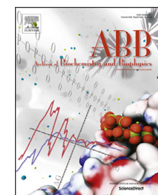
Archives of Biochemistry and Biophysics 606 (2016) 157–166

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi



Abnormal movement of tropomyosin and response of myosin heads and actin during the ATPase cycle caused by the Arg167His, Arg167Gly and Lys168Glu mutations in TPM1 gene

Yurii S. Borovikov [a, *], Nikita A. Rysev [a], Aleksey A. Chernev [a], Stanislava V. Avrova [a], Olga E. Karpicheva [a], Danuta Borys [b], Małgorzata Śliwińska [b], Joanna Moraczewska [b]

[a] *Institute of Cytology, Tikhoretsky Pr., 4, Saint Petersburg, 194064, Russia*
[b] *Kazimierz Wielki University in Bydgoszcz, Department of Biochemistry and Cell Biology, Poland*

ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form
27 July 2016
Accepted 28 July 2016
Available online 30 July 2016

Keywords:
Tropomyosin
F-actin
Myosin
Troponin
Ghost muscle fibres
Congenital myopathies
Fluorescence polarization

ABSTRACT

Amino acid substitutions: Arg167His, Arg167Gly and Lys168Glu, located in a consensus actin-binding site of the striated muscle tropomyosin Tpm1.1 (TM), were used to investigate mechanisms of the thin filament regulation. The azimuthal movement of TM strands on the actin filament and the responses of the myosin heads and actin subunits during the ATPase cycle were studied using fluorescence polarization of muscle fibres. The recombinant wild-type and mutant TMs labelled with 5-IAF, 1,5-IAEDANS-labelled S1and FITC-phalloidin F-actin were incorporated into the ghost muscle fibres to acquire information on the orientation of the probes relative to the fibre axis. The substitutions Arg167Gly and Lys168Glu shifted TM strands into the actin filament centre, whereas Arg167His moved TM towards the periphery of the filament. In the presence of Arg167Gly-TM and Lys168Glu-TM the fraction of actin monomers that were switched on and the number of the myosin heads strongly bound to F-actin were abnormally high even under conditions close to relaxation. In contrast, Arg167His-TM decreased the fraction of switched on actin and reduced the formation of strongly bound myosin heads throughout the ATPase cycle. We concluded that the altered TM-actin contacts destabilized the thin filament and affected the actin-myosin interactions.

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1. Introduction

Tropomyosin (TM) is an α-helical coiled coil protein, which polymerizes end-to-end along each chain of the actin filament (F-actin) and regulates actin interactions with myosin [1]. TM sequence is divided into semi equivalent periodic repeats about 40 amino acids long, which extend along consecutive actin monomers. The N-terminal halves of each repeat contain two groups of charged amino acids, which make direct electrostatic contacts with oppositely charged residues exposed on actin [2,3].

It is well established that efficient regulation of the actin-myosin interactions requires changes in TM azimuthal position on F-actin. When bound to actin alone TM is in equilibrium between closed and blocked positions, where it partially occludes myosin-binding sites on actin [4]. Initial attachment of the myosin heads to actin filament shifts TM further to the inner domain of actin (open position) and exposes the myosin binding sites on actin [5]. Isomerization of myosin heads into a rigor state allows for stabilization of TM in the open position, which involves specific interactions between TM and the tip of myosin head [6]. It appears that the exposure of the myosin-binding sites on actin occurs not only due to the movement of TM, but also due to the rotation of actin in the opposite direction, which allows an easier access for the myosin heads to their actin-binding sites on actin. It has been found [7–10] that when TM strands move from the periphery of actin filament to its centre (from the closed/blocked to the open position), actin monomers turn to meet TM. Thus, the movement of TM strands from the closed to the open position correlates with an increase in the proportion of switched on actin monomers and an enhanced

Abbreviations used: TM, tropomyosin; WTTM, wild-type tropomyosin; S1, myosin subfragment 1; FITC-phalloidin, phalloidin-fluorescein isothiocyanate; 1,5-IAEDANS, N-(iodoacetaminoethyl)-1-naphthyl-amine-5-sulfonic acid; 5-IAF, 5-iodoacetamidofluorescein; DTT, dithiothreitol; AMP-PNP, adenosine 5′-(*β*,*γ*-*imido*) triphosphate tetralithium salt hydrate.

* Corresponding author.
E-mail address: borovikov@incras.ru (Y.S. Borovikov).

http://dx.doi.org/10.1016/j.abb.2016.07.022
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ability of actin filament to stimulate the ATPase activity of myosin. Conversely, along with the shift of TM towards the outer edge of the filament (from the open to the blocked position) the proportion of the switched on actin monomers decreases and the ability of F-actin to stimulate the ATPase activity is reduced [7–10].

Structural elements of TM that are employed in the integration of the conformational changes associated with regulation of actin-myosin interactions are not fully understood. Mutations in specific regions of TM were found in patients suffering from various types of congenital skeletal muscle myopathies and cardiomyopathies [11,12].

In humans most of the disease-causing mutations were found in a specific tropomyosin gene. For example, in nemaline myopathy and congenital fiber type disproportion point mutations segregate with TPM3 gene, which encodes for Tpm3.12 isoform expressed in type 1 slow muscle fibers [13]. However, when introduced into TPM1 gene the mutations impair *in vitro* functions of fast skeletal Tpm1.1 in a way that is consistent with the effects observed in myopathy patients [14,15]. This suggests that the regions affected by mutations share similar functions in both TM isoforms. In the previous work we studied functional effects of Arg167His, Arg167Gly and Lys168Glu replacements in Tpm1.1 [14,15]. Arg167 is one of the positively charged residues, which form actin-binding site through electrostatic interactions with the negatively charged Asp25 exposed on the actin surface [16,17]. Although Lys168 does not interact directly with actin, is was postulated to maintain the proper position of Arg167 by electrostatic repulsion [18]. We have found that all three substitutions decreased the affinity of tropomyosin for actin, and suppressed the activation of the actomyosin ATPase activity and *in vitro* motility of actin filaments [14,15]. We hypothesized that the reduced capability of the thin filament to fully activate myosin cross-bridges was due to impaired TM interactions with actin causing an abnormal azimuthal movement of TM strands. To confirm this hypothesis and gain more insight into the phenomena associated with alteration on the thin filament caused by mutations in TM, we used the polarized fluorescence of muscle fibres reconstituted with recombinant TMs carrying the substitutions mentioned above. We have found that the mutations indeed disturbed the azimuthal movement of TM on the filament, but the direction of the movement of the three types of TM was not uniform. The presence of the mutant TMs had different effects on actin monomers ability to rotate in the filament, a process necessary to switch the monomers on. In addition, TM mutants affected the fraction of myosin heads strongly bound to actin. The results revealed that there is no simple mechanism which governs the TM-dependent regulation of actin-myosin interactions. Depending on the location and the type of amino acid substitution, the reduced activation of contraction is caused either by blocking the access of myosin heads to actin or by increasing the fraction of strongly bound myosin heads which cannot freely enter the cross-bridge cycle.

2. Materials and methods

2.1. Preparation of proteins

Myosin was separated from fast skeletal muscles of rabbits as described by Margossian and Lowey [19]. Myosin subfragment-1 (S1) was prepared by treatment of fast skeletal muscle myosin with α-chymotrypsin for 10 min at 25 °C according to Okamoto and Sekine [20]. Modification of the reactive residue Cys707 of S1 with 1,5-IAEDANS (Molecular Probes, actin-AEDANS) was carried out as described previously [21]. The degree of Cys707 modification was 0.90–0.95.

Recombinant wild type and mutant Tpm1.1 carrying Arg167His, Arg167Gly or Lys168Glu substitutions were expressed in BL21 (DE3) cells and purified as described before [22]. All tropomyosins had an extension of two additional amino acids (AlaSer), which compensated for the reduced affinity of recombinant non-acetylated skeletal TM to F-actin. TM labelling with 5-IAF at Cys190 was performed as described previously [10,23], producing a probe to protein molar ratio 0.8:1.

2.2. Preparation and labelling of ghost fibres

Experiments were performed at the animal care facility of the Institute of Cytology RAS. Adult male New Zealand white rabbits (3–4 kg) were killed by sodium pentobarbitone injection (200 mg/kg) in accordance with the official regulations of the community council on the use of laboratory animals, and the study was approved by the ethics committee for animal experiments. The psoas muscle was exposed ventrally and a bundle of about 100 fibres was gently separated from the muscle. The fibers were glycerinated using the method of Rome [24]. Ghost fibres were prepared by incubation of single glycerinated skeletal fibres for 1.5 h in 800 mM KCl, 1 mM $MgCl_2$, 10 mM ATP, 67 mM phosphate buffer, pH 7.0 [8]. Actin accounted for 80% of the overall protein content of the ghost fibres. S1 and TM were incorporated into pure actin filaments by incubation of the fibre in a standard solution containing 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 6.8, and the respective protein in concentration 1.0–2.5 mg/ml. The unbound proteins were washed out by incubation of the fibres in the same buffer without proteins. FITC-phalloidin was dissolved in methanol, pre-aliquoted and then methanol was evaporated. FITC-phalloidin was conjugated with F-actin of the fibres by their incubation in a standard solution containing 40 μM FITC-phalloidin for 2.5 h at room temperature [8,10].

The effectiveness of reconstitution of the filaments in ghost muscle fibres used for fluorescent measurements was verified by SDS-PAGE [25]. The fibres in the final preparations contained actin, S1, recombinant TM and Z-line proteins. The molar ratio of WTTM or mutant TMs to actin was 1:6.5 (±2) irrespective of whether TMs were modified by 5-IAF or not. In the absence of nucleotides and in the presence of MgADP, MgAMP-PNP, and MgATP the molar ratios of S1 to actin were 1:5 (±2), 1:5 (±2), 1:8 (±2), and 1:14 (±2), respectively.

2.3. Fluorescence polarization measurement

Steady-state fluorescence polarization measurements on single ghost muscle fibres were made using a flow-through chamber and polarized microfluorimeter [26]. The polarized fluorescence from 1,5-IAEDANS-labelled S1 was excited at 407 ± 5 nm, and from FITC-phalloidin-labelled actin and 5-IAF-labelled TM, at 437 ± 5 nm and recorded at 500–600 nm. The intensities of four components of polarized fluorescence $_{||}I_{||}$, $_{||}I_{\perp}$, $_{\perp}I_{\perp}$ and $_{\perp}I_{||}$ were detected by two photomultiplier tubes. The subscripts || and ⊥ designate the direction of polarization parallel and perpendicular to the fibre axis, the former denoting the direction of polarization of the incident light and the latter that of the emitted light (Fig. 1A). Fluorescence polarization ratios were defined as: $P_{||} = (_{||}I_{||} - _{||}I_{\perp})/(_{||}I_{||} + _{||}I_{\perp})$ and $P_{\perp} = (_{\perp}I_{\perp} - _{\perp}I_{||})/(_{\perp}I_{\perp} + _{\perp}I_{||})$. Measurements were carried out in a standard solution containing 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 6.8 in the absence or presence of 1.0 mM ADP, 16 mM AMP-PNP or 5 mM ATP. 10 mM creatine phosphate and 140 unit/ml creatine kinase were added to the solution containing ATP. The concentration of $MgCl_2$ was 3 mM when experimental medium contained ADP or no nucleotides, 8 mM in the presence of ATP, and 18 mM in the presence of AMP-PNP. The absence of nucleotides or the presence of MgADP mimicked strong binding of myosin heads to F-actin (AM and AM^·ADP states), and the presence of MgAMP-PNP or MgATP mimicked weak myosin binding (AM'·ADP and

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