The structural role of high molecular weight tropomyosins in dipteran indirect flight muscle and the effect of phosphorylation

ARTICLE *in* JOURNAL OF MUSCLE RESEARCH AND CELL MOTILITY · FEBRUARY 2006

Impact Factor: 2.09 · DOI: 10.1007/s10974-005-9044-3 · Source: PubMed

5 AUTHORS, INCLUDING:

Jesús Mateos
IDIBELL Bellvitge Biomedical Research Insti…
80 PUBLICATIONS **449** CITATIONS
SEE PROFILE

Raul Herranz
Spanish National Research Council
51 PUBLICATIONS **375** CITATIONS
SEE PROFILE

Alberto Domingo
University of Alcalá
31 PUBLICATIONS **642** CITATIONS
SEE PROFILE

Journal of Muscle Research and Cell Motility (2006)
DOI 10.1007/s10974-005-9044-3

The structural role of high molecular weight tropomyosins in dipteran indirect flight muscle and the effect of phosphorylation

JESÚS MATEOS[1], RAÚL HERRANZ[1], ALBERTO DOMINGO[1,2], JOHN SPARROW[3] and ROBERTO MARCO[1,*]
[1]*Departamento de Bioquímica (UAM) e Instituto "Alberto Sols" (UAM-CSIC), Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain;* [2]*Departamento de Bioquímica y Biología Molecular, Universidad de Alcalá de Henares, Madrid, Spain;* [3]*Department of Biology, University of York, YO10 5DD, York, UK*

Received 28 April 2005; accepted in revised form 18 October 2005

Abstract

In *Drosophila melanogaster* two high molecular weight tropomyosin isoforms, historically named heavy troponins (TnH-33 and TnH-34), are encoded by the *Tm1* tropomyosin gene. They are specifically expressed in the indirect flight muscles (IFM). Their N-termini are conventional and complete tropomyosin sequences, but their C-termini consist of different IFM-specific domains that are rich in proline, alanine, glycine and glutamate. The evidence indicates that in Diptera these IFM-specific isoforms are conserved and are not troponins, but heavy tropomyosins (TmH). We report here that they are post-translationally modified by several phosphorylations in their C-termini in mature flies, but not in recently emerged flies that are incapable of flight. From stoichiometric measurements of thin filament proteins and interactions of the TmH isoforms with the standard *Drosophila* IFM tropomyosin isoform (protein 129), we propose that the TmH N-termini are integrated into the thin filament structural unit as tropomyosin dimers. The phosphorylated C-termini remain unlocated and may be important in IFM stretch-activation. Comparison of the *Tm1* and *Tm2* gene sequences shows a complete conservation of gene organisation in other Drosophilidae, such as *Drosophila pseudoobscura*, while in *Anopheles gambiae* only one exon encodes a single C-terminal domain, though overall gene organization is maintained. Interestingly, in *Apis mellifera* (hymenopteran), while most of the *Tm1* and *Tm2* gene features are conserved, the gene lacks any C-terminal exons. Instead these sequences are found at the 3' end of the troponin I gene. In this insect order, as in *Lethocerus* (hemipteran), the original designation of troponin H (TnH) should be retained. We discuss whether the insertion of the IFM-specific pro-ala-gly-glu-rich domain into the tropomyosin or troponin I genes in different insect orders may be related to proposals that the IFM stretch activation mechanism has evolved independently several times in higher insects.

Introduction

In vertebrate striated muscle thick and thin filament interactions are regulated by the thin filament troponin-tropomyosin (Tn-Tm) protein complex (Geeves and Holmes, 1999; Gordon *et al.*, 2000). The troponin complex consists of a tropomyosin binding subunit, troponin T (TnT), an inhibitory subunit, troponin I (TnI), and a Ca^{2+}-binding subunit, troponin C (TnC). In skeletal muscle, Ca^{2+} binding to Tn-Tm promotes conformational changes which permit the movement of the continuous tropomyosin (Tm) filament across the F-actin surface, exposing the myosin-binding site of actin and allowing crossbridge cycling to begin (Craig and Lehman, 2001). The components of the Tn-Tm complex have been well characterised in the indirect flight muscles (IFM) of *Drosophila melanogaster* and the giant water-bug, *Lethocerus* (Wendt and Leonard, 1999). All the insect muscle thin filament proteins show considerable sequence homology to their vertebrate counterparts, but IFM-specific isoforms occur, and these usually contain sequences not found in vertebrate or the non-flight muscles of the insect. In *Drosophila* this variability is produced either by IFM-specific expression of one or more alternate gene copies, e.g. TnC (Qiu *et al.*, 2003; Herranz *et al.*, 2005a, b), or by IFM-specific splicing of gene transcripts from single genes, e.g., TnI (Barbas *et al.*, 1991; Herranz *et al.*, 2005a, b) and TnT (Fyrberg *et al.*, 1990; Herranz *et al.*, 2005a, b). Tropomyosin IFM isoforms are generated by alternate splicing of two transcripts of the *Tm1* and *Tm2* genes (Bautch *et al.*, 1982; Karlik and Fyrberg, 1986). Note that these genes were originally designated *TmII* and *TmI* (Hanke and Storti, 1988), but are now known in Flybase as *Tm1* and *Tm2*, respectively. Muscle contraction in insect IFM is oscillatory, but neural activation is asynchronous, i.e. not in phase with the contractions (Pringle, 1978; Thorson and White, 1983). Although Ca^{2+} is necessary for the initiation and

* To whom correspondence should be addressed. E-mail: roberto.marco@uam.es

maintenance of IFM contractions, it is the periodic, alternating strain applied from one set of IFM on the opposing fibres, transmitted through movements of the thorax cuticle that causes an oscillatory stretch-activation. The fibres respond to this strain by a delayed tension rise whose kinetics is species-specific and correlated with wing beat frequency and insect size (Molloy *et al*., 1987). This oscillatory system is an evolutionary adaptation in higher insects (Sparrow, 1995; Vigoreaux, 2001) that allows wing beat frequencies (>200 Hz) above the limiting frequency for nervous impulses, and also reduces the volume of muscle fibres that would otherwise be occupied by the sarcoplasmic reticulum and mitochondria for rapid Ca^{2+} pumping. Stretch-activation is an inherent property of the contractile apparatus (Peckham *et al*., 1990). The evolution of novel IFM-specific isoforms may have been important in this regard as proposed by Reedy *et al*. (Reedy *et al*., 1994) for the TnH C-terminal domains. More recently, phosphorylation of the *Drosophila* regulatory light chain, DmMLC-2, was shown to be necessary for flight, but not for the function of other muscles (Tohtong *et al*., 1995). The presence of an N-terminal extension in this protein led to proposals that it may be involved in establishing actomyosin cross-bridges (Irving *et al*., 2001). Proposals for the existence and role of other potential cross-links between thick and thin filaments involving IFM-specific proteins such as flightin (Vigoreaux *et al*., 1993), or IFM-specific variants of TnT, TnI (TnH) and Tm (TmH) remain to be examined critically.

Drosophila IFM proteins 33 and 34 were identified as potential members of the insect troponin complex because they reacted to monoclonal antibodies raised against *Lethocerus* TnH (Bullard *et al*., 1988). Qiu *et al*. (2003) mentioned that *Lethocerus* TnH is a TnI which contains a pro-ala-gly-glu rich extension, justifying its designation as troponin H. However, characterisation of the *Drosophila Tm1* gene showed that proteins 33 and 34 consist of a tropomyosin-homologous N-terminal domain with pro-ala-gly-glu rich C-terminal domains (Karlik and Fyrberg, 1986; Hanke and Storti, 1988) which share some sequence homology and epitopes within the *Lethocerus* pro-ala-gly-glu rich sequence and we now refer to them as heavy tropomyosins – TmH-33 and TmH-34. Apart from this pro-ala-gly-glu rich domain, the *Drosophila* and *Lethocerus* proteins are not homologous. The C-terminal domains of *D. melanogaster* IFM-specific TmH-33 and TmH-34 (Mogami *et al*., 1982) are encoded by exons 16 and 15 of the *Tm1* gene, respectively. The *Tm2* gene (Karlik and Fyrberg, 1986) encodes standard muscle tropomyosins, isoforms 127 and 129. By alternative splicing the *Tm1* gene also produces a standard muscle tropomyosin isoform, mTm1 (tropomyosin 128), found in several muscles, but not in the IFM, and a cytoskeletal tropomyosin (cTm1) expressed in non-muscle cells. The two genes located in tandem in chromosome region 88F, show a high degree of homology in those exons encoding the common tropomyosin sequences and in similar regulatory elements within intron 1 of both genes (Gremke *et al*., 1993; Meredith and Storti, 1993).

The location and role of TmH-33 and TmH-34 in the IFM sarcomeres are unknown. Tropomyosins normally form a continuous dimeric coiled-coil filament that extends along the thin filament. TmH-33 and TmH-34 may be able to substitute for or dimerize with the standard *Tm2* product (isoform 127) as has been suggested to explain the effects of some *Tm2* gene mutations (Kreuz *et al*., 1996).

In this paper we show that the TmH-33 and TmH-34 are phosphorylated in their C-terminal domains in fully mature flies. Stoichiometric analyses of the major sarcomeric components support the conclusion that all the Tm IFM isoforms, including Tm isoform 127, TmH-33 and TmH-34, are necessary to generate sufficient Tm dimers along the thin filament F-actin core. Moreover, the presence of cysteine residues in the N-terminal part of all these isoforms allowed us to show that TmH-33 and TmH-34 can form disulfide complexes between themselves and with the standard Tm isoform. Therefore, we propose that TmH-33 and TmH-34 are integrated into the thin filament by their N-terminal parts, forming homo- and heterodimers between themselves and with Tm127. We postulate that the C-terminal domains may project towards the thick filament. Such extensions could act either to maintain the thick/thin filament geometry out of phase before stretch as required for the model proposed by Wray (Wray, 1979) or to function as stretch sensors directly affecting the thin filament regulatory mechanism. In other holometabolous insects such as hymenopterans (*Apis*) and hemipterans (*Lethocerus*) where it has been proposed that a stretch-activation may have evolved independently (Pringle, 1981; Dudley, 2000) a similar mechanism may have been achieved by inserting a related sequence into the TnI gene.

Material and methods

Fly stocks

The *Oregon-R* strain was used as wild type *Drosophila* for all experiments.

2D electrophoresis

Single female *Drosophila* thoraces were dissected in cold acetone and homogenised in 8 M urea, 0.5% IGEPAL CA-630, 10 mM dithiothreitol and 0.1% ampholytes (pH range 3–10). Solubilization was performed by agitation at room temperature for 3 h. Samples were centrifuged at 13,000 rpm and the supernatants subjected to 2-D electrophoresis using an IPGphor system (Amersham Pharmacia Biotech). Second

dimension separations were performed on 10% SDS-PAGE gels.

In vivo *phosphorylation of TmH-33 and -34*

Flies were fed for 20 h with 1mCi of ^{32}P added in the food. Thoraces were dissected and processed as described above. The 2-D gels were developed using X-ray films.

Spots corresponding to TmH-33 and TmH-34 were cut from a dry 2-D gel and processed for phosphoaminoacid analysis as previously described (Boyle *et al*., 1991). The amino acid mixture was separated by TLC and the radioactivity detected using X-ray film.

Stains-all staining

2-D gels were fixed with 50% 2-propanol (Merck) with microwave heating and stained in the dark with 0.1% Stains–all (Serva) in 30% 2-propanol, 0.3 M Tris–HCl pH 8.8, 10% formamide (Riedel).

Mass spectrometry experiments

Spots corresponding to TmH-33 and -34 were excised from silver stained 2-D gels of single thoraces and trypsin digested *in gel*. The peptides were extracted several times with sonication in 50% acetonitrile (Sigma) and 0.5% trifluoroacetic acid (Pierce). Samples were concentrated in a Speedvac centrifuge prior to MALDI-TOF and nanospray mass spectrometric analyses.

MALDI-TOF of proteins extracted from 2-D gels was performed with either a Kompact Probe (Kratos-Shimadzu) or a BrukerReflexIII (Bruker-Franzen Analytic GmbH) mass spectrometer (Paradela *et al*., 2000), working in positive ion mode. Samples were microdesalted in a 300 μm diameter nanocolumn (Vydac C-18) in a Smart-HPLC (Amersham Pharmacia Biotech). Peptides with suspected phosphorylations were selected and sequenced by quadrupole nanoelectrospray-ionic trap in a LCQ instrument (Finnigan ThermoQuest) as described previously (Paradela *et al*., 2000). The peptide maps obtained were compared with the Swiss-Prot database.

Stoichiometry analyses

Thoraces and IFM from recently emerged flies were dissected and prepared for SDS gel electrophoresis. For ^{35}S-labelling, newly eclosed flies (with the meconium still visible) were anesthetised (di-ethyl ether) and injected with nanoliter quantities of a high specific activity (0.1 Ci/l) ^{35}S-methionine (Amersham) in *Drosophila* Ringer solution. The gels were stained with Coomassie blue and autoradiographed. The autoradiograph and the blue-stained gel bands were quantified by densitometry. Peaks were analysed taking into account their surface/height/width and the known quantities of methionines and/or lysines/arginines in the different thin filament proteins. Coomassie blue binds to positive charged amino acid residues. The fact that we know the amino acid composition of these proteins makes possible to relate the staining intensity to the protein quantity. Relative mass estimates of IFM and non-IFM thoracic muscles were made from drawings of the thoracic musculature (Miller, 1950) approximating each muscle as a regular geometric body.

Protein immunodetection in reducing/non-reducing 1-D and diagonal gels

Immunodetection was performed using two rat monoclonal antibodies developed and kindly provided by Dr Belinda Bullard (Clayton *et al*., 1998). Antibody MAC144 recognizes an epitope in the IFM-specific C-terminal domain of TmH-34, but not TnH-33 (Cripps and Sparrow, 1992) and a similar sequence in glutathione S-transferase-2, GST-2 (Clayton *et al*., 1998). MAC141 recognizes the common sequence shared by the standard Tm and the two Drosophila TmH.

Dissected thoraces were homogenised in 'York modified glycerol buffer' (Clayton *et al*., 1998) with or without dithiothreitol (Sigma). After centrifugation, pellets were solubilized in Laemmli sample buffer with or without β-mercaptoethanol and heated at 100°C for 5 min. Samples were run on 10% SDS-PAGE gels and transferred onto nitrocellulose in a semi-dry blotting apparatus (Pharmacia). Membranes were incubated with MAC 141 or MAC 144 antibodies, then with goat-anti-rat serum and developed using a chemiluminescent substrate for the ECL system (Amersham).

Diagonal gels were performed by running a non-reducing dimension in a tube gel and a reducing second dimension in a slab gel as described in (Creighton and Chothia, 1989). Detection of cross-linked complexes using MAC144 and MAC141 antibodies was carried out as indicated above.

Protein and genome sequence analysis and comparison

The ClustalW program from ExPASy (Combet *et al*., 2000) was used for protein sequence analysis. Secondary structure predictions were made with the PSI-pred program from ExPASy (Mcguffin *et al*., 2000). Tm gene sequence comparisons were performed using ClustalW or GeneJockey II. *D. melanogaster* and *Anopheles gambiae* sequences were obtained from the NCBI BLAST server (www.ncbi.nlm.nih.org). *D. pseudoobscura* and *Apis* sequences were obtained from the Baylor College of Medicine BLAST server (www.hgsc.bcm.tmc.edu).

Results

Properties of muscle isoforms TmH-33 and -34

Figure 1 shows the sequence comparison of TmH-33 and -34, as well as a secondary structure prediction of

the consensus sequence. The Tm-homologous N-terminal domain, encoded by the common exons, will have a α-helical structure. Cys residues appear within the first 27 amino acids involved in Tm dimerization (indicated with an arrowhead) and could be able to form disulphide bridges in vertebrate striated muscle isoforms (Holtzer, 1986). These conserved residues occur in all isoforms encoded by the *Tm1* and *Tm2* genes By comparison, the C-terminal domain is pro-ala-gly-glu rich and predicted to be an unstructured random coiled extension containing several Thr residues (indicated by asterisks).

The 2-D gels of *Drosophila* thoraces show that TmH-33 and TmH-34 are differentially stained blue with *stains-all* (Figure 2a), similar to other proteins such as MLC or TnT (Tohtong *et al.*, 1995; Domingo *et al.*, 1998). Phosphorylated proteins or Ca^{2+}-binding proteins stain blue with this cationic dye (Myers *et al.*,

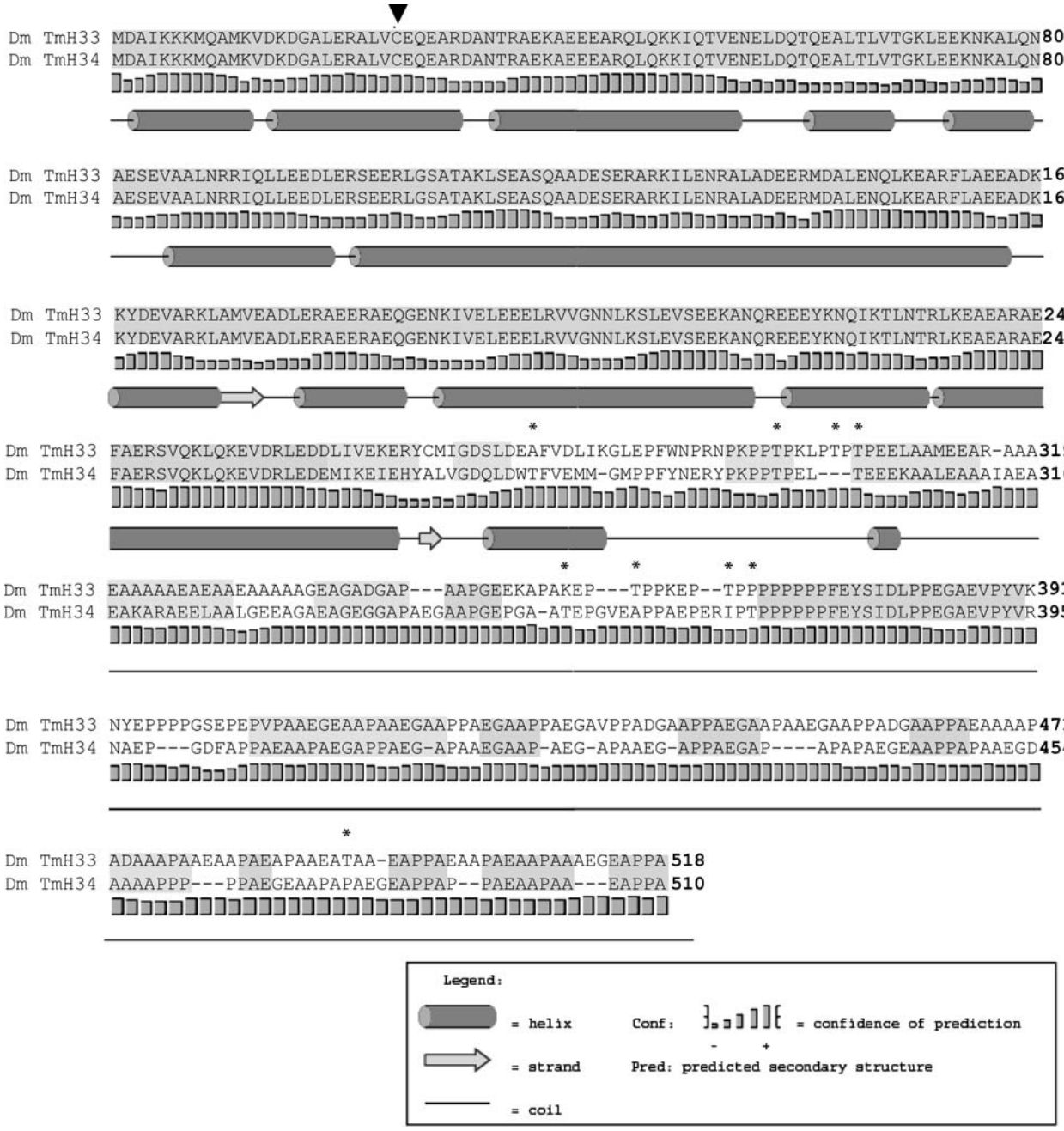

Fig. 1. Sequence comparison of TmH-33 and TmH-34 and secondary structure prediction for the consensus sequence. ClustalW and PSI-pred programs were used to align the sequences and to predict their secondary structure, respectively. For both isoforms, the N-terminal domain has a predicted α-helical structure similar to that of standard Tm; the C-terminal domains are predicted as unstructured random coiled sequences. Arrowheads indicate Cys residues in the N-terminal domain, while asterisks show potential Thr phosphorylation sites in the C-terminal domains. α-helical structures are indicated as cylinders, β-sheets as arrows and unstructured parts as plain lines. High homology sequences are shadowed.

1996; Domingo *et al*., 1998). Calcium overlay experiments did not show any signal from TmH-33 and TmH-34 (data not shown).

Both isoforms 33 and 34 are in vivo *phosphorylated*

The autoradiograph of a 2-D gel of thoraces from flies fed with ^{32}P shows a relatively strong signal from both isoforms TmH-33 and TmH-34 (inset in Figure 2a). This feature is shared with other proteins classically involved in the mechanism of contraction such as TnT (Domingo *et al*., 1998), the myosin regulatory light chains (Warmke *et al*., 1992; Tohtong *et al*., 1995), flightin (Vigoreaux and Perry, 1994) and paramyosin (Liu *et al*., 2005). The phospho-amino acid analysis of the protein spots by thin layer chromatography of the residues and comparison with non-radioactive markers showed that the phosphorylation occurs on threonine residues (data not shown).

TmH-33 and TmH-34 are phosphorylated in fully mature flies

Mass spectrometry techniques were used to identify the specific amino acid that is phosphorylated. Peptides maps (Figure 3) from MALDI-TOF analysis of the tryptic peptides from spots corresponding to TnH-33 and 34 excised from silver stained 2D gels (Figure 2c) and comparison of the maps to those derived from databases confirmed the proteins as TmH-33 and -34. In addition, to establish the sites of post-eclosional phosphorylation, comparisons were made between IFM proteins from recently emerged flies, minutes after eclosion, and 2-day-old, mature flies. The data from TmH-33 in mature flies showed two new peaks not present in the reference peptide set, with masses consistent with two contiguous peptides, Asn293-Lys300 and Leu301-Arg316, if each contained a single phosphate moiety. Only the non-phosphorylated forms of these peptides were detected in recently-emerged flies. Moreover, TmH-34 from fully mature flies produced a peak with a mass consistent with a Tyr292-Lys305 peptide, containing a single phosphorylation. The last peptide is similar in sequence position to the modified Asn293-Lys300 found in TmH-33 (See alignment in Figure 1). So these analogous peptides, Tyr292-Lys305 from TmH-34 and Asn293-Lys300 from TnH-33, are both phosphorylated after eclosion. Note that all three phosphorylated peptides are located in the IFM-specific domain of each protein. Several Thr residues occur in this domain (Figure 1), but only specific residues appear capable of being phosphorylated, namely, T297 and T302 in TmH33 and T297 in TmH34.

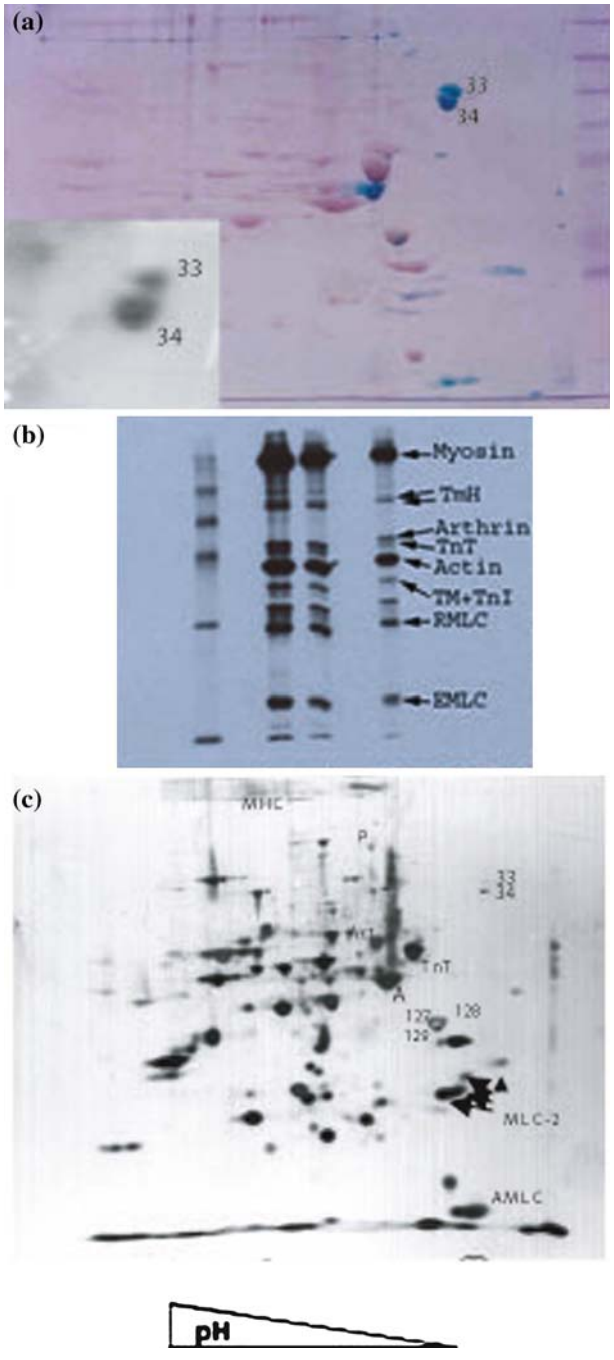

Fig. 2. TnH-33 and -34 phosphorylation analysis. (a) 2-D gel stained with *Stains-all*, showing TmH-33 and -34 staining blue, a differential staining property of phosphorylated and Ca^{2+}-binding proteins. Other phosphorylated isoforms including TnT and myosin light chains also appear differentially stained. Inset: Autoradiography of a 2-D gel of thoraces of flies fed with ^{32}P showing the *in vivo* phosphorylation of TmH-33 and TmH-34 (detail). Protein spots were excised from the gel, proteolysed, separated by TLC as described (Boyle *et al*., 1991)and the radioactivity confirmed as phosphorylation of threonine residues (data not shown). (b) S^{35}-Methionine labeling of the thorax proteins. The four lanes correspond to the molecular weight markers and three differentially loaded (4×, 2× and 1×) of the proteins. The label was normalized as described in the legend of Table 1. (c) 2-D electrophoresis (pH range 3–10) of single female *Drosophila* thorax stained with silver nitrate using the Mann method (36), compatible with mass spectrometry analyses. Main muscle proteins detected are myosin heavy chain (MHC), paramyosin (P), arthrin (Art), actin (A), troponin T (TnT), standard Tms – (127, 129), muscle Tm2 – (128), myosin light chains (MLC-2, AMLC), TmH-33 and TmH-34.

The residues that are phosphorylated in mature flies, but not in newly eclosed flies, were identified by nano-electrospray-ionic trapping of the phosphorylated peptides. This confirmed that the phosphorylations occur at specific Thr residues. The fragmentation spectra of each peptide are shown in Figure 3.

Stoichiometric *proportion of the sarcomere components in Drosophila IFM*

The IFM of dipterans almost completely fill the thorax. We estimate that they occupy at least 75% of the total muscle volume, even without taking into account the solidity of the fibrillar muscles vs. the more sparse organization of the non-fibrillar ones. The hypodermal musculature is insignificant by comparison and the major tubular muscle in the thorax, the tergal depressor of the trochanter (TDT) occupies at most only 15% of the volume. The protein bands in 1-D SDS-PAGE gels of dissected IFMs and whole thoraces of *Drosophila* are almost indistinguishable. Therefore, we used dissected thoraxes to continue the analysis. We have established the stoichiometry of the main thin filaments proteins of *Drosophila* IFM using two

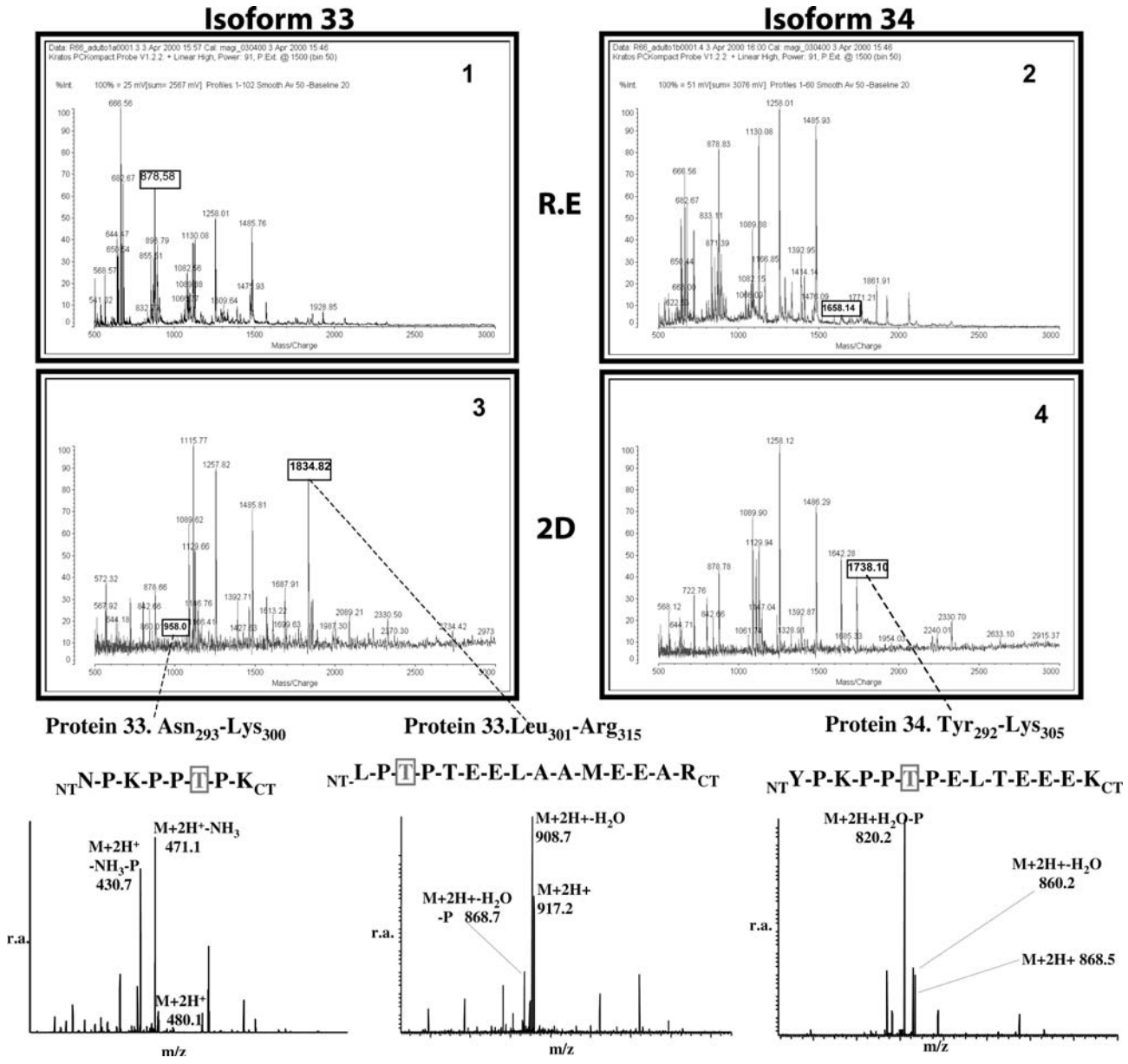

Fig. 3. Mass Spectrometry analysis of TmH-33 and TmH-34. MALDI-TOF analyses of spots 33 and 34 were excised from silver-stained 2-D gels of single thoraces from recently eclosed (RE) and mature (2D) female flies. Peptide maps from trypsin digestions of TmH-33 (1, 3) and TmH-34 (2, 4) of recently emerged (1, 2) and fully mature flies (3, 4) are shown. (1) $M/Z = 878.58$ fragment corresponds to the Asn293-Lys300 non-phosphorylated peptide. (2) $M/Z = 1568.14$ fragment, corresponds to the Tyr292-Lys305 non-phosphorylated peptide. (3) $M/Z = 958.0$ and $M/Z = 1834.82$ are the Asn293-Lys300 and Leu301-Arg316 phosphorylated peptides, respectively. (4) $M/Z = 1738.10$ fragment, corresponding to the Tyr292-Lys305 phosphorylated peptide. Fragmentation spectra of the three phosphorylated peptides are shown at the bottom. Phosphorylated Thr residues from fully mature flies peptides are in grey.

approaches: (a) by measuring the incorporation of ^{35}S-methionine into the sarcomeric proteins and normalising the data using their known contents of this amino acid, and (b) the intensity of Coomassie blue staining related to the known proportions of basic amino acids (lysine and arginine) in these proteins. The data (Table 1) show some variation between the experimental approaches and methods of analysis used, but overall indicate common stoichiometric ratios. When the numbers are normalized to 14 actin molecules, the number of actin monomers in the repeating unit of the thin filament (Filatov *et al*., 1999), the mean value gives two molecules of arthrin (ubiquitinated actin) for every 12 molecules of actin. The repeating unit should accommodate 4 molecules of Tm, one dimer in each F-actin groove, each pair associated with one troponin complex. This fits with a TnT number close to this value, but Tm does not reach a value of 4, unless the TmH-33 and 34 molecules are included in the calculation. The more important variations relate to the amounts of arthrin which range from less than 2 to 3 per 14 actins. The number of TmH-33 and -34 molecules approaches the stoichiometric levels given above, although usually, but not always, TmH-33 is more prevalent than TmH-34. The model suggested by these measurements is also supported by the following analysis.

TmH-33 and TmH-34 form protein complexes with standard tropomyosins in vivo

Sequence homology between the N-terminal domains of TmH isoforms and Tm127 argues that they will form homodimers or heterodimers and be integrated into the IFM thin filament structure as tropomyosin dimers. The stoichiometry results support both this proposal. Cysteine residues occur in the conserved N-terminal domain of these *Drosophila* Tms suggesting that, as has been described in vertebrate skeletal muscle (Holtzer, 1986), IFM Tm coiled-coils could be stabilized *in vivo* by intermolecular disulfide bridges. These have been established in insect muscle paramyosin (Vinos *et al*., 1991). In non-reducing gels but not in reducing gels (Figure 4a), higher apparent MW complexes are detected by the monoclonal antibodies MAC141 and MAC144 that recognize the common Tm part and the C-terminal domain of TmH-34, respectively.

Diagonal gel electrophoresis, with a non-reducing 'first dimension' followed by a reducing slab 'second dimension' was performed on IFM samples to determine which of the tropomyosins are forming disulphide complexes with each other. Under non-reducing conditions the disulfide bridge containing complexes appear delayed, but after the reducing second dimension these proteins will appear off the theoretical diagonal, given by a straight line from the origin of the gel and the signal from the monomeric forms (GST-2 and Tm). Immunodetection of two parallel diagonal gels using each monoclonal antibody are represented in Figure 4b. MAC144 reveals a large spot of a small molecular weight protein on the diagonal, which corresponds to GST-2 and a minor GST-2 fraction corresponding to the previously described interaction between TmH-33 and/or -34 and GST-2 (Clayton *et al*., 1998) that is apparently only released under reducing conditions. The MAC144 antibody further identifies dimers of TmH-33 and TmH-34. The MAC141 antibody clearly shows that a large fraction of tropomyosin 127 lies on the diagonal and must therefore derive from dimers in which cysteine bridges remained unformed in non-reducing conditions. This makes it impossible to completely track the whole dimeric Tm complement. The MAC141 antibody clearly detected heterodimers of TmH-33 or -34 with Tm127. The intensity of the spots detected off the diagonal with the antibodies indicates that, ignoring the monomeric Tm127, at least 25% of Tm (low and high molecular weight) is homodimers of Tm127 forms, 50% is present in heterodimers of Tm127 with the

Table I. Stoichiometry of thin filament components in IFM

Components	Methionines in the molecule	Exp 1	Exp 2	Exp 3	Exp 4	Lysines and arginines in the molecule	Exp 5	Exp 6	Mean ± standard deviation	Model
Actin[a]	15	11.92	12.01	12.02	12.14	45	10.06	9.56	11.29 ± 1.56	12
Arthrin[a]	16	2.08	1.9	1.98	1.86	57	2.94	4.44	2.53 ± 1.02	2
Tn-T	6	2.47	2.2	2.52	1.05	85	1.9	1.74	1.98 ± 0.55	2
Tm[b]	7	1.26[b]	1.54[b]	1.42[b]	2.66[b]	49	3.23[b]	2.84[b]	2.16 ± 0.85[b]	2
TnI[b]	4	2.47[b]	2.2[b]	2.52[b]	1.05[b]	54	1.9[b]	1.74[b]	1.98 ± 0.55[b]	2
Isoform 33	7	1.18	1.2	1.34	0.8	58	1.6	1.12	1.21 ± 0.26	1
Isoform 34	9	0.96	0.92	0.86	0.62	56	0.96	1.68	1 ± 0.36	1
Tm + 33 + 34		3.40	3.64	3.64	4.08		5.85	5.64	4.38 ± 1.09	4

[a]The quantitative information obtained from the densitometry of the autoradiographies (in the case of the ^{35}S-labelling (Figure 2b) or the Coomassie gels) was normalized so that the addition of the arthrin and actin numbers sum to 14). The actual value of methionines, lysines and arginines of arthrin were obtained by adding to actin the value found in *Drosophila* ubiquitin.

[b]The band which allows the quantification of the low molecular weight Tm also contains TnI (Kronert *et al*., 1995). To quantify the actual level of Tm in the different experiments, we have assumed that the actual number of TnI molecules match the number of TnT molecules and taken into account the number of amino acids, we have obtained the numbers of Tm molecules listed in the table by subtracting the contribution of the TnI from the total of the band.

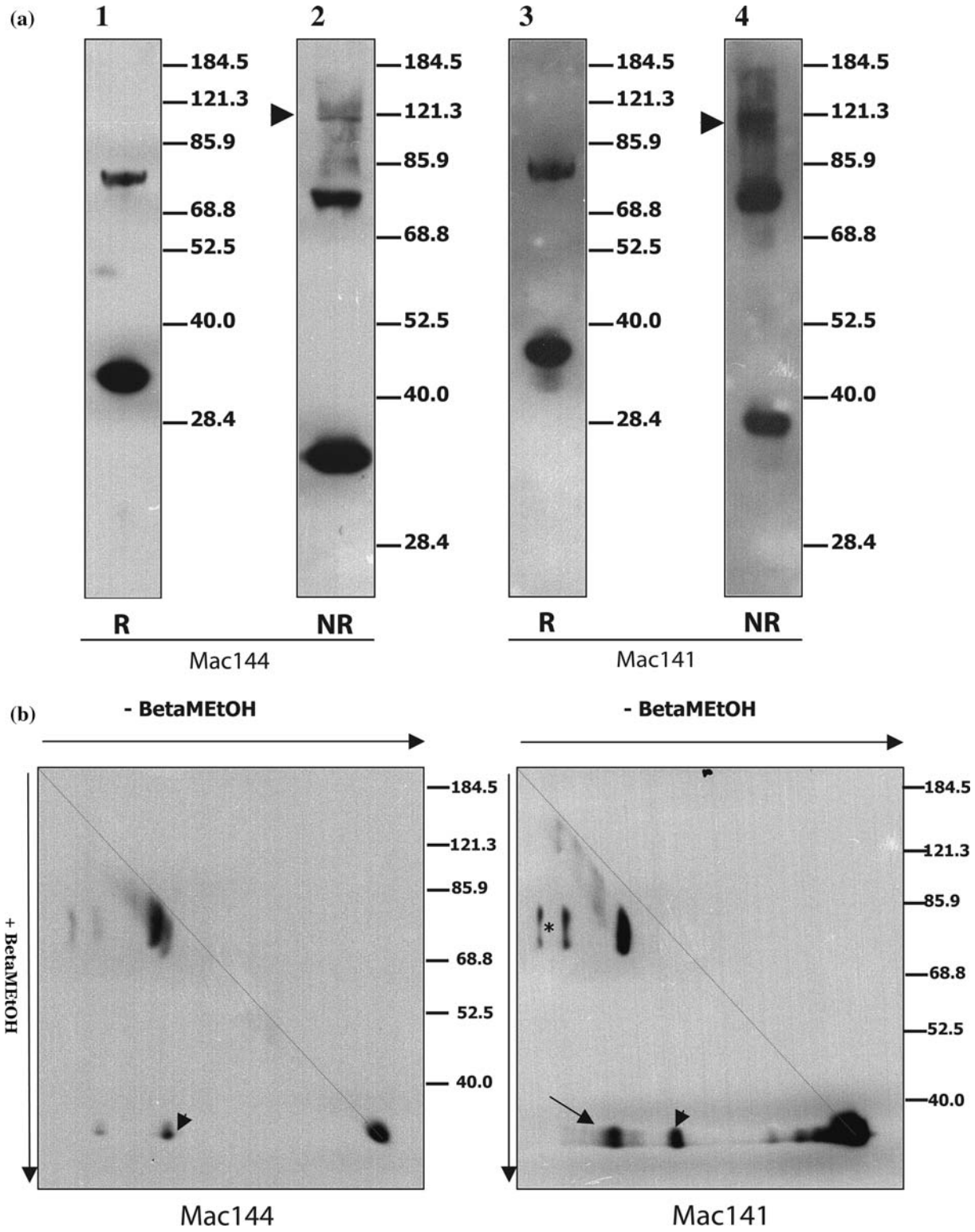

Fig. 4. Analysis of the tropomyosin complexes in non-reducing and diagonal gels. (a) Protein extracts from fully mature flies run in polyacrylamide gels in reducing (R, 1 and 3) and non-reducing (NR, 2 and 4) conditions. After transfer to membranes protein immunodetection used MAC144 (1, 2) or MAC141 (3, 4). MAC144, recognizes the IFM- GST-2 and the C-terminal domain of TnH-34 (1). Under non-reducing conditions it detected a larger complex (arrow head), apparent MW $\approx$ 120 kDa. MAC141, recognizes the common Tm sequence; TmH-33, TmH-34 and standard Tm (protein 129) were detected (3), but in non-reducing conditions a complex of apparent MW $\approx$ 120 kDa was also detected (arrow head). (b) For a more detailed analysis of these interactions, diagonal gel electrophoresis of protein extracts from fully mature flies was run. The gels were transferred and immunodetection was performed using MAC144 (1) and MAC141 (2). Several signals below the theoretic diagonal (given by a straight line from the origin of the gel to the msignal of the monomeric low MW isoform) were detected. Using MAC144, homodimers of standard Tm were detected (short arrow), as well as heterodimers between standard Tm and TmH-33 and/or -34 (long arrow). Dimers between both TmH-33 and -34 are also present (asterisk). In addition, the previously described interaction between GST-2 and TmH-33 and TmH-34 is detected (short arrow) using Mac141. No signal from monomeric TmH-33 and TmH-34 is detected, while a significant fraction of Tm127 appears to be in monomeric form.

TmH-33 and -34 IFM-specific isoforms, and 25% is present as a significative fraction of Tm forms complexes *in vivo* as either homodimers of Tm127 or heterodimers of Tm127 and TmH-33 and -34 or homodimers of these large isoforms.

The interactions determined in these experiments confirm that a significant fraction of TmH-33 and -34 is integrated into the thin filament as high molecular weight tropomyosins, as was already implied from the high homology of their N-terminal parts and secondary structure predictions of a high α-helical coiled coil N-terminal Tm domains for the TmH-33 and -34 (Figure 1). A model in which each Tm-Tn complex contains a heterodimeric tropomyosin (Tm127 + Tm isoform 33 or 34) is made untenable by our detection of Tm127 homodimers. Together with the existence of Tm127 + TmH-33/34 heterodimers and heavy Tm dimers, this observation means that Tm-Tn complexes along the thin filament will be non-identical. TmH-33 contains a cysteine within the C-terminal part of the Tm domain which could conceivably generate cysteine bridges in the Tm-overlap region and thereby some of the heterodimers, but in the absence of data on the structure of the overlap region, we consider this unlikely.

The Drosophila melanogaster *Tm gene organization is conserved in* Drosophila pseudooboscura *and* Anopheles gambiae *but not* Apis mellifera

If *Drosophila* TmH-33 and -34 play essential roles in the activation of IFM contraction, then the *Tm1* gene and its IFM-specific exons are likely to be conserved and functional in other dipterans. This idea is sustained by observations that TmH-like proteins occur in the flight muscles of many insects (Peckham *et al*., 1990; Reedy *et al*., 1994), but only in the Diptera are they detected by an antibody recognizing Tm sequences. By BLAST searches of the recently sequenced genomes of *A. gambiae* and *D. pseudoobscura* with the *D. melanogaster Tm1* and *Tm2* gene exons the entire *Tm1*/*Tm2* genomic muscle complement was identified in these two dipterans (Figure 5a). Homology between the exons encoding the tropomyosin sequences is very high and extends into the IFM-specific TmH exons 15 and 16. The overall *Tm1-Tm2* gene structure and sequences are similar though differences are detected. First, the sequence containing the two genes is larger in *D. pseudoobscura* (37 kb) and in *A. gambiae* (49 kb) than in *D. melanogaster* (33.6 kb), especially in *A. gambiae* where the *Tm1* gene homologue covers more than 38 kb. The larger gene size is due largely to longer introns and intergenic region between the two *Anopheles Tm* genes and may reflect the larger genome of *Anopheles* compared to *D. melanogaster*. Second, in *A. gambiae* there is only one IFM-specific exon, which is more closely related to the *D. melanogaster* exon 16 than to exon 15 and finally, in *Anopheles* the *Tm1* exons 2 (including the initiating codon) and 3 appear fused. The *D. melanogaster Tm1* gene organization and exon splicing of the cytoplasmic isoform (cTm1) and the muscle-specific isoform (mTm1) are conserved in the three Diptera. Organization of the dipteran *Tm2* genes is also conserved, as is their close chromosomal linkage with the *Tm1* gene. In *Anopheles*, the large first translated *Tm2* exon of the *Drosophilidae* is split into two exons.

In other insect's orders (Peckham *et al*., 1990), a high molecular weight protein containing the anti-TnH-34 epitope is not linked to a standard Tm sequence. BLAST searches of the *Apis mellifera* sequenced genome revealed two genes, one homologous to *Tm1* the other to *Tm2*. One contains specific exons homologous to the dipteran cytoplasmic Tm, as well as *Tm1* muscle-specific and common exons (Figure 5a). The *Tm2* orthologue shows a further division of exon 2 in *Apis* into three exons (2a, b and c). Using the completed *Apis mellifera* genome sequence we confirmed the absence of TmH-specific exons in the *AmTm1* and the *AmTm2* genes and the presence, but in the *AmTnI* gene (Herranz *et al*., 2005b), of additional exons encoding a C-terminal domain containing a repetitive APPAEGA motif identical to the one found in the dipteran TmH. Qiu *et al*. (2003) have reported a similar ala-pro-gly-glu rich sequence in TnI of *Lethocerus*. Interestingly, the *Apis Tm1* gene orthologue has two final muscle-specific exons, the first one (15) highly homologous to the initial polypeptide sequence of the *Drosophila* ala-pro-gly-glu rich exon 16, suggesting this sequence as the site for the evolutionary insertion of the longer ala-pro-gly-glu rich sequences specific to the dipteran flight muscles. The final exons of the bee *TnI* gene encode sequences homologous to the C-terminal domains of TmH-33 and -34 (Figure 5b). Although somewhat different from the dipteran C-terminal TmH domains, their similarity can easily be seen, including conservation of the phosphorylatable threonine residues (indicated by asterisks). The phosphorylated residues of the TmH C-terminal domain in *D. melanogaster* are conserved in the other two dipteran species, as are the predicted protein secondary structures (data not shown).

Discussion

Drosophila *troponin H or tropomyosin H?*

The IFM-specific proteins 33 and 34 were designated as TnH-33 and TnH-34 (Cripps and Sparrow, 1992) because of their interaction with a monoclonal antibody raised against heavy troponin (TnH) of the *Lethocerus* flight muscle troponin complex (Reedy *et al*., 1994). As these are clearly products of the *Tm1* gene, and we have described homologous proteins from two other diptera, this nomenclature is confusing. We propose that these 'heavy tropomyosin' products be referred to as TmH-33 and TmH-34 in

D. melanogaster, while in *Lethocerus* and *Apis* where the common domain sequences have been identified in TnI isoforms (Qiu *et al.*, 2003; Herranz *et al.*, 2005b), their designation as TnH should be retained.

Physiological roles of phosphorylation of TmH and other IFM proteins

We have described the *in vivo* phosphorylation of the two *Drosophila* IFM-specific proteins TmH-33 and -34. Phosphorylation is readily detectable in 2-day old mature flies but not in recently emerged ones incapable of flight. This suggests that phosphorylation of Thr residues in the C-terminal domains may be required for flight. Phosphorylation of many *Drosophila* muscle proteins such as the myosin regulatory light chain (Tohtong *et al.*, 1995), flightin (Vigoreaux and Perry, 1994; Vigoreaux, 2001), troponin T (Domingo *et al.*, 1998), [Nongthomba and Sparrow, pers. comm.], miniparamyosin (Maroto *et al.*, 1996) and projectin (Maroto *et al.*, 1992; Ayme-Southgate *et al.*, 1995) may be required for IFM function. Only in the case of the *Drosophila* regulatory light chain (DmMLC-2) have alanine substitutions of phosphorylatable serines (Tohtong *et al.*, 1995) demonstrated that phosphorylation may be required for normal power output and flight ability, although myofibrillogenesis and the calcium-activated force development appeared normal. The effects are probably due to an interaction between regulatory light chain and actin, similar to the essential light chain–actin interaction in vertebrates (Trayer *et al.*, 1987; Henkin *et al.*, 2004). Our observations of TmH phosphorylation after eclosion mirror the multiple phosphorylations of flightin during this period (Vigoreaux and Perry, 1994) and it is likely both are important for flight ability.

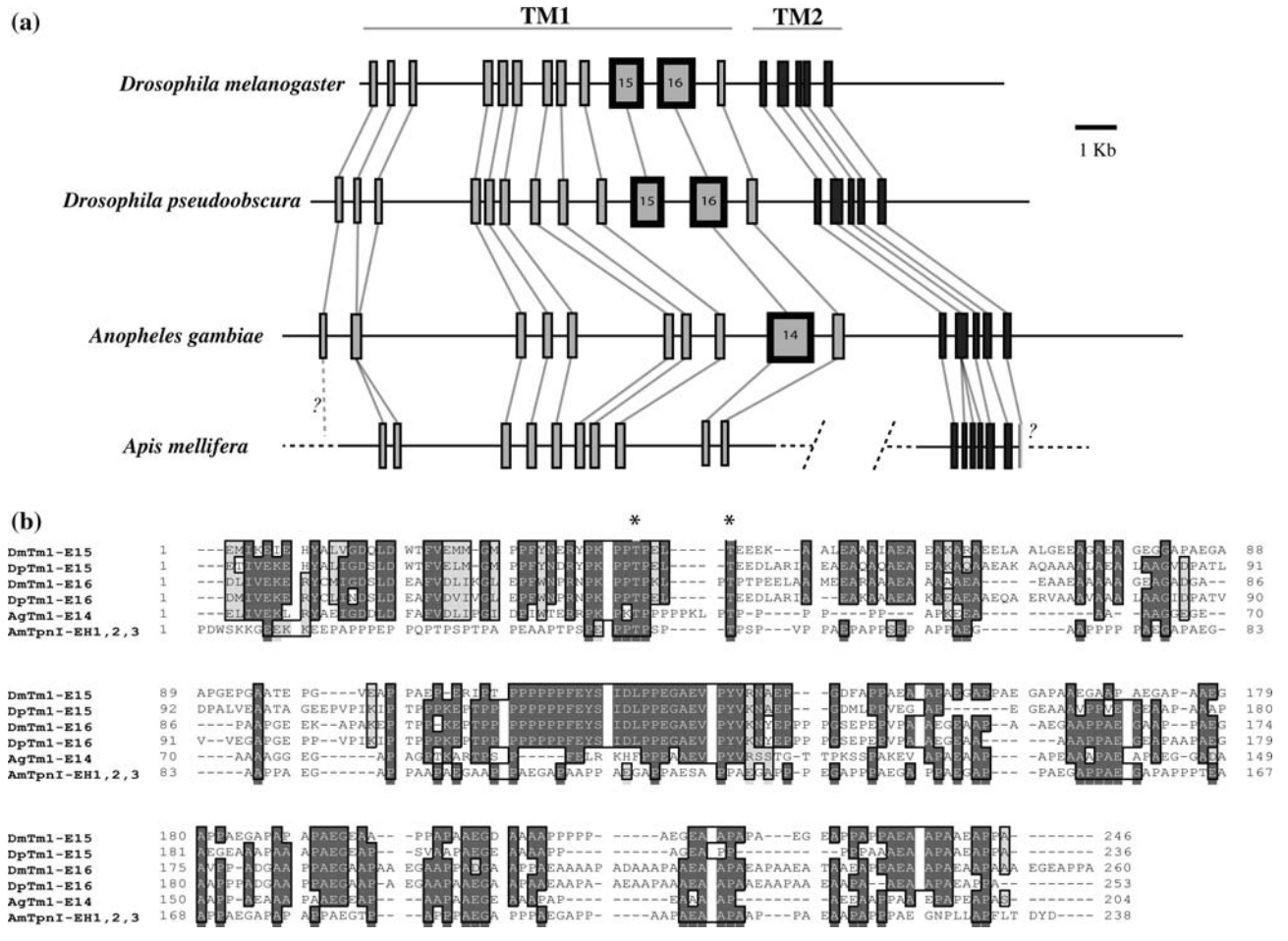

Fig. 5. The Tm gene complement is conserved in *Drosophila pseudoobscura* and *Anopheles gambiae* but not in *Apis mellifera*. (a) Comparison tropomyosin gene structures in *Diptera* and *Hemiptera*. Only muscle exons are shown for clarity. All genes contain a similar set of additional exons encoding cytoplasmic Tm (cTm-specific exons 4, 6, 10 and 14 of *Drosophila* are not shown). Exons common to both cytoplasmic and muscles isoforms are shown here (5, 10 and 13 of *Drosophilidae*). Even though gene structure is similar, slight differences are detected. In *Anopheles*, exons 2 and 3 are fused and there is only one IFM-specific exon. The genomic region is also bigger in this organism, due to its larger introns. *Apis* Tm genes lack IFM-specific domain on TmH; these occur in the *Apis* TnI gene (Herranz *et al.*, 2005b). The 3′ *Tm1* exons (2 in *Anopheles* and *Apis* and 3 in *Drosophilidae*) are alternatively spliced. The initial part of exon 16/14 of Diptera is highly analogous to the last but one exon of *Apis*. (b) Sequence comparison of *Anopheles* differential exon, *Drosophila* species exons 15 and 16 and exons H1, H2 and H3 of the *Apis* TnI gene. Boxes indicate high homology regions. Phosphorylated Thr residues (asterisks) are conserved in the other two species.

IFM thin filament stoichiometry and structure

Protein stoichiometry is important in defining the structure of a macromolecular complex. Our data confirm for the IFM thin filament that each F-actin 14-monomer unit contains two molecules of each troponin component (TnT, TnI, and probably, TnC). Homology to vertebrate thin filaments indicates there should be two Tm dimers per unit. Our stoichiometry data shows only two molecules of standard length Tm per repeat. However, there are two molecules per unit of the TmH-33 and -34, each with a standard Tm sequence as their N-terminal domains. This suggests that each IFM thin filament 14 actins repeat could be associated with two heterodimeric (Tm + TmH) tropomyosins. However, analysis of cysteine crosslinks between Tm and TmH isoforms suggest they do not just form heterodimers, but homodimers of Tm and TmH isoforms occur as well as was argued by Kreuz *et al.* (1996). So non-identical thin filament repeats in terms of Tm isoform content are probable.

The TmH-Tm interactions demonstrated in this report imply that the TmH N-terminal domains of function as *bona fide* tropomyosins. The role of the TmH C-terminal domains remains unidentified. The occurrence of novel proteins in the IFM sarcomeres (e.g. flightin), or unusual forms of proteins (TmH, arthrin, TnT, TnI) with otherwise distinct vertebrate striated muscle protein homologies, in muscles (IFM) that are stretch-activated leads inevitably to speculation that these two observations are functionally related. Recent studies suggest that stretch-activation is due to stretch-induced distortion of attached crossbridges relieving the steric blocking by tropomyosin of myosin binding sites on actin (Linari *et al.*, 2004). These authors propose that the stretch distorted, calcium-activated crossbridges displace tropomyosin by IFM-specific structural interactions within the tropomyosin-troponin regulatory complex. If so, it still remains unclear what the specific role TmH, and the C-terminal extension may be in stretch activation. No *Drosophila Tm1* gene mutant affecting flight muscle function has been recovered so far. From genetic studies using a deletion which removes both *Tm1* and *Tm2* genes and nonsense mutations within the *Tm2* gene, Kreuz *et al.* (1996) concluded that while flies heterozygous for the deletion affected important IFM functions (from wing beat frequency, myofibrillar organization, to skinned fibre mechanics), by comparison to the *Tm1* mutant heterozygotes most of these effects were explicable by a 50% gene dose reduction of *Tm2* rather than a similar *Tm1* reduction. This suggests that a single *Tm1* gene copy is sufficient to cover the role(s) of TmH-33 and TmH-34 in asynchronous muscle contraction.

The location and function of the C-terminal domain is much less clear. The related domain in *Lethocerus* TnI is readily cleaved and released from skinned myofibrils by mild calpain treatment (Bullard *et al.*, 1988) and immunogold labelling showed a close association of the *Lethocerus* TnH epitope with the rear myosin chevron in rigor muscle (Reedy *et al.*, 1994). These observations suggest that this domain may be extended outwards from the thin filament raising the possibility of inter-filament cross-links, perhaps additional to the actomyosin cross-bridges. The *Drosophila* TmH C-terminal domain also binds GST-2 (Clayton *et al.*, 1998) as also detected in our diagonal gels (Figure 4B left). This interaction may be structural, but GST-2 enzymatic activity towards lipid substrates (Singh *et al.*, 2001) may also provide protection against the deleterious effects of oxidative stress in IFM.

Comparative evolution of the Tm1/Tm2 gene complements in holometabolous insects

Comparison of the Tm sequences within the *Tm1* and *Tm2* genes shows that they likely arose by duplication from a single gene, although the situation with the *Tm1* gene is complex as different gene products, cytoplasmic and several muscle specific isoforms, are coded by both common and alternatively spliced exons. By sequence homology, we have identified the entire Tm gene complement in *D. pseudoobscura*, *A. gambiae*, and *Apis mellifera*. In the dipterans the *Tm1* genes are highly conserved and encode isoforms similar in sequence and predicted structure to TmH-33 and -34 (Figure 5).

The *Apis* genome contains two tropomyosin genes, *Tm1* and *Tm2*. Compared to the diptera the *Apis Tm2* gene is subdivided into more exons. Interestingly, neither gene contains the pro-ala-gly-glu rich sequences typical of dipteran TmH but the *Tm1* gene includes two final small exons, one highly homologous to the initial residues in the *Drosophilidae* exon 16 and *Anopheles* exon 14. In *Apis* the pro-ala-gly-glu rich sequences are found in three exons (H1, H2 and H3) at the end of the TnI gene (Figure 5B) (Herranz *et al.*, 2005b) in agreement with earlier data (Peckham *et al.*, 1990) and information from the hemipteran *Lethocerus* (Qiu *et al.*, 2003).

The data suggest an evolutionary pathway for the acquisition of these pro-ala-gly-glu rich sequences in holometabolous insects. The primitive tropomyosin genes may have been similar to that currently found in *Apis*. In dipterans the extra sequence was initially inserted coupled to the penultimate muscle exon producing a configuration similar to that now found in *Anopheles*. Finally in the *Drosophilidae*, this modified exon was duplicated producing the TmH-33 and TmH-34 isoforms. The functional implications of this putative duplication in drosophilid evolution are unknown.

The distribution of asynchronous flight muscles in different insect taxa suggests that stretch-activation mechanisms may have evolved independently several times (Pringle, 1981). The appearance of multiple IFM-specific components and isoforms (Vigoreaux, 2001; Herranz *et al.*, 2005b) may be an obvious consequence

of this, though the structure–function relationship between these proteins and stretch-activation remains elusive. Similar physiological mechanisms may have been achieved by different evolutionary pathways. An example may be the conservation of the pro-ala-gly-glu rich domain in *Apis* and *Lethocerus* TnI and dipteran TmH. Such conservation argues for an important function for these domains in IFM physiology, but with flexibility as to whether this domain is associated with TnI (*Apis*, *Lethocerus*) or Tm (Diptera). It remains unclear whether these IFM-specific pro-ala-gly-glu rich extensions are involved in interfilament crosslinks, or with specific cross-bridges as shown by the close proximity of this domain and the rear myosin rigor chevron in *Lethocerus* muscle. The importance of completing the description of the molecular properties of the IFM sarcomere components and testing realistic structural models is clear if wish to understand the subtleties involved in the structure–function relationship in these high performance muscles.

Acknowledgements

We thank Dr Belinda Bullard for supplying the monoclonal antibodies, Dr. Jesús Vázquez and Anabel Marina for help with the MALDI-TOF analysis and Aida Villa for technical help. This research was supported by grants from the Spanish Government PB96-0069, ESP1999-0379-C02, ESP-2001-4521-PE, ESP2002-11913-E and ESP2003-09475-C02-01. JCS was funded by the BBSRC (UK). R. Herranz was supported during this work by a FPI fellowship of the Spanish Ministery of Science and Technology.

References

Ayme-Southgate A, Southgate R, Saide J, Benian GM and Pardue ML (1995) Both synchronous and asynchronous muscle isoforms of projectin (the Drosophila bent locus product) contain functional kinase domains. *J Cell Biol* **128:** 393–403.

Barbas JA, Galceran J, Krah-Jentgens I, de la Pompa JL, Canal I, Pongs O and Ferrus A (1991) Troponin I is encoded in the haplolethal region of the Shaker gene complex of Drosophila. *Genes Dev* **5:** 132–140.

Bautch VL, Storti RV, Mischke D and Pardue ML (1982) Organization and expression of Drosophila tropomyosin genes. *J Mol Biol* **162:** 231–250.

Boyle WJ, Geer P and Hunter T (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Meth. Enzymol.* **201:** 110–149.

Bullard B, Leonard K, Larkins A, Butcher G, Karlik C and Fyrberg E (1988) Troponin of asynchronous flight muscle. *J Mol Biol* **204:** 621–637.

Clayton JD, Cripps RM, Sparrow JC and Bullard B (1998) Interaction of troponin-H and glutathione S-transferase-2 in the indirect flight muscles of Drosophila melanogaster. *J Muscle Res Cell Motil* **19:** 117–127.

Combet C, Blanchet C, Geourjon C and Deleage G (2000) NPS@: network protein sequence analysis. *Trends Biochem Sci* **25:** 147–150.

Craig R and Lehman W (2001) Crossbridge and tropomyosin positions observed in native, interacting thick and thin filaments. *J Mol Biol* **311:** 1027–1036.

Creighton TE and Chothia C (1989) Protein structure. Selecting buried residues. *Nature* **339:** 14–15.

Cripps RM and Sparrow JC (1992) Polymorphism in a Drosophila indirect flight muscle-specific tropomyosin isozyme does not affect flight ability. *Biochem Genet* **30:** 159–168.

Domingo A, Gonzalez-Jurado J, Maroto M, Diaz C, Vinos J, Carrasco C, Cervera M and Marco R (1998) Troponin-T is a calcium-binding protein in insect muscle: in vivo phosphorylation, muscle-specific isoforms and developmental profile in Drosophila melanogaster. *J Muscle Res. Cell. Motil.* **19:** 393–403.

Dudley R (2000) The evolutionary physiology of animal flight: paleobiological and present perspectives. *Annu Rev Physiol* **62:** 135–155.

Filatov VL, Katrukha AG, Bulargina TV and Gusev NB (1999) Troponin: structure, properties, and mechanism of functioning. *Biochemistry (Mosc)* **64:** 969–985.

Fyrberg E, Fyrberg CC, Beall C and Saville DL (1990) Drosophila melanogaster troponin-T mutations engender three distinct syndromes of myofibrillar abnormalities. *J Mol Biol* **216:** 657–675.

Geeves MA and Holmes KC (1999) Structural mechanism of muscle contraction. *Annu Rev Biochem* **68:** 687–728.

Gordon AM, Homsher E and Regnier M (2000) Regulation of contraction in striated muscle. *Physiol Rev* **80:** 853–924.

Gremke L, Lord PC, Sabacan L, Lin SC, Wohlwill A and Storti RV (1993) Coordinate regulation of Drosophila tropomyosin gene expression is controlled by multiple muscle-type-specific positive and negative enhancer elements. *Dev Biol* **159:** 513–527.

Hanke PD and Storti RV (1988) The Drosophila melanogaster tropomyosin II gene produces multiple proteins by use of alternative tissue-specific promoters and alternative splicing. *Mol Cell Biol* **8:** 3591–3602.

Henkin JA, Maughan DW and Vigoreaux JO (2004) Mutations that affect flightin expression in Drosophila alter the viscoelastic properties of flight muscle fibers. *Am J Physiol Cell Physiol* **286:** C65–C72.

Herranz R, Mateos J and Marco R (2005a) Diversification and independent evolution of troponin C genes in insects. *J Mol Evol* **60:** 31–44.

Herranz R, Mateos J, Mas JA, García-Zaragoza E, Cervera M. and Marco R (2005b) The Co-Evolution of Insect Muscle TpnT and TpnI Gene Isoforms. *Mol Biol Evol* **22:** 1131–1142.

Holtzer A (1986) Phenomenological analysis of the kinetics of the production of interchain disulfide cross-links in two-chain, coiled-coil proteins by reaction with 5,5'-dithiobis(2-nitrobenzoate). *Biochemistry* **25:** 3008–3012.

Irving T, Bhattacharya S, Tesic I, Moore J, Farman G, Simcox A, Vigoreaux J and Maughan D (2001) Changes in myofibrillar structure and function produced by N-terminal deletion of the regulatory light chain in *Drosophila*. *J Muscle Res Cell Motil* **22** (8): 675–683.

Karlik CC and Fyrberg EA (1986) Two Drosophila melanogaster tropomyosin genes: structural and functional aspects. *Mol Cell Biol* **6:** 1965–1973.

Kreuz AJ, Simcox A and Maughan D (1996) Alterations in flight muscle ultrastructure and function in Drosophila tropomyosin mutants. *J Cell Biol* **135:** 673–687.

Kronert WA, O'Donnell PT, Fieck A, Lawn A, Vigoreaux JO, Sparrow JC and Bernstein SI (1995) Defects in the Drosophila myosin rod permit sarcomere assembly but cause flight muscle degeneration. *J Mol Biol* **249:** 111–125.

Linari M, Reedy MK, Reedy MC, Lombardi V and Piazzesi G (2004) Ca-activation and stretch-activation in insect flight muscle. *Biophys. J.* **87:** 1101–1111.

Liu HSMM, Swank DM, Kronert WA, Maughan DW and Bernstein SI (2005) Paramyosin phosphorylation site disruption affects indirect flight muscle stiffness and power generation in Drosophila melanogaster. *Proc Natl Acad Sci USA* **102:** 10522–10527.

Maroto M, Vinos J, Marco R and Cervera M (1992) Autophosphorylating protein kinase activity in titin-like arthropod projectin. *J Mol Biol* **224:** 287–291.

Maroto M, Arredondo J, Goulding D, Marco R, Bullard B and Cervera M (1996) Drosophila paramyosin/miniparamyosin gene products show a large diversity in quantity, localization, and isoform pattern: a possible role in muscle maturation and function. *J Cell Biol* **134:** 81–92.

McGuffin LJ, Bryson K and Jones DT (2000) The PSIPRED protein structure prediction server. *Bioinformatics* **16:** 404–405.

Meredith J and Storti RV (1993) Developmental regulation of the Drosophila tropomyosin II gene in different muscles is controlled by muscle-type-specific intron enhancer elements and distal and proximal promoter control elements. *Dev Biol* **159:** 500–512.

Miller A (1950) *The internal anatomy and histology of the imago of Drosophila*. In: Demerec M (ed.) Biology of *Drosophila* (pp. 420–534). John Wiley, New York.

Mogami K, Fujita SC and Hotta Y (1982) Identification of Drosophila indirect flight muscle myofibrillar proteins by means of two-dimensional electrophoresis. *J Biochem (Tokyo)* **91:** 643–650.

Molloy JE, Kyrtatas V, Sparrow JC and White DCS (1987) Kinetics of flight muscles from insects with different wingbeat frequencies. *Nature* **328:** 449–451.

Myers JM, Veis A, Sabsay B and Wheeler AP (1996) A method for enhancing the sensitivity and stability of stains-all for phosphoproteins separated in sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem* **240:** 300–302.

Paradela A, Alvarez I, Garcia-Peydro M, Sesma L, Ramos M, Vazquez J and De Lopez Castro JA (2000) Limited diversity of peptides related to an alloreactive T cell epitope in the HLA-B27-bound peptide repertoire results from restrictions at multiple steps along the processing-loading pathway. *J Immunol* **164:** 329–337.

Peckham M, Molloy JE, Sparrow JC and White DC (1990) Physiological properties of the dorsal longitudinal flight muscle and the tergal depressor of the trochanter muscle of Drosophila melanogaster. *J Muscle Res Cell Motil* **11:** 203–215.

Pringle JWS (1978) The Croonian Lecture, 1977. Stretch activation of muscle: function and mechanism. *Proc R Soc Lond B Biol Sci* **201:** 107–130.

Pringle JWS (1981) The evolution of fibrillar muscle in insects. *J. Exp. Biol* **94:** 1–14.

Qiu F, Lakey A, Agianian B, Hutchings A, Butcher GW, Labeit S, Leonard K and Bullard B (2003) Troponin C in different insect muscle types: identification of two isoforms in *Lethocerus, Drosophila* and *Anopheles* that are specific to asynchronous flight muscle in the adult insect. *Biochem J* **371:** 811–821.

Reedy MC, Reedy MK, Leonard KR and Bullard B (1994) Gold/Fab immuno electron microscopy localization of troponin H and troponin T in Lethocerus flight muscle. *J Mol Biol* **239:** 52–67.

Singh SP, Coronella JA, Benes H, Cochrane BJ and Zimniak P (2001) Catalytic function of Drosophila melanogaster glutathione S-transferase DmGSTS1–1 (GST-2) in conjugation of lipid peroxidation end products. *Eur J Biochem* **268:** 2912–2923.

Sparrow JC (1995) Muscle. Flight and phosphorylation. *Nature* **374:** 592–593.

Thorson J and White DC (1983) Role of cross-bridge distortion in the small-signal mechanical dynamics of insect and rabbit striated muscle. *J Physiol* **343:** 59–84.

Tohtong R, Yamashita H, Graham M, Haeberle J, Simcox A and Maughan D (1995) Impairment of muscle function caused by mutations of phosphorylation sites in myosin regulatory light chain. *Nature* **374:** 650–653.

Trayer IP, Trayer HR and Levine BA (1987) Evidence that the N-terminal region of A1-light chain of myosin interacts directly with the C-terminal region of actin. *A proton magnetic resonance study*. *Eur J Biochem* **164:** 259–266.

Vigoreaux JO, Saide JD, Valgeirsdottir K and Pardue ML (1993) Flightin, a novel myofibrillar protein of Drosophila stretch-activated muscles. *J Cell Biol* **121:** 587–598.

Vigoreaux JO and Perry LM (1994) Multiple isoelectric variants of flightin in Drosophila stretch-activated muscles are generated by temporally regulated phosphorylations. *J Muscle Res Cell Motil* **15:** 607–616.

Vigoreaux JO (2001) Genetics of the Drosophila flight muscle myofibril: a window into the biology of complex systems. *Bioessays* **23:** 1047–1063.

Vinos J, Domingo A, Marco R and Cervera M (1991) Identification and characterization of Drosophila melanogaster paramyosin. *J Mol Biol* **220:** 687–700.

Warmke J, Yamakawa M, Molloy JE, Falkenthal S and Maughan D (1992) Myosin light chain-2 mutation affects flight, wing beat frequency, and indirect flight muscle contraction kinetics in Drosophila. *J Cell Biol* **119:** 1523–1539.

Wendt T and Leonard K (1999) Structure of the insect troponin complex. *J Mol Biol* **285:** 1845–1856.

Wray JS (1979) Filament geometry and the activation of insect flight muscles. *Nature* **280:** 325–326.