isoform       | TM alone   | TM-actin  | TM-actin-S1 |
|------------------|------------|-----------|-------------|
| skTM/A269C       | 0.073 ± 0.008 | 0.086 ± 0.008 | 0.123 ± 0.012 |
| TM2/S269C        | 0.072 ± 0.002 | 0.106 ± 0.005 | 0.137 ± 0.004 |
| TM5a/S232C       | 0.066 ± 0.003 | 0.096 ± 0.004 | 0.124 ± 0.004 |
| TM1b9a/A232C     | 0.095 ± 0.008 | 0.104 ± 0.003 | 0.127 ± 0.009 |
Table 4 (on next page)

Spectral parameters of FRET between AEDANS-labeled tropomyosins and DABMI-labeled actin

Conditions as given under Table 3. Average values ± S.E. were taken from 10 to 14 independent experiments.
| TM isoform         | $Q_0$ | $J \times 10^{14}$ nm$^4$ M$^{-1}$ cm$^{-1}$ | $R_0$ (Å) | $E$    | $R$ (Å) |
|-------------------|-------|----------------------------------|-----------|-------|--------|
| skTM/A269C        | 0.14  | 6.838                            | 34.7      | 0.40 ± 0.01 | 37.3 ± 0.21 |
| TM2/S269C         | 0.08  | 6.924                            | 32.1      | 0.32 ± 0.01 | 36.4 ± 0.21 |
| TM5a/S232C        | 0.20  | 7.012                            | 37.1      | 0.32 ± 0.03 | 42.7 ± 1.20 |
| TM1b9a/A232C      | 0.12  | 7.087                            | 34.3      | 0.13 ± 0.02 | 48.1 ± 1.45 |
Table 5 (on next page)

S1-induced changes in FRET between AEDANS-labeled TMs and DABMI-labeled actin

Conditions as given under Table 3. Average values ± S.E. were taken from 6 to 9 independent experiments. S1/actin molar ratio is the ratio of strongly bound myosin heads required for half-maximal change in FRET. In parentheses, S1/TM molar ratio obtained by multiplying S1/actin by the number of actin subunits bound by one molecule of each TM isoform.
| TM isoform         | Q₀  | R₀   | E    | R (Å) | ΔRₛ₁ | S₁/actin molar ratio |
|--------------------|-----|------|------|-------|------|----------------------|
| skTM/A269C         | 0.15| 35.0 | 0.28 ± 0.01 | 41.0 ± 0.1 | 3.7  | 0.15 ± 0.02 (1.05)   |
| TM2/S269C          | 0.09| 32.5 | 0.26 ± 0.02 | 38.8 ± 0.2  | 2.4  | 0.12 ± 0.01 (0.84)   |
| TM5a/S232C         | 0.22| 37.6 | 0.44 ± 0.02 | 38.9 ± 0.4  | -3.8 | 0.08 ± 0.02 (0.48)   |
| TM1b9a/A232C       | 0.14| 35.0 | 0.24 ± 0.03 | 42.6 ± 1.2  | -5.5 | 0.14 ± 0.03 (0.84)   |