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The long C-terminal extension of insect flight muscle-specific troponin-I isoform is not required for stretch activation

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ABSTRACT

Stretch-induced enhancement of active force (stretch activation, SA) is observed in striated muscles in general, and most conspicuously in insect flight muscle (IFM). It remains unclear whether a common mechanism underlies the SA of all muscle types, or the SA of IFM relies on its highly specialized features. Recent studies suggest that IFM-specific isoforms of thin filament regulatory proteins (troponin and tropomyosin) are implicated in SA. Among others, IFM-specific troponin-I (troponin-H or TnH), with an unusually long Pro-Ala-rich extension at the C-terminus, has been speculated to transmit the mechanical signal of stretch to the troponin complex. To verify this hypothesis, it was removed by a specific endoproteinase in bumblebee IFM, expecting that it would eliminate SA while leaving intact the capacity for Ca^{2+}-activated isometric force. Electrophoretic data showed that the extension was almost completely (97%) removed from IFM fibers after treatment. Unexpectedly, SA force was still conspicuous, and its rate of rise was not affected. Therefore, the results preclude the possibility that the extension is a main part of the mechanism of SA. This leaves open the possibility that SAs of IFM and vertebrate striated muscles, which lack the extension, operate under common basic mechanisms.

1. Introduction

Stretch-induced delayed enhancement of active force (stretch activation or SA) is widely observed in cross-striated muscles, including vertebrate skeletal and cardiac muscles. Owing to this property, the muscle produces positive work (exhibits negative viscosity) when sinusoidal oscillation is applied in a certain range of frequencies. Insect flight muscle (IFM) makes maximal use of this property to cause autonomous high-frequency oscillation needed to drive the wings [1,2].

Structurally, IFM is one of the cross striated muscles. Its myofibrils consist of sarcomeres connected in series, and the basic architecture of each sarcomere is not different from that in vertebrate skeletal muscle. However, IFM is positioned in the highly specialized end of cross striated muscles: it is not readily extensible as vertebrate skeletal muscle. The constituent proteins of IFM are mostly isoforms or homologs of those found in vertebrate skeletal muscle, but many of them are expressed as peculiar flight muscle-specific isoforms. These IFM-specific isoforms include those of actin [3], myosin [4] and troponin subunits [5,6]. Among others, IFM troponin-I is one of the most peculiar of the IFM-specific protein isoforms. It has a long Pro-Ala-rich extension at the C-terminus, and its apparent molecular mass on the gel is 70–80 kDa as opposed to ~25 kDa for non-IFM isoforms. Because of its large mass, it is often called troponin-H or TnH (H stands for heavy). Then the question is whether these features are essential for SA in IFM, or the basic mechanism for SA is common among IFM and other types of striated muscle in spite of all these peculiarities. The present study specifically addresses the question of whether the long extension of TnH is essential for SA.

Although the molecular mechanism of SA has not been clarified, increasing evidence suggests that the thin filament regulatory proteins (troponin and tropomyosin, which usually turn on and off the contraction in a calcium-dependent manner) are involved in each SA event, despite that the intracellular calcium concentration is held constant during repetitive IFM oscillations. Evidence for this comes from (a) the discovery of an IFM-specific troponin-C isoform which lacks calcium binding sites on the N-lobe and without which SA is not observed [6], (b) the X-ray observation that the second actin layer line reflection (reporting tropomyosin movement on actin) is enhanced during SA [7,8], and (c) demonstrations that tropomyosin can move fast enough to regulate each wing-beat [8] in fast-beating bumblebee and that tropomyosin actually moves ahead of force during sinusoidal oscillation in giant waterbug [9].

Abbreviations: SA, stretch activation; IFM, insect flight muscle; GST, glutathione S-transferase; GPx, glutathione peroxidase; TnH, troponin-H.

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One of the ways for the thin filament regulatory proteins to participate in each SA event would be to transmit stretch signal from the thick filaments to the thin filaments (the thin filaments have free ends on the M-line side of the half sarcomere). The candidates for the structure to transmit the signal include the troponin bridge, which may be either a myosin head [9] or the TnH extension. The latter has repeating PAEGEAP motifs or the like, similar to SH3 (src homolog 3) domain-binding motifs, and could bind to the SH3 domain on myosin head [10]. In addition, the extension is known to bind a class of glutathione-S-transferase [11], which may also be involved in SA [11] or alternatively, counteract the oxidative stress caused by high mitochondrial activity [12,13].

The most straightforward way to address the role of the TnH extension would be to examine the effects of its enzymatic removal. In *Drosophila* IFM, a specific endoproteinase (Igase) is known to sever the extension [11](in *Drosophila*, the sequence is not associated with troponin-I but with tropomyosin). Here we applied Igase to bumblebee IFM and confirmed that the extension is effectively removed. By using this system, we examined the effects of the removal of extension on the mechanical properties to test if the capacity for SA is eliminated. A brief account of a part of the present study has appeared [14].

2. Materials and methods

2.1. Muscle fiber specimens

The bumblebee (*Bombus* sp.) IFM fibers were prepared as described elsewhere [15]. Each single fiber was split to 2–4 strips before mounting for mechanical measurements. The samples were 4–5 mm long.

2.2. Solutions

The compositions of the solutions were as described earlier [8,15–17]. The relaxing, pre-activating and contracting solutions contained 20 mM imidazole, 5 mM $MgCl_2$ (free $[Mg^{2+}] = 0.75$ mM), 4 mM Na_2ATP, 20 mM creatine phosphate and 125–700 U/ml creatine phosphokinase (pH 7.2), ionic strength was adjusted to 193 mM by adding varying concentrations of K-propionate. In addition, the relaxing and activating solutions contained 10 mM EGTA, which was reduced to 0.1 mM in the pre-activating solution. The activating solution contained 10.4–10.1 mM of $CaCl_2$ to make pCa = 4.0–4.5. The rigor solution contained 120 mM K-propionate, 20 mM imidazole, and 5 mM each of EGTA and EDTA.

2.3. Enzymatic removal of troponin-H extension

To enzymatically sever the TnH extension of bumblebee IFM, the fibers were treated by endoproteinase Pro-Pro-Y-Pro (Igase, MoBiTec, Goettingen, Germany) [11]. Fibers were first transferred to the rigor solution, and then to a 1:10 mixture of the endoproteinase solution as provided (in 50% glycerol) and the rigor solution. The treatment was carried out in a refrigerator (4 °C) overnight. After treatment, the fibers were washed thoroughly in the rigor solution. The control fibers were incubated for the same period in the rigor solution without the endoproteinase but with 5% glycerol.

2.4. Gel electrophoresis and immunoblot analysis

SDS–PAGE was performed by using 5–20% gradient gels (Atto, Tokyo, Japan), and they were stained with Coomassie Brilliant Blue. For immunoblot analysis, the gels were electroblotted to nitrocellulose membranes and the band for TnH was detected by using an anti-TnH rat monoclonal antibody (MAC143 [5]). The secondary antibody was either horse radish peroxidase- or alkaline phosphatase-conjugated, and the cross-reacted bands were visualized by using Easy West Blue (Atto) or BCIP/NBT (Sigma–Aldrich, St. Louis, MO, USA).

2.5. Mechanical measurement

The setup for mechanical measurement was as described [16]. Briefly, the IFM fiber was calcium-activated in the activating solution, after placing in the pre-activating solution, and then step-stretched and released by ~1% of its length by using a servo motor (G120D, General Scanning, Watertown, MA, USA). Force was measured by a semiconductor-type transducer (AE801, Akers, Horten, Norway). The data were collected by a digital data acquisition system (either hand-made or USB-6210, National Instruments, Austin, TX, USA). Experiments were done at 20 °C.

3. Results

3.1. Effect of Igase treatment on electrophoretic pattern

The SDS–PAGE patterns from bumblebee IFM fibers show that only one band, at ~70 kDa, was visibly weakened after Igase treatment (Fig. 1A, lanes a and b). At the same time, a number of protein bands appeared in the lane for the supernatant (lanes c and d), ranging from 16 to 65 kDa. Under the current digesting condition, the band intensity of the ~70 kDa protein decreased with a rate constant of ~0.24 h^{-1} (Fig. 1B), meaning that ~97% of the ~70 kDa protein is removed after overnight treatment (12 h). The antibody directed against TnH (MAC143) strongly cross-reacted with the ~70 kDa component in the untreated fiber (lane e), indicating that the Igase-digestable 70-kDa protein is bumblebee TnH. After Igase treatment, its staining is visibly weaker (lane f) and the cross-reactivity is transferred to the supernatant (~38 and ~28 kDa bands). After the supernatant is further incubated with Igase, the intensities of the two bands reversed (lanes h and i), suggesting that the ~38 kDa peptide is the primary cleavage product.

The epitope for MAC143 is likely to be the repeating PAEG motifs, because MAC143 also cross-reacts with *Drosophila* GST-2, and the motif is the only sequence shared by GST-2 and the epitope-containing stretch of *Drosophila* extension [11]. The motifs are distributed along the entire length of the extension but are not present in the TnI proper. After Igase treatment, TnH epitopes are exclusively found in the supernatant. This observation indicates that Igase removes the bumblebee extension from its base.

Although the bumblebee TnH sequence has not been determined, the TnH sequence of allied honeybee (Both bees belong to the family Apidae) has a single susceptible sequence (PPTP) at the base of the Pro-Ala-rich extension [18]. If the two bees share this sequence, Igase is expected to remove most of the extension (195 a.a.), leaving the rest of the TnH (235 a.a.; predicted molecular mass, ~28 kDa) with only a short segment of the extension (<30 a.a.). As expected from this, a new faint band is observed at ~30 kDa in lanes for Igase-treated fibers (lane b). The cleavage patterns of honeybee IFMs are very similar to that of bumblebee IFM (see Supplementary data). Taken together, it is very likely that the bumblebee extension also has a susceptible sequence very close to its base.

3.2. Mechanical properties

We have shown that the Igase treatment removes the long Pro-Ala-rich extension from ~97% of TnH molecules from bumblebee IFM fibers, even without leaving a shortest segment of TnH epitope

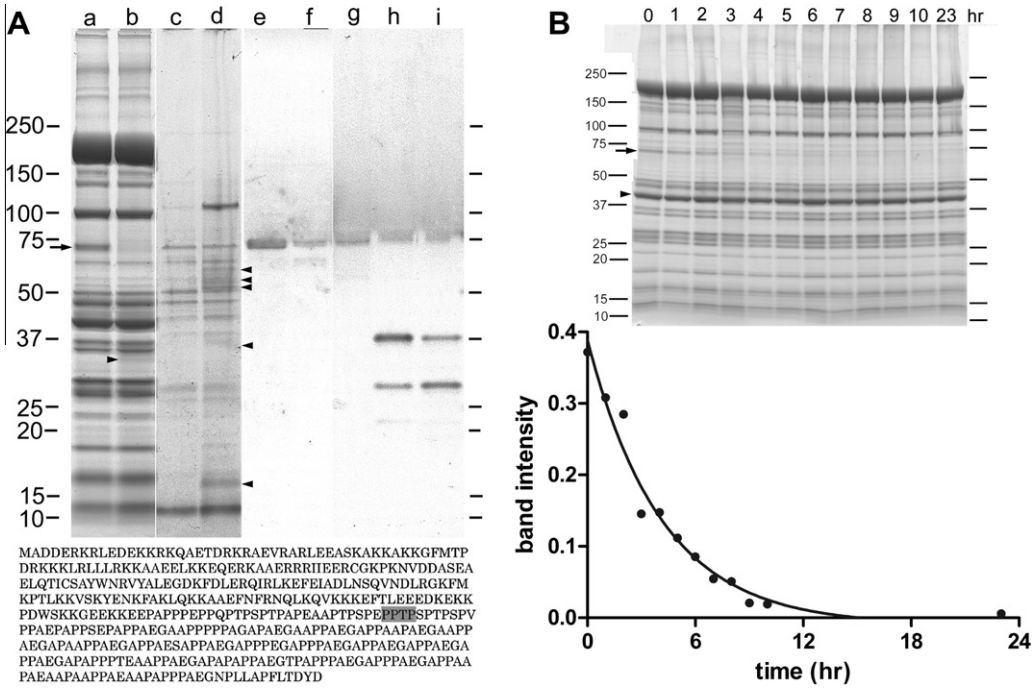

Fig. 1. SDS–PAGE pattern and Western blot of control and Igase-treated bumblebee IFM specimens. (A), patterns from control and fibers treated with Igase overnight. Lanes a–d, Coomassie Brilliant-Blue-stained patterns; lanes e–i, immunoblot. Lanes a and b, control and Igase treated fibers, respectively, c and d, proteins released to the solutions from fibers in a and b, respectively. Note that the ~70 kDa band in a (arrow) disappears after Igase treatment and a new band appears at ~30 kDa in b (right-pointing arrowhead). In extract (d), a number of new bands appear (left-pointing arrowheads). The ~100 kDa band is Igase (106 kDa). Lanes e and f, cross-reaction of blotted proteins in lanes a and b with anti-TnH antibody (MAC143), respectively; lanes g and h, cross-reaction of specimens in lanes c and d, respectively. The extract with Igase (lane h) was incubated for an extended period (lane i). Note that the intensities of the two stained bands are reversed. The bottom is the TnH sequence of honeybee, *Apis mellifera* ([18]; Accession: Genbank BK005282.1). The highlighted letters indicate the susceptible sequence. (B), Time course of digestion of TnH extension by Igase. The band density of the TnH extension (arrow) in the Coomassie Brilliant Blue-stained gel was determined by using commercial software (1D, Eastman Kodak Co., Rochester, NY, USA), normalized against the band density of actin (arrowhead), and plotted against digestion time. Data are fitted with a single exponential decay function, $d = 0.388 \exp(-0.241t) - 0.01$, where t is time in hour. This equation indicates that only ~3% of TnH molecules remain intact after overnight (12-h) digestion.

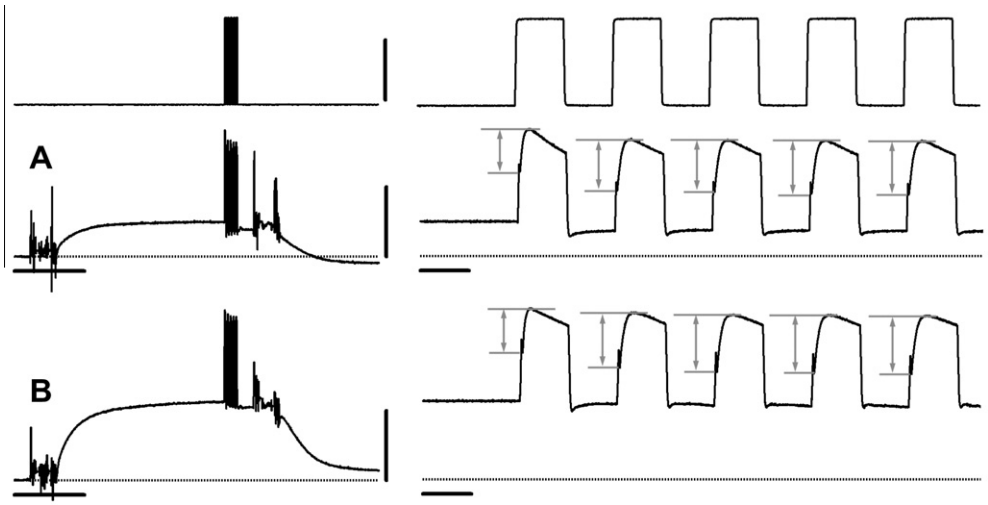

Fig. 2. Response of activated bumblebee IFM fibers to repeated stretches and releases in slow (left) and fast (right) time bases. (A), force of control fiber; (B), force of Igase-treated fiber. The control fiber (A) was incubated in the rigor solution without Igase overnight. Top traces show fiber length. Fibers had been prestretched so that they exhibited a small amount of passive force before activation. The gray arrows in the right traces represent the amplitude of stretch-activated force, which is defined as the difference between the peak force after a stretch and the force extrapolated to the onset of stretch. Scale bars for length, force, and time in slow and fast time bases are 1% fiber length, 0.5 mN, 5 s and 100 ms, respectively.

in fibers. Then the question is how the treatment affects the mechanical properties of bumblebee IFM fibers, especially the capacity for SA, because the extension has long been regarded as a primary candidate for the structure that transmits the stretch signal to the troponin complex.

Fig. 2 shows the responses of a control or an Igase-treated, maximally calcium-activated IFM fiber to repeated stretches and releases. The control fibers show typical SA in response to stretch (Fig. 2A), as reported earlier [8]. Unexpectedly, the capacity for SA largely remains intact after Igase treatment (Fig. 2B).

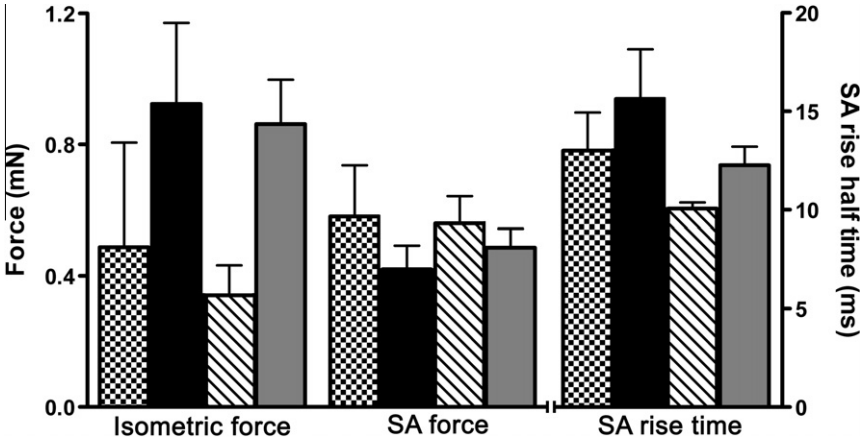

Fig. 3. Summary of mechanical properties of bumblebee IFM fibers. Checkered and filled columns, data before and after Igase-treatment, hatched and gray columns, data for control fibers before and after incubation in rigor solution. For the results of statistical analysis see text ($n = 6$ in all cases). Error bars represent standard deviation.

The results of mechanical measurements are summarized in Fig. 3. After mechanical analysis of each fiber, it was incubated in a rigor solution with or without Igase and subjected to the same mechanical analysis. In both Igase-treated and control groups, the isometric force tended to increase (by 140% and 168% for Igase-treated and control groups, respectively), the SA force tended to decrease (to 25% and 13%), and the rate of rise of SA force tended to decrease slightly (by 20% and 22%). The changes after incubation were all statistically significant ($p < 0.02$, $n = 6$ each, paired t-test). Although the decrease of SA force of Igase-treated fibers was significantly greater than in control ($p \sim 0.02$, $n = 6$, unpaired t-test), the 25% reduction in SA force as opposed to 97% removal of the TmH extension clearly indicate that the extension is not essential for SA. Moreover, the reduction of SA force is also observed in control fibers. Thus, the decrease of the SA force should rather be ascribed to the reciprocal relationship between isometric and SA forces [19]; the more the myosin heads are recruited for isometric force, the less are available for SA. For the changes of isometric force or the rate of SA rise after incubation, there was no statistical difference between Igase-treated and control groups ($p > 0.6$).

4. Discussion

Although stretch activation (SA) is most conspicuously observed in insect flight muscles (IFMs), this phenomenon is observed in various types of muscles, and is considered to play an important role for the Frank–Starling's law of the heart. Then the question is whether IFM can serve a model material to study the molecular mechanism of SA. The answer to this question rests on whether common molecular mechanisms underlie the SA of various muscle types. However, the answer will be in the negative if the SA of IFM relies on its highly specialized features. In this respect, the long Pro-Ala-rich extension of troponin-I (troponin-H) is one of the most specialized features of proteins in IFM. In this study, its physiological role was examined by thoroughly removing this extension by using a specific endoproteinase (Igase). The consequences of such thorough removal are discussed below.

4.1. Effect on stretch activation

The long Pro-Ala-rich extension of TnH is unique to IFM, and its repeated PAEG motifs seem to be conserved among various species. In Diptera the extension has translocated to tropomyosin, but is still retained by all species examined. Insects are known as bold experimenters in evolution (e.g., modification of the common 9 + 2 architecture of flagellar axoneme, which is conserved from single-cell algae to vertebrates. See [20] and references therein), but no insect species has been reported to lack the TnH extension in its IFM. Such strong genetic conservation suggests that this structure is essential for the function of IFM, and it would be natural to consider that its function may be related to SA, another striking feature of IFM.

As mentioned earlier, there is increasing evidence that the regulatory proteins on the thin filament are involved in SA, and a most straightforward way to be so is to sense the stretch signal by themselves. If so, there must exist a structure that transmits the mechanical signal to the regulatory proteins, and the TnH extension seems to be the most suitable candidate to perform this function [21]. However, there are also pieces of evidence that are not quite supportive of this role. One is that the extension is also found in IFMs of synchronous flight muscles, which do not show conspicuous SA [22]. The other is the *Drosophila* mutant in which the expression of the IFM-specific tropomyosin isoform (with extension) was reduced [23]. The authors found "an unexpectedly mild effect on IFM structure and function".

Here we also unexpectedly found that the removal of the extension had little effect on the capacity for SA. The amplitude of the SA force tended to decrease after Igase treatment, but it is likely to be due to the reciprocal relationship between SA and isometric forces [19]. The isometric force increased after Igase treatment, but it was also observed in control fibers, which were incubated overnight in the rigor solution without Igase. The isometric force may increase after incubation in a rigor solution because a small number of myosin heads may irreversibly form rigor bridges, which may further activate the thin filaments.

Two questions arise from this result. (a) If the TnH extension does not transmit the stretch signal to the regulatory proteins, what structure does it, or maybe the regulatory proteins do not sense the signal after all? (b) If it is not to support SA, what is the true function of the TnH extension? Clearly, we need more experiments to answer the first question, but the second question can be discussed here, as described below.

4.2. TnH extension as a scaffold for other proteins

In *Drosophila* IFM, it has been reported that an isoform of glutathione S-transferase (GST-2) is stably associated with the extension [11]. This isoform exhibits a high glutathione peroxidase (GPx) activity. Here we have shown that a number of proteins are released to solution after Igase treatment, and not all of them cross-react with the MAC143 antibody. One cannot rule out the possibility that they are hydrolytic products of other proteins, but evidence from X-ray diffraction studies suggests that a

substantial mass is lost from the thin filament (details will be presented elsewhere), and it would better be interpreted to mean that the extension of bumblebee TnH binds a substantial amount of proteins by serving as a scaffold for them.

The skinned bumblebee IFM fibers exhibited substantial GPx activity even after through washing, indicating that the enzymes with GPx activity (presumably GST-2) is associated with myofibrillar proteins (data not shown). However, substantial GPx activity remained after removal of the extension. In *Drosophila*, the GST-2 molecule has a short extension that contains the PAEG motif, and it has been suggested that the molecule is associated with the TnH extension by means of this motif [11]. Honeybee or bumblebee GST-2 lacks such an extension [24]. Therefore, the bee GST-2 molecules may be still associated with myofibrillar proteins but not specifically with the TnH extension.

4.3. Comments on cleavage sites by Igase

Igase is an endoproteinase that specifically cleaves a peptide sequence PPXP, where X can be T, S or A (and exceptionally PAPSP), and several proteins, including Igase itself, are known substrates [25]. As mentioned earlier, the extension of tropomyosin in *Drosophila* IFM contains this susceptible sequence [11]. Like in allied honeybee, the extension of bumblebee TnH also seems to contain a susceptible sequence at its base, and as expected, a new band of ~30 kDa mass appeared in the SDS–PAGE lane for treated fibers (Fig. 1). Unlike in honeybee (Supplementary data), two bands that were released to the solution cross-reacted with the MAC143 antibody, and the lower-mass band was intensified after prolonged incubation with Igase. This means that there is a second susceptible site in the bumblebee extension, but the cleavage at this site seems somewhat slower than at the first site. Therefore, there could be sequence-dependent differences in susceptibility. Determination of bumblebee genome will solve the question in the near future.

The TnH extension of the giant waterbug *Lethocerus indicus* (Accession: GenBank AJ621044.1) does not contain the PPXP sequence, but in Japanese species (*Lethocerus deyrollei*), the TnH band disappears from the SDS gel pattern after the same overnight treatment (the capacity for SA also remains intact. Data not shown). These observations suggest that there is a substantial interspecies difference in sequences, and that the sequence of *L. deyrollei* contains at least one susceptible sequence.

4.4. Conclusion

It is an unproven hypothesis that the conspicuous SA in IFM is a manifestation of its high degree of specialization. Another unproven hypothesis is that the SAs of IFM and other muscle types, including vertebrate striated muscle, operate under a common molecular mechanism. It may eventually turn out that the two hypotheses are not mutually exclusive, but the present finding provides an answer to one of a vast number of questions lying before these hypotheses are verified. The present finding is that the TnH extension is not a part of the mechanism of SA. Rather, its suggested functions are to serve as a scaffold for other proteins. Unlike in *Drosophila*, the TnH extension of bumblebee IFM is not a specific host for GST-2, but for a number of currently unidentified proteins. Characterization of these proteins will help better defining the role of this IFM-specific conspicuous structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.101.

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