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

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The indirect flight muscles (IFMs) of *Lethocerus* (giant water bug) and *Drosophila* (fruitfly) are asynchronous: oscillatory contractions are produced by periodic stretches in the presence of a Ca^{2+} concentration that does not fully activate the muscle. The troponin complex on thin filaments regulates contraction in striated muscle. The complex in IFM has subunits that are specific to this muscle type, and stretch activation may act through troponin. *Lethocerus* and *Drosophila* have an unusual isoform of the Ca^{2+}-binding subunit of troponin, troponin C (TnC), with a single Ca^{2+}-binding site near the C-terminus (domain IV); this isoform is only in IFMs, together with a minor isoform with an additional Ca^{2+}-binding site in the N-terminal region (domain II). *Lethocerus* has another TnC isoform in leg muscle which also has two Ca^{2+}-binding sites. Ca^{2+} binds more strongly to domain IV than to domain II in two-site isoforms. There are four isoforms in *Drosophila* and *Anopheles* (malarial mosquito), three of which are also in adult *Lethocerus*. A larval isoform has not been identified in *Lethocerus*. Different TnC isoforms are expressed in the embryonic, larval, pupal and adult stages of *Drosophila*; the expression of the two IFM isoforms is increased in the pupal stage. Immunoelectron microscopy shows the distribution of the major IFM isoform with one Ca^{2+}-binding site is uniform along *Lethocerus* thin filaments. We suggest that initial activation of IFM is by Ca^{2+} binding to troponin with the two-site TnC, and full activation is through the action of stretch on the complex with the one-site isoform.

Key words: EF hand, Ca^{2+} binding, insect flight, phylogeny, stretch activation.

INTRODUCTION

Muscle contraction is regulated by changes in the concentration of Ca^{2+} in the fibres. In many invertebrates, including insects, control is through Ca^{2+}-binding proteins on both thick and thin filaments [1,2]. Thin filaments in skeletal muscles are activated when Ca^{2+} binds to the tropomyosin–troponin complex, producing a shift in the position of tropomyosin, which partially exposes the myosin-binding site on actin [3–7]. In many species of insect, the wing-beat frequency is too high for individual contractions to be activated by Ca^{2+}. These asynchronous flight muscles respond very little to Ca^{2+}, but are fully activated by periodic stretches synchronized with the wing beat [8]. The wings are moved by resonant changes in the shape of the thorax produced by indirect flight muscles (IFMs). The action of stretch on the IFM may be through the tropomyosin–troponin regulatory system on thin filaments. Two insects with asynchronous muscle have been studied most intensively: *Lethocerus*, the giant water bug, used in studies of structure and mechanics, and the fruitfly *Drosophila*, which has the advantage that the genome has been sequenced and mutants are available. Troponin components in vertebrate skeletal muscle have been well characterized [9–12] and some properties of the insect complex can be predicted from what is known about vertebrate troponin. All the troponin subunits have been characterized and sequenced in *Lethocerus*, and the sequence of the *Drosophila* subunits is known. In both insects, the tropomyosin-binding subunit, TnT, has a negatively charged extension to the C-terminus not present in vertebrate TnT, but found in other arthropod TnTs. *Drosophila* troponin consists of TnT, the inhibitory subunit TnI, the Ca^{2+}-binding subunit TnC and two isoforms of an extra component, troponin H (TnH33 and TnH34) in which tropomyosin is fused to a proline-and-alanine-rich C-terminal extension [13–17]. *Lethocerus* troponin consists of TnT, TnC and a TnH in which TnI is fused to a proline-and-alanine-rich extension similar to the one in *Drosophila* TnH [14] (F. Qiu, K. Leonard and B. Bullard, unpublished work). Ca^{2+}-binding proteins specific to IFM and non-IFM have been identified in *Drosophila*, but have not been characterized [18].

The crystal structure of TnC in vertebrate fast skeletal muscle shows that the molecule has two globular regions linked by a central helix. There are two Ca^{2+}-binding domains in both N-terminal and C-terminal regions, each of which has an EF-hand motif made up of two perpendicular helices connected by a loop [19]. Residues in the loop co-ordinate Ca^{2+} or Mg^{2+}, depending on the sequence. TnC in vertebrate fast skeletal muscle has four Ca^{2+}-binding sites: two low-affinity sites (I and II) in the N-terminal region of the molecule, which are Ca^{2+}-specific, and two high-affinity sites (III and IV) in the C-terminal region, which bind both Ca^{2+} and Mg^{2+} [20]. The N-terminal sites bind Ca^{2+}

Abbreviations used: AgTnC, *Anopheles gambiae* TnC (troponin C); DmTnC, *Drosophila melanogaster* TnC; DsTnC, *Drosophila silvestris* TnC; E69A mutant (etc.), glutamic acid^{69}→alanine (etc.); EST, expressed sequence tag; His_6, hexahistidine; IFM, indirect flight muscle; LiTnC, *Lethocerus indicus* TnC; Ni-NTA, Ni^{2+}-nitrilotriacetate; RabTnC, rabbit TnC; RACE, rapid amplification of cDNA ends; Tm, tropomyosin; TnH, troponin H; TnI, troponin I; TnT, troponin T; TEV, tobacco etch virus; WT, wild-type.

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reversibly during regulation, and the C-terminal sites are needed to bind TnC to the rest of the troponin complex. The isoform of TnC in vertebrate slow skeletal and cardiac muscle has only one low-affinity site in the N-terminal region, owing to sequence changes in site I. In these muscles, Ca^{2+} binding to site II alone regulates contraction [21].

Sequences of TnCs from invertebrate muscles have the four potential EF-hand Ca^{2+}-binding domains, but mutation of essential Ca^{2+}-co-ordinating residues means that two or more of the domains do not bind Ca^{2+}. The TnC of the nematode worm *Caenorhabditis elegans* [22,23] and TnC isoforms of the arthropods studied so far: barnacle (*Balanus nubilis*) [24–26] and crayfish (*Astacus lepodactylus*) [27,28] have two Ca^{2+}-specific binding sites at positions II and IV; TnC in Old World horseshoe crab (*Tachypleus tridentatus*) muscle and two isoforms in lobster (*Homarus americanus*) tail muscle are also predicted to have Ca^{2+}-binding sites II and IV [29,30]. Molluscs are unusual in having TnC with only one Ca^{2+}-binding site at position IV, which is Ca^{2+}-specific. Scallop (*Platinopecten yessoensis*) TnC is active in regulating actomyosin [31,32], and squid (*Todarodes pacificus*) TnC is predicted to be regulatory [33]. However, molluscan muscles are mainly regulated through the thick filament, and thin-filament regulation may only be functional at low temperature [34]. *Drosophila* is the only insect for which TnC sequences have been published. Three genes have been identified coding for isoforms expressed in embryo and adult (TnC73F), in larva (TnC47D) and in adult only (TnC41C), but no prediction was made about which sites might bind Ca^{2+} or in which muscles the adult forms might be [17]. Here we identify TnC isoforms of *Lethocerus* flight and non-flight muscles and compare these with similar isoforms in *Drosophila*. The malarial mosquito *Anopheles gambiae* also has asynchronous flight muscle, and the recent completion of the genome [35] has allowed us to identify the same TnC isoforms in this insect. The characteristics of TnC isoforms in flight muscle compared with those in other muscles are consistent with a function for troponin in the activation of the IFM thin filament by stretching.

MATERIALS AND METHODS

Fly stocks

Wild-type *Drosophila melanogaster* were *Oregon-R* strain. A mutant lacking IFM actin and thin filaments was $Act88F^{KM88}$ (*KM88* [36]), and a mutant lacking IFM myosin and thick filaments was Mhc^{7} (*Ifm(2)2* [37]).

Isolation of TnC from *Lethocerus* flight muscle and preparation of antibody

Giant water bugs (*Lethocerus indicus*) were obtained from Thailand. A fraction containing the tropomyosin–troponin complex was isolated from glycerol-extracted washed myofibrils from 20 bugs which had been stored at −20 °C [14]. The components were separated on a DEAE-cellulose column (Whatman DE52) equilibrated in 6 M urea/50 mM Tris/HCl (pH 8)/ 0.5 mM EGTA/1 mM dithiothreitol; fractions were eluted with a gradient of 0–0.5 M NaCl in the same buffer. A peak eluted at 0.24 M NaCl contained TnT and TnC. These were separated on a DEAE-Sephadex A25 column equilibrated with 0.2 M NaCl/ 20 mM Tris/HCl (pH 7.8)/5 mM EDTA/1 mM dithiothreitol (no urea) and eluted with a gradient up to 1.0 M NaCl. Female LOU rats were immunized with isolated TnC, and monoclonal antibodies were raised as previously described [14] using the rat Y3Ag1.2.3 plasmacytoma fusion partner. Monoclonal antibodies MAC 352 and MAC 414 are subclass IgG1.

Molecular cloning and sequencing

A λgt11 expression library was prepared from 4 g of IFM dissected from 9 *Lethocerus* and the library was screened with monoclonal antibody MAC 352 to TnC, as described in [38]. Immunopositive clones were subcloned into M13 (a single-strand plasmid for sequencing) and sequenced. For rapid-amplification-of-cDNA-ends (RACE) reactions, mRNA was prepared from 100 μg of total RNA isolated from *Lethocerus* IFM or leg muscle (whole legs), using an Oligotex™ purification kit from Qiagen. Double-stranded cDNA was prepared using a Marathon cDNA amplification kit from Clontech, according to the manufacturer's instructions. *Drosophila melanogaster* cDNA was prepared from adult mRNA obtained from Clontech.

To clone the full-length *L. indicus* TnC1 (LiTnC1) and TnC4 (LiTnC4) cDNA from IFM, two sets of oligonucleotides (3′-RACE CGGAGGTTCTACGAGAGATTCTG, 5′-RACE CCTCCGTTATGTATCCATTCC for LiTnC1 and 3′-RACE CTGGGGGCGGAATTCAGCAGAC, 5′-RACE CAACAGTTCCAGAACCATCAGCG for LiTnC4) were designed from the sequence of inserts in the positive clones LiTnC1 and LiTnC4 from the λgt11 cDNA library screened with the monoclonal antibody MAC 352. The PCR products of both 5′-RACE and 3′-RACE were gel-purified and cloned in the pCR2.1TA™ vector (Invitrogen) and sequenced. Full-length cDNAs of both IFM isoforms were cloned by PCR from 5′- and 3′-RACE ends. *L. indicus* leg TnC (LiTnC3) was cloned by designing a set of degenerate primers based on the conserved peptide sequences of invertebrate TnC (EAFRLYDK and DGSGTVDF). The full-length sequence of LiTnC3 cDNA was obtained with the two primers (3′-RACE GGAAGGCAATGGTTACATCCC and 5′-RACE TTTGTGAGCTGGTCATCAAGC) based on the sequences of degenerate PCR products.

Drosophila melanogaster TnC1 (DmTnC1) and TnC2 (DmTnC2) cDNA were cloned by PCR amplification using P47947 and P47948 cDNA sequences. The full-length cDNA of a *D. melanogaster* TnC4 (DmTnC4) was cloned by RACE using partial sequence CG12408, with 3′-RACE primer GGTAAAGGCCCTTATTAAAGAGGTCG and 5′-RACE primer TTGCAACTGTCAGGTATCCTTTCCCCTC. cDNA sequences were checked after cloning. Amino acid sequences were aligned with the ClustalX program, and Ca^{2+}-binding sites were predicted with the UWGCG (University of Wisconsin Genetics Computer Group) software package (version 10). Sequences of *Anopheles* TnC isoforms were obtained from genomic and expressed-sequence-tag (EST) sequences [35].

Accession numbers are: DmTnC4, AJ512938; LiTnC1, AJ512940; LiTnC3, AJ512941; LiTnC4, AJ512939.

Preparation of mutants and expression of recombinant protein

Lethocerus or *Drosophila* TnC cDNA was inserted into the *Nco*I/*Eco*RI or *Nco*I/*Hin*dIII sites of a modified pET24d (M11) expression vector (Novagen) containing an N-terminal hexahistidine (His_6) tag followed by a tobacco-etch-virus (TEV)-protease cleavage site. Site-directed mutagenesis of glutamic acid to alanine was performed with a QuikChange™ site-directed mutagenesis kit (Stratagene). Mutations in LiTnC1 were: LiTnC1-mII with glutamic acid changed to alanine in Ca^{2+}-binding site II (E69A); LiTnC1-mIV (E148A in site IV)

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and LiTnC1-mII+mIV (E69A and E148A in sites II and IV). Mutations in LiTnC4 were: LiTnC4-mIV (E148A) and LiTnC4-ΔC with the last 18 residues deleted from the sequence, made by PCR amplification of LiTnC4 residues 1–140 and insertion into the same pET cloning site. Recombinant proteins were grown in a modified *Escherichia coli* strain BL21(DE3)pJY2 (Stratagene) and the soluble TnC was purified from the lysate of sonicated cells on Ni^{2+}-nitrilotriacetate (Ni-NTA)–agarose columns (Qiagen). The His_6 tag was cleaved from purified proteins with TEV protease, and the proteins were run through the column again to remove the His_6 tag.

RNA preparation and Northern-blot analysis

Lethocerus IFM (1 g) or legs (2 g) were frozen in liquid N_2 and ground with a pestle and mortar. The powder was dissolved in guanidine isothiocyanate lysis solution and total RNA prepared by CsCl sedimentation [39]. Northern-blot analysis was performed using standard methods [39] and Hybond-N membrane (Amersham). Membranes were hybridized with gel-purified coding region inserts of full-length cDNA of IFM isoforms LiTnC1 and LiTnC4 and leg LiTnC3. DNA was labelled with [α-^{32}P]dCTP (Amersham;10 mCi/ml) using the prime DNA labelling system (Promega). Hybridization was performed at 42 °C overnight. After exposure to X-ray film, the cDNA probe was stripped according to the Amersham instructions and the same membrane was reprobed with the next cDNA.

Ca^{2+} binding assays

The stoichiometry of Ca^{2+} binding to the *Lethocerus* flight-muscle TnC isoforms was measured by atomic-absorption spectroscopy. Defined amounts of Ca^{2+}-free LiTnC4 (1.8–21.7 μM) or LiTnC1 (2–20 μM) were added in 0.5 ml final volume of 15 mM Mops (pH 7.2)/105 mM KCl buffer containing 25 or 40 μM $CaCl_2$ respectively. Ca^{2+} binding was measured for six concentrations of each protein. The mixtures were incubated for at least 30 min, transferred to the top chambers of Ultrafree (Millipore) filtering units pre-washed with Ca^{2+}-free buffer and centrifuged through a membrane with 5 kDa cut-off for 20 min at 2000 ***g***. Total Ca^{2+} in the flowthrough was measured by atomic absorption using an AAS vario 6 FL atomic-absorption spectrophotometer (Analytik Jena AG, Jena, Germany), thus allowing calculation of the amount of Ca^{2+} bound to the proteins. Atomic-absorption values were corrected for Ca^{2+} contamination in the buffer and proteins, which was usually < 2 μM. Buffers were made using ultra-pure water (Fluka) and a 0.1 M Ca^{2+} standard (Orion Research, now Thermo Orion, Beverly, MA, U.S.A.). Proteins were decalcified using a Chelex-100 (Bio-Rad) column equilibrated in buffer.

Ca^{2+} binding by wild-type and mutant *Lethocerus* TnC was determined by a modification of the slot-filtration method [40]. Recombinant protein (15 μg) in 200 μl of sample buffer (60 mM KCl/5 mM $MgCl_2$/10 mM imidazole/HCl, pH 6.8) was bound to a nitrocellulose membrane (BA83; Schleicher und Schüll) using a Bio-Dot$^{(R)}$SF microfiltration apparatus (Bio-Rad). After washing twice with sample buffer, the membrane was incubated with 10 mM imidazole/HCl/5 mM EGTA, pH 6.8, for 10 min and washed with Milli-Q water three times for 5 min. The membrane was incubated with 2 ml of sample buffer containing 5 μCi/ml $^{45}CaCl_2$ (Amersham Pharmacia Biotech) at a total Ca^{2+} concentration of 5 μM, in a 50 ml Falcon tube in a roller hybridization oven at 25 °C for 15 min. The membrane was washed twice with deionized water, dried, and autoradiographed. Autoradiographs were scanned and integrated using the NIH (National Institutes of Health) public-domain image processing and analysis program *Image*).

Drosophila TnC gene expression

The expression patterns of DmTnC1, DmTnC2, DmTnC3 and DmTnC4 were determined using a gene expression array containing cDNA from different developmental stages (RAPID-Scan™; OriGene Technologies, Inc., Rockville, MD, U.S.A.). PCR reactions were allowed to proceed on this cDNA using the following primers: 5′-ATGAGCGATGAATTGACTAAGGAG-3′ and 5′-GATATTGTTTAGTCGCCACC-3′ for DmTnC1; 5′-GATCTGGTGAACAACTAGCACAG-3′ and 5′-TTATGAG-ATACGTTGGGTCCAC-3′ for DmTnC2; 5′-ATGAGCAGC-GTCGATGAAGATC-3′ and 5′-TTACTCGCCAGTCATCATC-TCCAT-3′ for DmTnC3; 5′-ATGGCCGATGGAGAATAC-3′ and 5′-TCAACCTGTCATAACTTGC-3′ for DmTnC4. Primers for the *Drosophila* housekeeping gene RP49 were used to check that the amount of cDNA was approximately the same for different developmental stages. The PCR products were detected on a 1.5 % (w/v)-agarose gel with ethidium bromide staining.

Electrophoresis and immunoblotting

Dorsal longitudinal flight-muscle fibres and leg-muscle fibres (posterior tergocoxal) were dissected from *Lethocerus* thorax that was stored in relaxing solution [0.1 M NaCl/20 mM sodium phosphate (pH 6.8)/5 mM $MgCl_2$/5 mM EGTA/5 mM ATP/ 5 mM NaN_3] with 75 % (v/v) glycerol at −80 °C [41], then dissolved in Laemmli sample buffer and heated to 56 °C. About 20 *Drosophila* thoraces were removed from wild-type and mutant flies and immediately put into liquid N_2; legs were removed from about 100 wild-type flies and put into liquid N_2. Thoraces and legs were homogenized in an Eppendorf tube while frozen and dissolved in Laemmli sample buffer. Flight muscle was dissected from about 30 half-thoraces of wild-type and mutant *Drosophila* in rigor solution (relaxing solution without ATP) with 50 % (v/v) glycerol. Samples were run on SDS/PAGE gels, which contained 12 or 15 % (w/v) acrylamide and were either 5 or 8 cm long. TnC samples for SDS/PAGE were heated to 95 °C in Laemmli sample buffer. Proteins were transferred to nitrocellulose with a semi-dry blotting apparatus (ATTO; Genetic Research Instrumentation Ltd., Felsted, Dunmow, Essex, U.K.) for 1 h at 900 mA. Blots were incubated in MAC 352 or MAC 414 and goat-anti-rat second antibody (Sigma) and developed with a chemiluminescent substrate [ECL® (enhanced chemiluminescence), Amersham]. The relative affinity of MAC 352 for the two flight muscle TnC isoforms was tested by running two gels, each having serially diluted LiTnC1 and LiTnC4; one gel was stained with Coomassie Blue and the other was blotted on to nitrocellulose which was incubated with MAC 352. The gel and the blot were scanned and the area of peaks measured using NIH *Image*. The peaks on the blot of the two TnC isoforms were normalized to equal initial protein concentrations by comparison with the peaks on the stained gel.

Immunoelectron microscopy

Strips were dissected from the dorsal longitudinal muscle of a *Lethocerus* thorax stored in 75 % (v/v) glycerol at −80 °C; a bundle of about five fibres was rinsed in rigor buffer (relaxing solution without ATP) and fixed with 4 % paraformaldehyde in rigor buffer for 30 min on ice. Leg-muscle fibres were dissected

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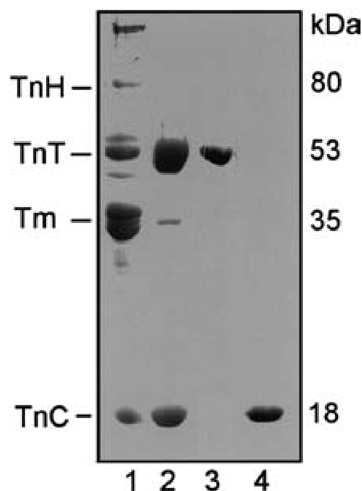

Figure 1 Isolation of TnC from *Lethocerus* IFM

An extract containing tropomyosin (Tm) and troponin was separated on a DEAE-cellulose column equilibrated with urea buffer [6 M urea/50 mM Tris/HCl (pH 8.0)/0.5 mM EGTA/1 mM dithiothreitol]. Lane 1, initial extract applied to the column; lane 2, a peak eluted with 0.24 M NaCl containing TnT and TnC; lane 3 and 4, separation of TnT and TnC on a DEAE-Sephadex column. The SDS/PAGE gel contained 12 % (w/v) acrylamide, which fails to separate the two isoforms of TnC.

from the same thorax and treated similarly. Fibres were infused with 2.1 M sucrose in rigor buffer at room temperature for 30 min and frozen on Cu^{2+} stubs in liquid N_2. Cryosections were labelled with rat monoclonal antibody MAC 352 (affinity-purified IgG, 0.1 mg/ml) or MAC 414 (hybridoma supernatant diluted 1 : 10), followed by an anti-rat secondary antibody (Cappel Laboratories) and Protein A linked to 10 nm gold particles [42]. To control for non-specific labelling, MAC 352 and MAC 414 were absorbed with LiTnC1 and LiTnC4 by adding the proteins at 0.5 mg/ml to the IgG or hybridoma supernatant and incubating at 23 °C for 1 h. The immune precipitate was removed by centrifuging at 12 000 ***g***. Images were taken at 25 000× magnification and 100 kV on a Philips Biotwin microscope and recorded on a Gatan CCD (charge coupled device) camera. Gold particles were measured using NIH *Image*, and the positions across the sarcomere displayed as a histogram using Kaleidagraph software. Images shown are representative of those obtained from 12 or more sections from three different specimens of IFM or leg muscle fibres.

RESULTS

Isoforms of TnC in *Lethocerus* muscles

Lethocerus TnC was isolated from a fraction containing IFM tropomyosin and troponin by ion-exchange chromatography (Figure 1). Monoclonal antibodies were raised to the IFM TnC and one high-affinity antibody (MAC 352) was used to screen an expression library prepared from *Lethocerus* IFM. Positive clones had one of two different sequences. The complete sequence of these two IFM TnCs was obtained by RACE reactions from a *Lethocerus* IFM cDNA library. In order to isolate cDNA corresponding to other isoforms of TnC in *Lethocerus* muscles, PCR reactions were done using degenerate primers based on conserved regions of invertebrate TnC sequences with a cDNA library prepared from IFM or leg muscles. A third

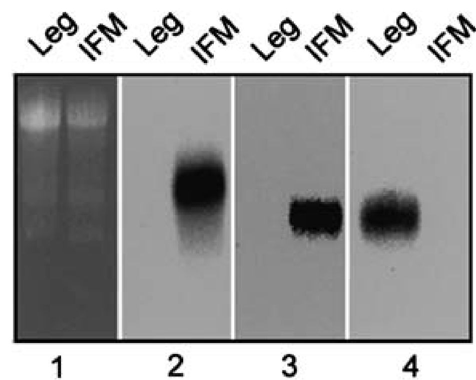

Figure 2 Northern blot of RNA from *Lethocerus* flight and leg muscles with cDNAs of TnC isoforms

Panel 1, total RNA from leg and IFM on an agarose gel. A membrane blotted from a similar gel was hybridized successively with different TnC cDNAs labelled with ^{32}P. After autoradiography, the membrane was stripped before hybridizing with the next probe: panel 2, LiTnC4 cDNA; panel 3, LiTnC1 cDNA; and panel 4, LiTnC3 cDNA.

TnC isoform was amplified from the leg-muscle cDNA library and the two TnC isoforms identified from the expression library were cloned from the IFM cDNA library. The IFM isoforms are LiTnC1 and LiTnC4, and the leg muscle isoform is LiTnC3 (see below).

Northern blots confirmed that two of the *Lethocerus* TnC isoforms are in IFM and the third one is in leg muscle. cDNA probes from both LiTnC1 and LiTnC4 hybridized with RNA from IFM but not with RNA from leg tissue, and a LiTnC3 cDNA probe only hybridized with RNA from legs (Figure 2).

Comparison of insect TnC sequences and predicted Ca^{2+}-binding sites

Drosophila TnC genes were previously cloned using primers from *Lethocerus* TnC sequences [17]; however, the *Drosophila* homologue of one of the *Lethocerus* IFM TnC isoforms (LiTnC4) was not identified. Searching the *Drosophila melanogaster* genomic sequence (FlyBase) revealed a gene (CG12408) with strong similarity to LiTnC4. CG12408 is predicted to code for a TnC [43]. The *Drosophila* isoform (called DmTnC4) was cloned and the complete sequence obtained by RACE from a cDNA library prepared from adult flies. A homologue of DmTnC4 was also found in cDNA sequence of the Hawaiian picture-wing fly, *Drosophila sylvestris* (DsTnC4, see Table 1). The genomic and EST sequences of the mosquito, *Anopheles gambiae*, contain sequences homologous with the *Lethocerus* and *Drosophila* TnC isoforms (Table 1). All three insects have asynchronous flight muscle.

Sequence alignment of the insect TnC isoforms is shown in Figure 3(A), together with the sequence of rabbit skeletal muscle TnC for comparison. Predicted Ca^{2+}-binding domains which have the residues necessary to co-ordinate Ca^{2+} are shown. The numbers of the isoforms are based on sequence homologies between species, which are illustrated in the phylogenetic tree (Figure 3B). All isoforms are predicted to have two Ca^{2+}-binding sites at positions II and IV, except those similar to the *Lethocerus* IFM isoform, LiTnC4, for which a single site at position IV is predicted. The nomenclature of TnC sequences used here is compared with other nomenclatures in Table 1, which also gives accession numbers and chromosome positions.

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Table 1 Nomenclature of troponin-C sequences

Swiss-prot sequences given in parentheses have not yet been named, although the accession numbers are confirmed. Fyrberg nomenclature is from [17].

Organism	Nomenclature used in the present paper	Fyrberg nomenclature	Accession or gene number	Swiss-prot nomenclature	Chromosome position
D. melanogaster	DmTnC1	TnC41C	P47947, FBgn0013348	TPC1_DROME	41E
	DmTnC2	TnC47D	P47948, FBgn0010423	TPC2_DROME	47E
	DmTnC3	TnC73F	P47949, FBgn0010424	TPC3_DROME	73E
	DmTnC4		AJ512938, FBgn0033027	(TPC4_DROME)	41F
D. sylvestris	DmTnC4		AAC04873, FBgn0023624	(TPC4_DROSI)	
L. indicus	LiTnC1		AJ512940	(TPC1_LETIN)	
	LiTnC2*		–	–	
	LiTnC3		AJ512941	(TPC3_LETIN)	
	LiTnC4		AJ512939	(TPC4_LETIN)	
A. gambiae	AgTnC1		BM648607	(TPC1_ANOGA)	22B–25D
	AgTnC2		BM637791	(TPC2_ANOGA)	25D–28D
	AgTnC3		BM576019	(TPC3_ANOGA)	22B–25D
	AgTnC4		BM646855	(TPC4_ANOGA)	33D–34A

* The LiTnC2 sequence was not identified in adult *Lethocerus* cDNA; it is assumed that a homologue of the larval isoform of *Drosophila* DmTnC2 will exist in *Lethocerus*.

The phylogenetic tree of insect TnC sequences shows three groups of isoforms (Figure 3B). Those with two predicted Ca^{2+}-binding sites, like the *Lethocerus* IFM LiTnC1, derive from a single branch, and other isoforms with two Ca^{2+}-binding sites, like the *Lethocerus* leg LiTnC3, derive from a second branch. The isoforms like the *Lethocerus* IFM LiTnC4 with a single predicted Ca^{2+}-binding site, derive from a third branch. Thus two probable IFM isoforms of TnC can be identified in different insects on the basis of sequence homology with *Lethocerus* isoforms LiTnC1 and LiTnC4. The relatively large distance of the isoforms with a single predicted Ca^{2+}-binding site from the branching point is probably due to variation in non-conserved parts of the sequence outside domain IV. The mosquito isoform, AgTnC3, which is predicted to have two Ca^{2+}-binding sites, has been excluded from the group predicted to have one site. Elucidation of the *Anopheles* genome is recent, and the derivation of the AgTnC3 sequence is uncertain.

Expression of *Drosophila* TnC isoforms at different developmental stages

Differential expression of *Drosophila* TnC isoforms in embryo, larva, pupa and adult was determined using a panel of cDNAs from different developmental stages (Figure 4). DmTnC1 is only expressed in pupa and adult. DmTnC2 is expressed in the late embryo, throughout the larval stages and in the pupa, but not in the adult. DmTnC3 appears in the embryo within the first 4 h and is expressed in appreciable amounts in larval stages; lower levels were detected in the adult. DmTnC4 is first expressed in the early larva and is not present in the embryo; expression is at a high level in the pupa and in the adult body (which excludes the head). The largest muscle mass in the adult body is IFM, so high expression levels correspond to high levels of DmTnC4 in this muscle. Amplification of the 'housekeeping' gene, RP49, showed that the amount of cDNA from different developmental stages used in the assay was approximately constant. These results are in general agreement with estimates of mRNA levels of three of the isoforms at different developmental stages [17]: DmTnC1 is an adult isoform; DmTnC2 is an embryonic, larval and pupal isoform; DmTnC3 is an embryonic, larval and adult isoform, but we detected DmTnC3 at an earlier embryonic stage than Fyrberg and colleagues [17] and detected less in the pupa.

Expression of *Lethocerus* and *Drosophila* wild-type TnC and *Lethocerus* mutants

The predicted Ca^{2+}-binding sites in the *Lethocerus* IFM isoforms of TnC were mutated to abolish the Ca^{2+}-binding at site II or IV or both. An essential Ca^{2+}-co-ordinating glutamic acid residue at the –Z position in site II and site IV was replaced by alanine (Figure 3A) and, in addition, a truncated mutant of LiTnC4 was produced by removing most of site IV. Figure 5(A) shows SDS/PAGE of recombinant wild-type LiTnC1, LiTnC3, LiTnC4 and mutant proteins used for immunoblotting and Ca^{2+}-binding assays. Figure 5(B) shows recombinant proteins of three wild-type isoforms of *Drosophila* TnC, DmTnC1, DmTnC4 and the larval form, DmTnC2, which were used for immunoblotting. Gels were run in the presence of background levels of Ca^{2+} (no added EGTA).

TnC isoforms in *Lethocerus* and *Drosophila* muscles

Different TnC isoforms were detected in IFM and leg muscles by incubating blots with monoclonal antibodies to *Lethocerus* TnC. The affinity of MAC 352 for LiTnC1 and LiTnC4, measured by antibody binding on blots, differed by less than 10%. The mobility of the bands labelled by antibody was compared with that of isoforms expressed *in vitro*. *Lethocerus* IFM had two isoforms that were labelled by MAC 352: a major one with mobility similar to recombinant LiTnC4 and a minor one with mobility similar to recombinant LiTnC1 (Figure 6A). Leg muscle had a single isoform with a mobility slightly lower than that of the other isoforms and similar to that of recombinant LiTnC3. The major isoform in IFM could be distinguished from the minor one and from the leg isoform because MAC 414 reacted specifically with this isoform; MAC 414 also reacted with recombinant LiTnC4 (Figure 6A). These results are in agreement with the Northern blots: LiTnC4 and LiTnC1 are in IFM and LiTnC3 is in other muscles, including leg muscle.

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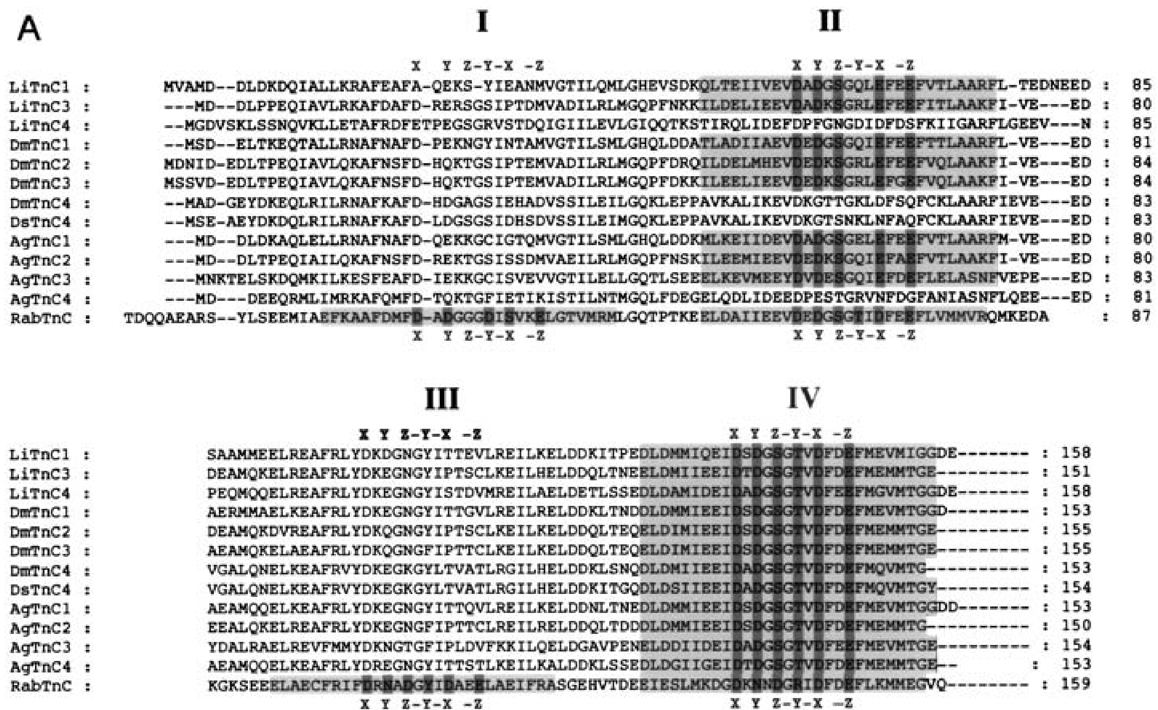

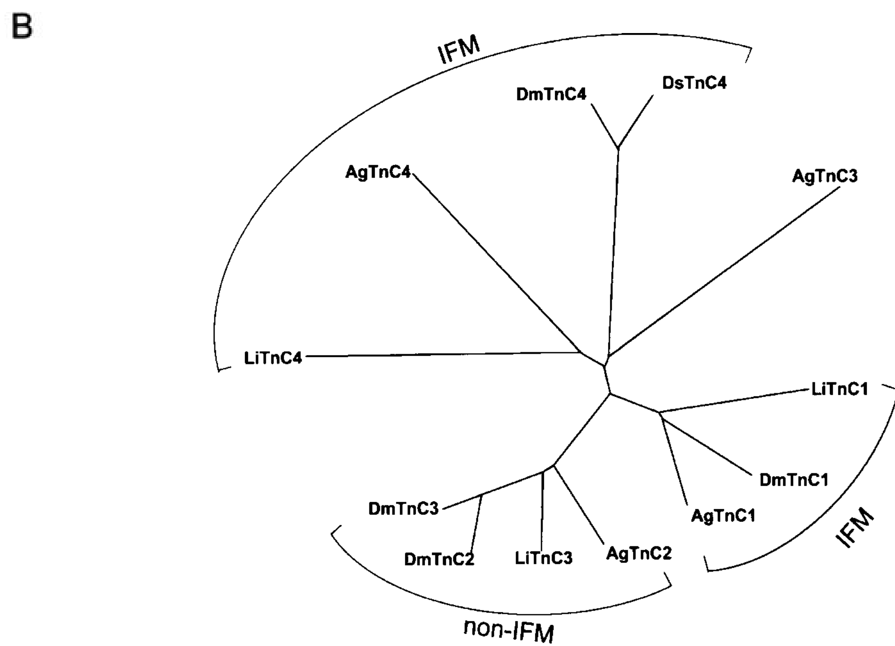

Figure 3 Comparison of insect TnC sequences

(**A**) Alignment of TnC sequences. The numbering of isoforms is based on homologies shown in the phylogenetic tree (**B**, below). The positions of potential Ca^{2+}-binding EF-hand domains I –IV are marked, and those predicted to bind Ca^{2+} are highlighted in pale grey. Residues at Ca^{2+}-co-ordinating positions are labelled X, Y, Z, –Y, –X and –Z and are highlighted in dark grey in the rabbit sequence; conserved Ca^{2+} coordinating residues are highlighted in dark grey in the predicted Ca^{2+}-binding domains of the insect sequences. Rabbit skeletal TnC has four Ca^{2+}-binding domains. All the insect TnC isoforms are predicted to have two Ca^{2+}-binding domains at positions II and IV, except the isoforms homologous with *Lethocerus* IFM LiTnC4, which are predicted to have one Ca^{2+}-binding domain at position IV. (**B**) Phylogenetic tree showing relationship of insect TnC isoforms. The sequences fall into three main groups: one with homology with the *Lethocerus* IFM LiTnC1, a second with homology with *Lethocerus* leg LiTnC3 (these have two Ca^{2+}-binding sites); and a third, less closely related, group with homology with IFM LiTnC4, which has one Ca^{2+}-binding site. Abbreviations: Rab, rabbit skeletal; Li, *L. indicus*; Dm, *D. melanogaster*; Ds, *D. sylvestris*; Ag, *A. gambiae*.

Drosophila thorax had two bands reacting equally strongly with MAC 352 and the mobilities were similar to those of recombinant DmTnC4 and DmTnC1 (Figure 6B). The IFM, like *Lethocerus* IFM, had major and minor isoforms with mobilities that were similar to those of recombinant DmTnC4 and DmTnC1 respectively. *Drosophila* legs had a single isoform with a mobility similar to that of DmTnC1. In the case of *Drosophila* it is not possible to distinguish the leg isoform from the minor IFM

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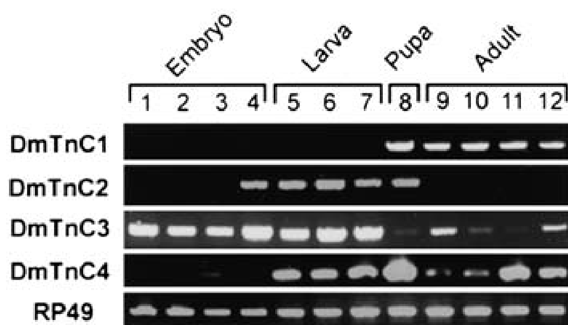

Figure 4 Expression of *Drosophila* TnC isoforms at different developmental stages

Primers derived from the coding region of the different isoforms were used in PCR reactions with an array made up of cDNA from the developmental stages. PCR products run on an agarose gel are shown. cDNA from the housekeeping gene RP49 was used to check that amounts of cDNA are approximately the same for different stages. Embryonic stages are: 0–4 h, 4–8 h, 8–12 h and 12–24 h (lanes 1–4). Larval stages are: first instar, second instar and third instar (lanes 5–7). Lane 8 is the pupal stage. Adult body parts are: male head, female head, male body and female body (lanes 9–12). The apparently inconsistent levels of expression of DmTnC3 in pupa and male and female adults is probably because the amounts of cDNA are close to the limits of detection.

isoform by the mobility. The thorax (including the legs) contains IFM and other thoracic muscles as well as leg muscle and the relatively strong labelling of the higher-mobility band is probably due both to the minor IFM isoform and to non-IFM isoforms with the same mobility. Recombinant DmTnC2 had a lower mobility than DmTnC4 or DmTnC1 and this isoform was not present in the thorax or legs, which is consistent with the lack of expression of this isoform in the adult fly (Figure 4). *Drosophila* TnCs did not react with MAC 414.

The exclusive expression of DmTnC4 in IFM was confirmed by immunoblots of *Drosophila* mutants lacking either thick filaments (*Mhc*^{7}) or thin filaments (*KM88*) in the IFM. The composition of non-IFMs is normal in these mutants. MAC 352 reacted with TnC isoforms in both upper and lower bands on a blot of wild-type and *Mhc*^{7} thoraces, but only reacted with the lower band in the *KM88* thorax (Figure 6C). IFMs isolated from *Mhc*^{7} had major and minor TnC isoforms similar to those in wild-type IFM, whereas IFMs from *KM88* had no TnC (Figures 6B and 6C). The absence of a TnC isoform with mobility characteristic of DmTnC4 in *KM88* shows that this isoform is only in IFM thin filaments. The band of higher mobility in *KM88* thorax is likely to be due to a TnC isoform in other muscles of the thorax.

Ca^{2+} binding by *Lethocerus* TnC isoforms and mutants

The stoichiometry of Ca^{2+} binding to the two flight-muscle isoforms of *Lethocerus* TnC was determined by atomic-absorption spectroscopy. The number of Ca^{2+} ions bound to LiTnC4 was 1.01 ± 0.37 and to LiTnC1 was 1.86 ± 0.58 (mean $\pm$ S.D. for six estimations). This confirms the prediction from the sequences of the two isoforms, namely that LiTnC4 binds one Ca^{2+} ion and LiTnC1 binds two.

Predictions of Ca^{2+}-binding sites from the sequence analysis were tested by measuring Ca^{2+} binding in mutant proteins by a blot assay. The essential glutamic acid residue at co-ordinating position –Z in a predicted EF-hand domain was changed to alanine in site II or site IV or both; for LiTnC4, the C-terminal Ca^{2+}-binding site IV was deleted. The flight-muscle isoform, LiTnC4, is predicted to bind Ca^{2+} at site IV only. The wild-type protein showed strong Ca^{2+} binding, which decreased to appproximately the background level in the E148A mutant (mIV) and decreased further in the truncated mutant (ΔC) (Figures 7A and 7B). The second flight-muscle isoform, LiTnC1, is predicted to bind Ca^{2+} at sites II and IV. The E69A mutant (mII) showed no decrease in Ca^{2+} binding compared with the wild-type, whereas Ca^{2+} binding to the E148A mutant (mIV) was decreased to approximately the background level. If both sites were mutated (mII+mIV), Ca^{2+} binding was decreased to a similar extent. The leg-muscle isoform, LiTnC3, is also predicted to bind Ca^{2+} at sites II and IV, and the effect of mutations at these sites was the same as for LiTnC1 (results not shown). The Ca^{2+} binding is consistent with a high-affinity site at position IV in both isoforms and a lower-affinity site at position II in LiTnC1, which binds Ca^{2+} weakly under the conditions of the blot assay.

Position of TnC isoforms in *Lethocerus* flight and leg myofibrils

The distribution of TnC isoforms in *Lethocerus* IFM and leg muscle was determined by immunogold labelling of cryosections with MAC 352 and MAC 414. MAC 352, which reacts with the two IFM isoforms LiTnC4 and LiTnC1 and the leg isoform LiTnC3, labelled the IFM and leg-muscle sarcomere uniformly

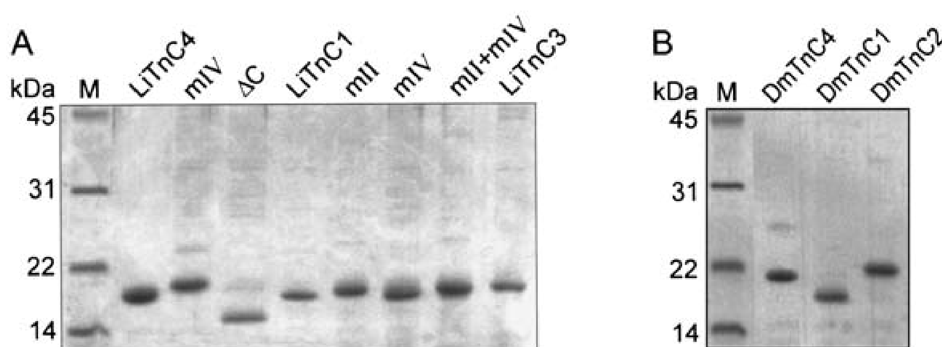

Figure 5 Recombinant TnC isoforms and mutants

The proteins were purified on an Ni-NTA–agarose column and His_6 tags removed. Mutations are in Ca^{2+}-binding sites II or IV or both. (**A**) *Lethocerus* TnC, lanes from left to right: molecular-mass markers ('M'); LiTnC4 wild-type ('LiTnC4'); LiTnC4-mIV mutated at site IV ('mIV'); LiTnC4-ΔC with 18 residues deleted from the C-terminus ('ΔC'); LiTnC1 wild-type ('LiTnC1'); LiTnC1-mII mutated at site II ('mII'); LiTnC1-mIV mutated at site IV ('mIV'); LiTnC1-mII+mIV mutated at both sites II and IV ('mII+mIV'); leg LiTnC3 wild-type ('LiTnC3'). (**B**) *Drosophila* TnC, lanes from left to right: molecular-mass markers ('M'); DmTnC4; DmTnC1; DmTnC2. SDS/PAGE gels contained 15 % acrylamide and were 5 cm long in both (**A**) and (**B**).

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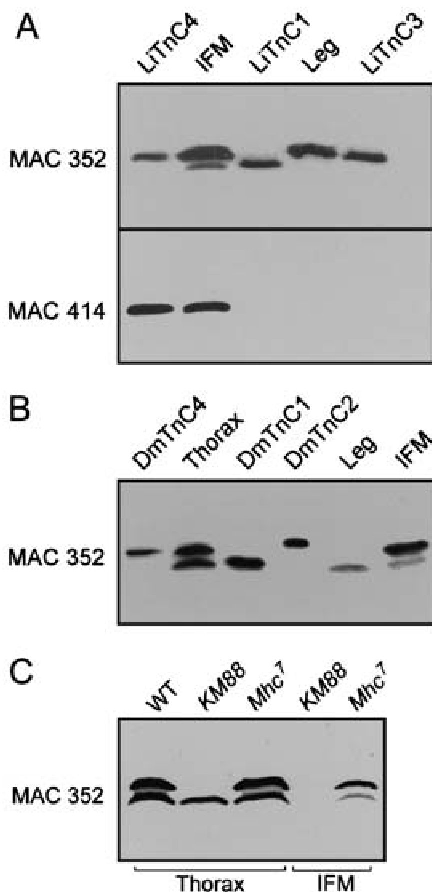

Figure 6 Isoforms of TnC in flight and leg muscles

Immunoblots of *Lethocerus* or *Drosophila* muscle samples and recombinant isoforms of TnC were incubated with antibodies MAC 352 or MAC 414. (**A**) TnC in *Lethocerus* IFM and leg compared with recombinant LiTnC1 and LiTnC4, and leg LiTnC3. The two IFM isoforms have mobilities similar to those of LiTnC4 and LiTnC1, and the leg isoform has a mobility similar to that of LiTnC3. MAC 414 reacts specifically with LiTnC4 and the larger IFM isoform. (**B**) TnC in *Drosophila* thorax, IFM and leg compared with recombinant DmTnC1, DmTnC2 and DmTnC4. The two isoforms in the thorax have mobilities similar to those of DmTnC4 and DmTnC1 and there are isoforms of similar mobility in IFM; the leg isoform also has mobility similar to that of DmTnC1. DmTnC2 has lower mobility than the other isoforms and is not present in thorax, IFM or legs. (**C**) TnC in *Drosophila* mutants. Thoraces of wild-type (WT) and a mutant without IFM thick filaments (*Mhc*^{7}) have two TnC isoforms; the thorax of a mutant without IFM thin filaments (*KM88*) only has the higher mobility isoform. Isoforms in *Mhc*^{7} IFM are like wild-type, but *KM88* IFM has no TnC. Blots were from SDS/PAGE gels which contained 15 % acrylamide and were 8 cm long in (**A**) and 5 cm long in (**B**) and (**C**).

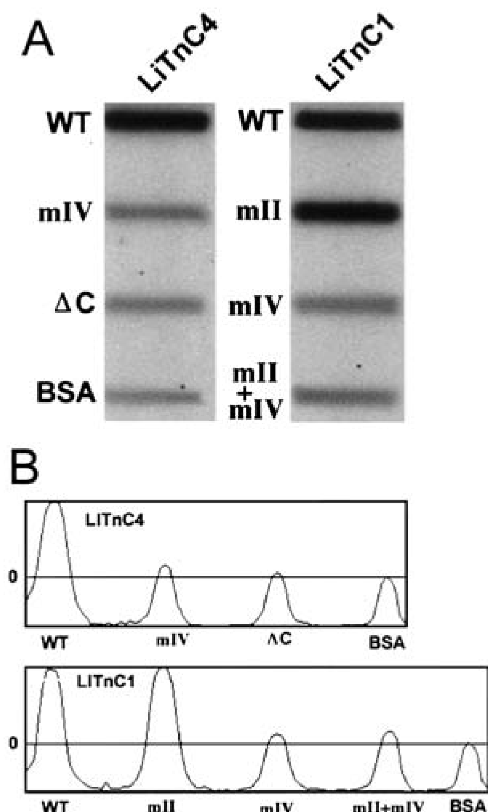

Figure 7 Ca^{2+} binding by *Lethocerus* IFM TnC isoforms and mutants

(**A**) Autoradiographs of $^{45}Ca^{2+}$ bound to TnC on nitrocellulose. Left panel, wild-type (WT) LiTnC4 and mutants; right panel, wild-type LiTnC1 and mutants. mIV has a mutation E148A in site IV; ΔC has 18 C-terminal residues deleted from site IV; mII has a mutation E69A in site II; mII+mIV has both mutations. TnC was bound to nitrocellulose in slots and membranes were incubated in $^{45}Ca^{2+}$. BSA was used to estimate non-specific labelling. (**B**) Densitometer scans of autoradiographs. The horizontal line indicates non-specific binding to BSA.

in the position of thin filaments (Figures 8A and 8B). MAC 414, which reacts specifically with LiTnC4, labelled IFM more sparsely, with the same distribution as MAC 352 and did not label leg-muscle sections, as expected (Figures 8C and 8D). All IFM myofibrils examined on an electron microscope grid were labelled by MAC 414, showing that there are not two different populations of myofibrils with different TnC isoforms. Thus the IFM isoform, LiTnC4, is uniformly distributed in the sarcomere and is not confined to a particular region; the leg isoform, LiTnC3 is also uniformly distributed. A control using MAC 352 absorbed with LiTnC4 and LiTnC1 gave low background labelling (Figure 8E); similar background was observed with absorbed MAC 414. It was not possible to determine the distribution of LiTnC1 in IFM because none of our monoclonal antibodies reacted specifically with this isoform.

DISCUSSION

Ca^{2+} binding to TnC is the trigger which activates skeletal muscle thin filaments. Even in dual-regulated muscles controlled by proteins on both thick and thin filaments, activation cannot occur unless tropomyosin moves from a blocking position on actin. We have investigated isoforms of insect TnC in IFM and other muscles to find out if some property of the TnC could account for the insensitivity of IFM to Ca^{2+} and the requirement for stretch to activate the muscle fully. The major isoform of TnC in *Lethocerus* IFM has only one EF-hand domain, at position IV, with the residues necessary for co-ordinating Ca^{2+}. This isoform was shown to bind a single Ca^{2+} ion. A second minor isoform in IFM has two domains predicted to bind Ca^{2+} at positions II and IV. This isoform was shown to bind two Ca^{2+} ions. *Drosophila* and *Anopheles*, both dipteran insects with asynchronous muscle, have similar isoforms.

The IFM isoform with one Ca^{2+}-binding site was unexpected, because the TnCs of arthropods generally have two sites [44]: although barnacle and crayfish have two or more isoforms, all bind Ca^{2+} at sites II and IV [25,26,27]. Non-IFM TnC isoforms in *Lethocerus* leg and *Drosophila* leg and larval muscles and the *Anopheles* homologues have the expected two sites at positions II and IV. However, Ca^{2+} binding to site IV is stronger than to site II in the two-site isoforms of *Lethocerus* IFM and leg; this differs from the situation in barnacle TnC isoforms, where sites II and

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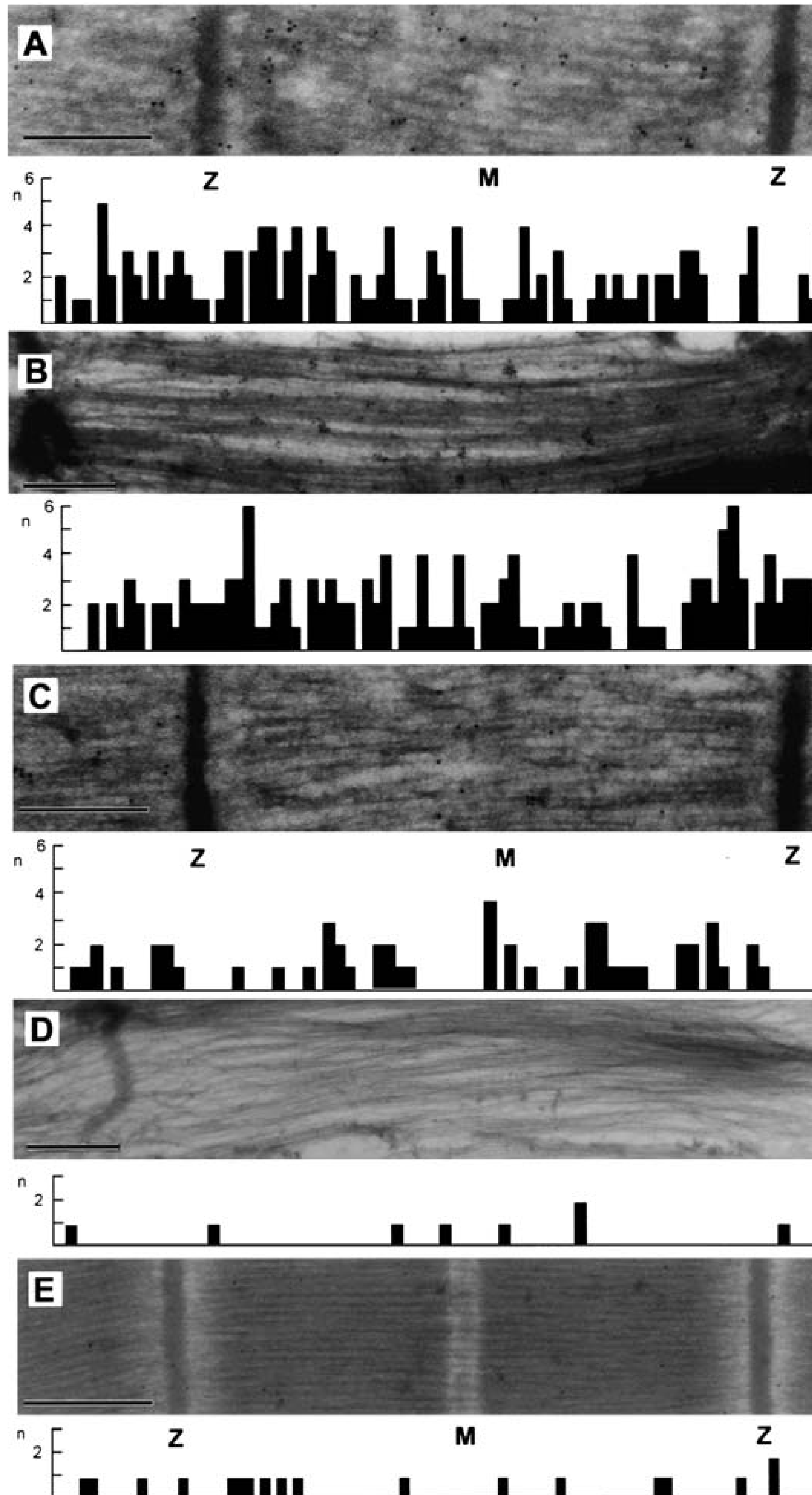

Figure 8 Distribution of TnC isoforms in *Lethocerus* flight and leg muscle

Cryosections were labelled with anti-TnC and Protein A–gold. Electron micrographs of (**A**) IFM and (**B**) leg muscle labelled with MAC 352, which reacts with IFM TnC isoforms LiTnC1 and LiTnC4 and with leg isoform LiTnC3; (**C**) IFM and (**D**) leg muscle labelled with MAC 414, which reacts specifically with LiTnC4. Representative strips across the IFM sarcomere are shown, with a bar diagram for the number (*n*) of gold particles across the whole width. Labelling by MAC 352 across the IFM sarcomere shows that one or both IFM TnC isoforms are distributed along the whole thin filament; the leg isoform is also distributed uniformly. LiTnC4 in IFM is more lightly labelled by MAC 414 and the distribution is uniform; the leg muscle is not labelled by MAC 414. (**E**) Background labelling of IFM by MAC 352 absorbed with LiTnC4 and LiTnC1. The scale bars represent 0.8 μm.

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IV bind Ca^{2+} with equal affinity and both are regulatory [24]. In one isoform of crayfish TnC, Ca^{2+} binds more strongly to site IV than II, and it was suggested that site II alone is regulatory in two isoforms in crayfish tail muscle [27].

The appearance of TnC isoforms in *Drosophila* at different developmental stages is consistent with the identification of IFM and non-IFM isoforms based on homology with *Lethocerus* sequences (Figure 4). The IFM isoforms, DmTnC1 and DmTnC4, are both expressed in the adult fly; in the case of DmTnC4, there is a particularly high level in the pupa, at which stage larval muscle is broken down and adult muscle constructed. The dorsal longitudinal muscles, unlike other IFMs, form on a template of persistent larval muscle [45], and it is possible that the DmTnC4 present in larval stages derives from these particular larval muscles. DmTnC2 is predominantly a larval form and is expressed during the time that the first myofibrils are recognized at around 15 h after fertilization [45,46]. DmTnC2 is replaced by DmTnC1 during the pupal stage. The appearance of DmTnC3 in the very early embryo, before the fusion of myoblasts at 9–12 h after fertilization and before the appearance of kettin (a high-molecular-mass modular protein found in invertebrates) at 7 h and myosin at 10 h, suggests this TnC isoform has a function in addition to muscle regulation. This isoform is present in all stages; the low levels in the pupa and adult, compared with the IFM isoforms, may be due to expression in minor muscles, such as wing adjustor muscles and those in head and legs. DmTnC2 is the only isoform not present in the adult of this species: it therefore seems likely that *Lethocerus* expresses a fourth TnC isoform homologous with this one which would not have been represented in the adult cDNA libraries that we screened (see Table 1).

The difference in the mobility of the flight-muscle TnC isoforms on SDS/PAGE was used to identify isoforms in IFM and non-IFM using an anti-TnC monoclonal antibody reacting with all isoforms and another specific to LiTnC4. The IFM-specific expression of DmTnC4 was shown by means of the *KM88* mutant, which lacks IFM thin filaments and does not have this TnC isoform. IFM in both *Lethocerus* and *Drosophila* has much more of the isoform with one Ca^{2+}-binding site (LiTnC4 and DmTnC4) than the isoform with two (LiTnC1 and DmTnC1). A preliminary estimate of the ratio of LiTnC4 to LiTnC1 is about 5 : 1. The isoform with a single Ca^{2+}-binding site appears to be uniformly distributed within the IFM myofibril. The position of the two-site isoform relative to the one-site isoform in the thin filament is not known. However, expression of both the IFM isoforms is increased at the same time in the *Drosophila* pupa, when IFMs are formed, and it is likely the TnCs are incorporated into troponin complexes at the same time; thin filaments probably have a mixture of troponins.

Oscillatory contraction of IFM is initiated by an increase in Ca^{2+} concentration in the fibres and is maintained by periodic stretches at constant Ca^{2+}. The initial activation may be due to reversible Ca^{2+} binding to troponin complexes having the two-site TnC isoform, and oscillations may be maintained by the action of stretch on troponin with the one-site TnC isoform. Site IV in the C-terminal domain of both IFM isoforms binds Ca^{2+} with higher affinity than does site II in the N-terminal domain of the two-site isoform. Analogy with vertebrate troponin suggests that Ca^{2+} binding to the C-terminal domain is needed to bind TnC to the rest of the troponin complex, and the lower-affinity site II in the N-terminal domain of the two-site isoform is regulatory. Troponin complexes with the isoform lacking the N-terminal Ca^{2+} site would not be expected to be regulated by Ca^{2+}. In the vertebrate model, the N-terminal domain of TnC is exposed on the thin filament in the absence of Ca^{2+} and binds to TnI in the presence of Ca^{2+}, reversing the inhibitory effect [10]. According to this model, IFM troponin with the one-site TnC would remain inhibitory in the presence of Ca^{2+}.

It appears there is not enough two-site TnC isoform to activate the entire IFM thin filament. In vertebrate striated muscle, the number of actin monomers in the thin filament that can be activated co-operatively when myosin binds in the presence of Ca^{2+} is 11–12 [47]. Therefore each two-site TnC would activate less than two tropomyosin repeats (14 actin monomers) on the thin filament, and this isoform only occupies about one in six of the troponin sites. This may account for the partial activation of fibres at rest length [8]. However, the size of the co-operative unit may be different in IFM, owing to differences in the other troponin subunits, such as the proline-and-alanine-rich sequence in TnH. Stretch may fully activate the thin filament by acting on troponin with one-site TnC, perhaps through the exposed N-terminus of the TnC. The protein interactions involved in activation by stretch are not known.

The presence of a major isoform of TnC lacking an N-terminal Ca^{2+}-binding site in the flight muscle of three insects using the asynchronous mechanism suggests this TnC is essential to the regulation of this muscle type. The effect of different isoforms of TnC on the properties of the whole troponin complex, including the IFM isoforms of TnT and TnH, must be determined before we can have a complete picture of the function of the different TnCs.

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