Drosophila Muscle Regulation Characterized by Electron Microscopy and Three-Dimensional Reconstruction of Thin Filament Mutants

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ABSTRACT Wild-type and mutant thin filaments were isolated directly from "myosinless" *Drosophila* indirect flight muscles to study the structural basis of muscle regulation genetically. Negatively stained filaments showed tropomyosin with periodically arranged troponin complexes in electron micrographs. Three-dimensional helical reconstruction of wild-type filaments indicated that the positions of tropomyosin on actin in the presence and absence of Ca^{2+} were indistinguishable from those in vertebrate striated muscle and consistent with a steric mechanism of regulation by troponin-tropomyosin in *Drosophila* muscles. Thus, the *Drosophila* model can be used to study steric regulation. Thin filaments from the *Drosophila* mutant *heldup*^{2}, which possesses a single amino acid conversion in troponin I, were similarly analyzed to assess the *Drosophila* model genetically. The positions of tropomyosin in the mutant filaments, in both the Ca^{2+}-free and the Ca^{2+}-induced states, were the same, and identical to that of wild-type filaments in the presence of Ca^{2+}. Thus, cross-bridge cycling would be expected to proceed uninhibited in these fibers, even in relaxing conditions, and this would account for the dramatic hypercontraction characteristic of these mutant muscles. The interaction of mutant troponin I with *Drosophila* troponin C is discussed, along with functional differences between troponin C from *Drosophila* and vertebrates.

INTRODUCTION

Contraction in all muscles results from the relative sliding of thick and thin filaments. In a repetitive cycle of attachment and dissociation, myosin cross-bridges that project from thick filaments advance along the actin-based molecular track of thin filaments, driving the contractile process (Huxley, 1969). Mechanisms that switch contraction on and off prevent muscles from being permanently contracted or non-functional. In all muscle types, contraction is initiated by an increase in the sarcoplasmic concentration of free Ca^{2+} ions, which bind to regulatory proteins. During relaxation, Ca^{2+} is sequestered and dissociates from these target proteins (reviewed in Tobacman, 1996). Although different protein systems regulate the activity of different muscle types, the thin filament-linked troponin-tropomyosin complex is involved in controlling most, if not all, striated muscles, including the asynchronous indirect flight muscles (A-IFM) of insects (Lehman and Szent-Györgyi, 1975). (The A-IFM are additionally modulated by stretch (Pringle, 1978) and myosin phosphorylation (Takahashi et al., 1990).) It is generally accepted that a ''steric'' regulatory mechanism governs the activity of these muscles, where a change in position of tropomyosin strands running along thin filaments acts as a molecular switch (Huxley, 1972; Haselgrove, 1972; Parry and Squire, 1973; Lehman et al., 1994). In relaxed muscles at low Ca^{2+}, tropomyosin is constrained by troponin (Tn) I and T of the troponin complex in a position blocking myosin binding sites on actin. Ca^{2+} binding to Tn C releases this constraint and allows tropomyosin movement to occur and actin and myosin to interact (Tobacman, 1996; McKillop and Geeves, 1993; Vibert et al. 1997). The initial binding of myosin heads on actin causes an additional shift in tropomyosin position, further increasing the probability of myosin binding cooperatively and leading to myosin cross-bridge cycling and contraction. (Tobacman, 1996; McKillop and Geeves, 1993; Vibert et al., 1997).

The indirect flight muscles of insects are an excellent model system to investigate the structural basis of muscle contraction and its regulation. A-IFM are composed of the same major myofibrillar proteins found in other muscles, and the precise lattice arrangement of thick and thin filaments in these striated muscles facilitates structural investigation (Reedy et al., 1965; Ashhurst and Cullen, 1977; Bullard et al., 1988; Ruiz et al., 1998). A-IFM of the fruit fly, *Drosophila melanogaster*, in particular, are amenable to genetic manipulation, and mutations specific for *Drosophila* A-IFM protein isoforms typically affect flight, but not the viability, of laboratory bred flies (Bernstein et al., 1993). However, the steric mechanism of thin filament regulation has never been demonstrated directly in the A-IFM of *Drosophila* or any other insect (cf. Ruiz et al., 1998), and hence the genetic advantages of this system could not be fully exploited to investigate troponin-tropomyosin regulation. In this study, we have developed a simple method to isolate thin filaments

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from *Drosophila* A-IFM and have demonstrated by electron microscopy (EM) and three-dimensional (3D) reconstruction that the positions of tropomyosin in the presence and absence of Ca^{2+} are indistinguishable from those in vertebrate muscle thin filaments (Lehman et al., 2000). We have also demonstrated the great potential of *Drosophila* A-IFM as a genetic model for studying thin filament regulation by characterizing the underlying structural basis for the dramatic hypercontraction in the flightless *heldup* (hdp^2) *Drosophila* strain, a mutant with a single amino acid conversion in Tn I (Beall and Fyrberg, 1991).

MATERIALS AND METHODS

Thin filament isolation

Thirty thoraces dissected from either anesthetized myosin heavy chain mutation Mhc^7 or hdp^2; Mhc^{12} ''myosinless'' *Drosophila* served as starting material. The genetic alteration in the myosinless Mhc^{12} *Drosophila* strain is similar to that in Mhc^7 *Drosophila*; in each strain, myosin heavy chains fail to accumulate in the A-IFM and therefore thick filament assembly does not occur. The Mhc^{12} strain available has identifiable phenotypic markers, *b*, *el*, and *cn* flanking the myosin gene, and these markers were useful in following the Mhc^{12} mutation in subsequent crosses. Crosses of *b el* Mhc^{12} *cn Drosophila* with hdp^2 flies possessing an *f* marker were carried out by standard procedures to obtain a stable double mutant hdp^2; Mhc^{12} stock. Balancer chromosomes were used to suppress chromosome recombination.

To solubilize cell membranes and extract nonfilamentous soluble proteins and nucleotides, the thoraces were ''chemically skinned'' in 0.1% saponin overnight by gentle agitation in a 10-ml ''rigor solution'' consisting additionally of 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 1 mM NaN_3, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM sodium phosphate/PIPES buffer (pH 7.0) at 4°C. The skinned material was then rinsed with the same solution lacking saponin, and homogenized in 0.8 ml fresh buffer in a glass homogenizer. Homogenates were centrifuged at 16,000 × *g* for 25 min to sediment particulate material, including trace amounts of thin filaments still bound in rigor to thick filaments and presumably derived from non-A-IFM muscles. A-IFM thin filaments were collected from the supernatant by sedimentation at 100,000 × *g* for 30 min and thin filament pellets resuspended in 0.2 ml rigor buffer with a 25-gauge syringe needle. Before preparation for EM, samples were diluted 2- to 10-fold either with the above buffer or the same buffer with 0.1 mM $CaCl_2$ added in excess of the EGTA present.

Rabbit skeletal muscle F-actin and troponin complexes containing mutant CBMII cardiac Tn C but wild-type cardiac Tn I and Tn T were prepared as before (Spudich and Watt, 1971; Tobacman et al., 1999; Morris et al., 2001). Stoichiometric amounts of the troponin complex were mixed with bovine cardiac tropomyosin (Tobacman and Adelstein, 1986) and the troponin-tropomyosin added to a suspension of F-actin (10 μM) in the above buffer in a 2:7 molar ratio (troponin-tropomyosin/actin), and diluted 10 times for EM.

Electron microscopy and 3D reconstruction

Negative staining and electron microscopy

Five μl of thin filaments in either EGTA or Ca buffer were applied to carbon-coated EM grids (at ~25°C), negatively stained with 1% (w/v) uranyl acetate and dried at 80% relative humidity to aid in spreading the stain (Lehman et al., 1994; Vibert et al., 1997). EM images were recorded at 80 kV on a Philips CM120 EM (Eindhoven, The Netherlands) at 60,000× magnification under low dose conditions (~12 $e^-/Å^2$) at a defocus of 0.5 μm.

3D reconstruction

Micrographs were digitized using a Zeiss SCAI scanner (Carl Zeiss, Thornwood, NY) at a pixel size corresponding to 0.7 nm in the filaments, and well-preserved regions of the filaments were selected and straightened as previously (Lehman et al., 1994; Vibert et al., 1997). Helical reconstruction, which resolves actin monomer structure and tropomyosin strands, but not troponin position or structure (Lehman et al., 2001), was carried out by standard methods (Owen et al., 1996). The statistical significance of densities in reconstructions was evaluated from the standard deviations associated with contributing points (Milligan and Flicker, 1987; Trachtenberg and DeRosier, 1987).

RESULTS AND DISCUSSION

Thin filament isolation from *Drosophila* A-IFM

Extraction of A-IFM thin filaments from insect muscle homogenates by routine procedures is ineffective, even though thin filaments are easily prepared from other insect muscle types (Lehman et al., 1974). This is, in part, because I-bands of A-IFM sarcomeres are extremely short, and interdigitating thick and thin filaments overlap almost completely. We therefore exploited the genetic advantages of the *Drosophila* system to isolate A-IFM thin filaments from myosinless (Mhc^7 or Mhc^{12}) strains that lack A-IFM thick filaments but are otherwise normal (Chun and Falkenthal, 1988; Bernstein et al., 1993). Although thick filaments do not accumulate in the flight muscles, thin filaments are apparently unaffected, develop normally, form I-bands and can be isolated directly and prepared free of contaminants with minimal processing (see Material and Methods section). Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (not shown) confirmed the purity of these preparations, and we and others (Chun and Falkenthal, 1988; Bernstein et al., 1993; Ruiz et al., 1998) demonstrated that actin, tropomyosin, and troponin components are present in thin filaments of myosinless A-IFM in normal ratios.

Electron microscopy of isolated thin filaments

Negative staining demonstrated that purified extracts of Mhc^7 A-IFM were replete with thin filaments and free of thick filaments and other particulate material (Fig. 1). A-IFM thin filaments (Fig. 1), isolated in EGTA and either maintained in EGTA or treated with Ca^{2+}, showed the characteristic double helical distribution of actin subunits and occasionally exhibited longitudinally oriented elongated tropomyosin strands. They also displayed periodic bulges, representing the globular end of the troponin complex that repeated at characteristic 38-nm intervals (Lehman et al., 1994, 2000, 2001). Although we have not quantified the mass or the dimensions of the troponin bulges in micrographs of A-IFM filaments, they are visibly larger than those present in vertebrate thin filaments (cf. Lehman et al., 1995), consistent with biochemical and structural evidence first

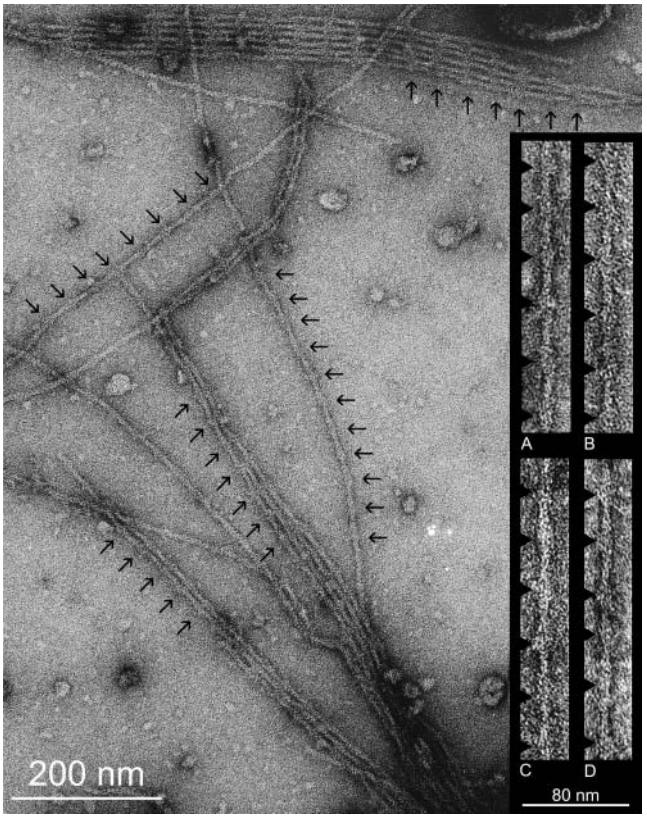

FIGURE 1 Electron micrographs of negatively stained *Drosophila* IFM thin filaments. A low magnification field showing single thin filaments, paired filaments, and filament rafts isolated from Mhc^7 myosinless flies. The globular ends of troponin (indicated by *black arrows*) are evident as bulges repeating at 38-nm intervals and are most easily seen by viewing filaments at a glancing angle. Filament pairs and rafts apparently are joined together by troponin molecules. (*Inset*) Examples of filaments used for image processing from the IFM of (*A* and *B*) Mhc^7 or (*C* and *D*) hdp^2*;* Mhc^{12} mutants isolated in EGTA (*A* and *C*) or treated with Ca^{2+} (*B* and *D*). Single filaments typically were chosen for image processing, but reconstruction of loosely paired filaments was also possible, and invariably both filament types yielded the same results.

presented by Bullard, Leonard, and colleagues (Bullard et al., 1988; Wendt and Leonard, 1999). Filaments were often linked together laterally by an apparent association of their troponin complexes, and sometimes as many as 20–30 thin filaments interacted to form ''rafts'' composed of a single layer of filaments.

3D reconstruction of A-IFM thin filaments

To determine the 3D structure of the negatively stained A-IFM thin filaments, 3D reconstructions were calculated for filaments in EGTA or Ca^{2+} using helical analysis (Owen et al., 1996). Density maps determined for pure F-actin served as controls. Surface views and helical projections of averaged data showed typical two-domain actin monomers that could be further divided into subdomains (Figs. 2 and 3).

In addition, longitudinally continuous strands of density followed the long-pitch actin helices of all thin filaments processed. These strands were not present in the F-actin control and were assumed to represent primarily tropomy-

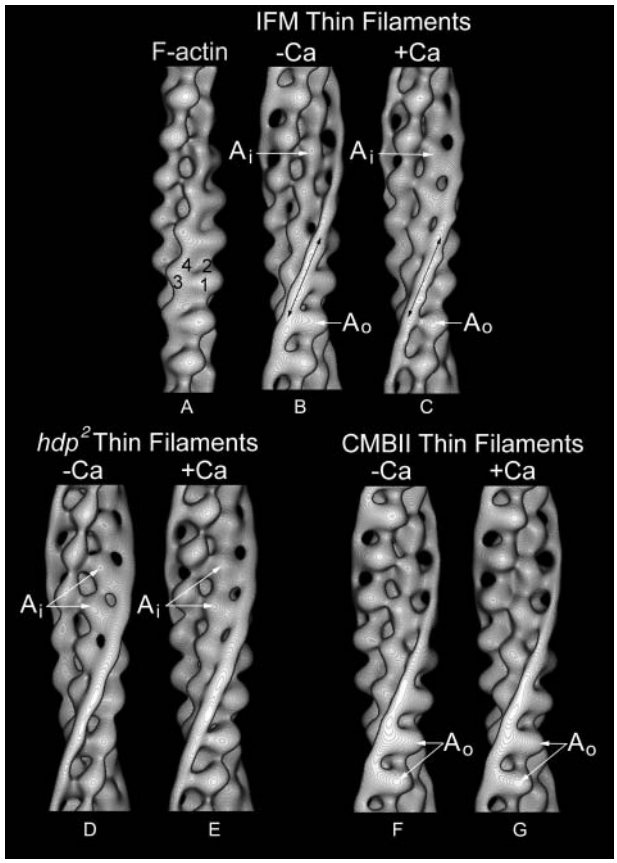

FIGURE 2 Surface views of reconstructed thin filaments showing the position of tropomyosin strands on actin. (*A*) F-actin control (actin subdomains numbered on one monomer); (*B–E*) *Drosophila* filaments from Mhc^7 (*B* and *C*) and hdp^2*;* Mhc^{12} (*D* and *E*) strains; (*F* and *G*) reconstituted thin filaments containing mutant CBMII Tn C and otherwise wild-type vertebrate cardiac troponin-tropomyosin components. Reconstructions are of samples maintained in the absence of Ca^{2+} (*B*, *D*, and *F*) or treated with Ca^{2+} (*C*, *E*, and *G*). Tropomyosin strands indicated by black arrows in *B* and *C*. Note that the tropomyosin density in *B*, *F*, and *G* is associated with subdomain-1 of each actin monomer and bridges over subdomain-2 of successive monomers along the long-pitch actin helix (on the inner edge of the outer domain of actin (A_o)). In *C*, *D*, and *E*), tropomyosin is associated with subdomain-3 and bridges over subdomain-4 of successive actin monomers (on the outer edge of the inner domain of actin (A_i)). Thus, Ca^{2+}-dependent movement of tropomyosin occurs in filaments from Mhc^7 IFM, but not from hdp^2; Mhc^{12} mutant IFM or from filaments containing CBMII Tn C. In the case of hdp^2; Mhc^{12}, tropomyosin is trapped on the inner domain of actin and, in the CMBII mutant, on the outer actin domain. The average phase residuals (±SD), a measurement of the agreement among filaments generating reconstructions, for the 15 filaments in each *Drosophila* data set were 58.0 ± 5.6°, 56.2 ± 7.0°, 55.8 ± 6.0°, and 54.8 ± 5.8°, respectively. The average up-down phase residuals, a measure of filament polarity, were 23.9 ± 6.6°, 24.4 ± 8.2°, 19.1 ± 5.5°, and 28.5 ± 7.9°, respectively, values comparable to those previously found. The average angular rotations between adjacent actin monomers along the genetic helix of the *Drosophila* filaments were 167.1 ± 0.7°, 167.0 ± 0.9°, 167.3 ± 1.4°, and 167.1 ± 1.3°, respectively, consistent with a 28/13 helix.

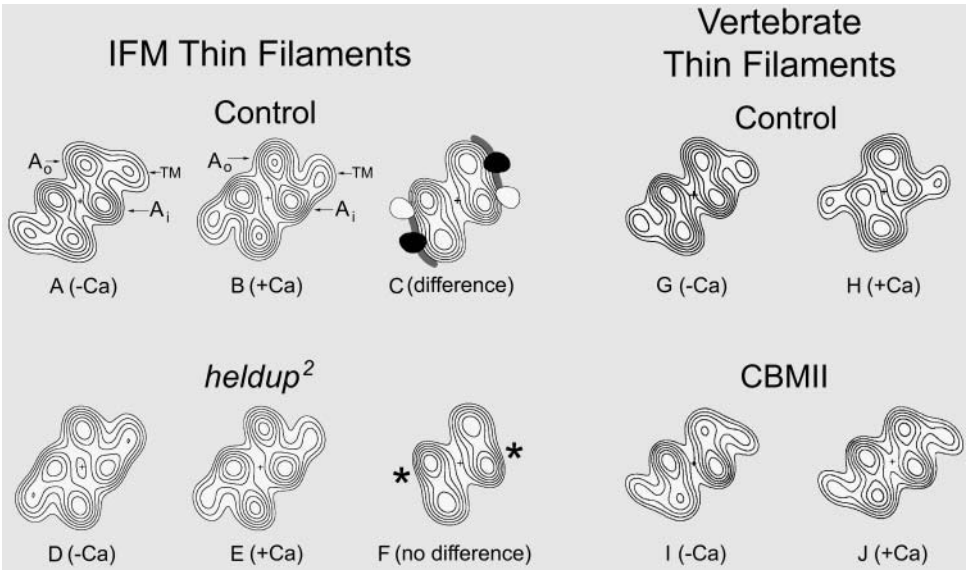

FIGURE 3 Helical projections (formed by projecting component densities in reconstructions down the long-pitch actin helices onto a plane perpendicular to the thin-filament axis) showing axially averaged positions of F-actin and tropomyosin (*TM*) that appear symmetrical on either side of the filament. Densities in all maps shown were significant over background noise at greater than the 99.95% confidence levels. *Drosophila* filaments from *Mhc*^{7} (*A* and *B*) and *hdp*^{2}*; Mhc*^{12} mutants (*D* and *E*); vertebrate filaments containing wild-type troponin (*G* and *H*); or mutant CBMII troponin (*I* and *J*). Reconstructions are of samples maintained in the absence of Ca^{2+} (*A*, *D*, *G*, and *I*) or treated with Ca^{2+} (*B*, *E*, *H*, and *J*). Again, note that Ca^{2+}-dependent movement of tropomyosin occurs in filaments from *Mhc*^{7} IFM, but not in those from *hdp*^{2}*; Mhc*^{12} mutants or from reconstituted filaments containing CBMII Tn C instead of control vertebrate troponin. The statistical significance of the differences observed between maps of *Mhc*^{7} IFM filaments in EGTA or Ca^{2+} conditions was computed. The regions of the two maps that were significantly different from each other at greater than the 99.95% confidence level are displayed as single contour envelopes superimposed on the control map of F-actin (*C*); densities specific to EGTA (*solid black*) or Ca^{2+} (*open*) highlight the tropomyosin movement and the tropomyosin binding positions on actin. The myosin-binding sites on actin are indicated by gray bands in *C*. No significant differences were detected between contributing densities forming the EGTA and Ca^{2+} maps of the *hdp*^{2}*; Mhc*^{12} mutants (*F*), confirming that tropomyosin movement did not occur; an asterisk marks the tropomyosin-binding site on actin in *hdp*^{2} filaments.

osin, as they were virtually identical to tropomyosin strands observed in actin-tropomyosin (no troponin) controls and thin filaments reconstructed from other muscles (Lehman et al., 1994, 2000). In the presence of EGTA, the tropomyosin strands contacted actin monomers along the inner edge of their outer domains (Figs. 2 and 3). In contrast, in Ca^{2+}, tropomyosin contacted the inner domains of actin monomers (Figs. 2 and 3). Difference density analysis, subtracting maps of one set of data from the other, demonstrated that the Ca^{2+}-dependent change in the association of tropomyosin with actin was significant at better than the 99.95% confidence level (Fig. 3). In the absence of Ca^{2+}, A-IFM tropomyosin was located over known myosin binding sites on actin (Milligan et al., 1990) in a position that could block myosin binding in relaxed muscles. Our results showing tropomyosin movement away from the blocking position on addition of Ca^{2+} are consistent with the presence of the steric mechanism of regulation in A-IFM, and the two distinctive positions for tropomyosin in A-IFM filaments are indistinguishable from those found in vertebrate striated muscle filaments (Lehman et al., 2000).

hdp^2 A-IFM thin filaments

Drosophila has a single gene that encodes Tn I (Barbas et al., 1993). A point mutation at residue 116 (Ala to Val) in constitutive exon 5 of Tn I causes the *hdp*^{2} *Drosophila* flightless phenotype characterized by abnormal ''heldup'' wing posture. This results from a hypercontraction that shears apart the A-IFM (Beall and Fyrberg, 1991; Nongthomba et al., 2003), and the ensuing muscle destruction can be easily visualized by polarized light microscopy as in Nongthomba et al. (2003). To study the effects of the *hdp*^{2} mutation on thin filament regulation, filaments were extracted from the A-IFM of *hdp*^{2}; *Mhc*^{12} ''double mutants'' by the same procedure used above. EM of these thin filaments showed normal actin filament substructure, troponin bulges, and tropomyosin strands, and confirms the results of others that thin filament ultrastructure and protein composition appear unaffected by the *hdp*^{2} mutation (Beall and Fyrberg, 1991; Nongthomba et al., 2003). However, in marked contrast to our results on normal thin filaments, 3D reconstruction of filaments isolated from the double mutants showed that Ca^{2+} had no significant effect on tropomyosin position (Figs. 2 and 3). Tropomyosin was in the ''Ca^{2+}-induced'' position on the inner domain of the actin whether Ca^{2+} was present or not. Hence, steric regulation is disabled in *hdp*^{2} filaments, and myosin binding sites on actin are exposed even in relaxing conditions. Thus, in A-IFM with thick filaments and ATP, unimpeded cross-bridge cycling would account for the hypercontraction in *hdp*^{2} flies.

How *hdp*^{2} leads to defective steric regulation is unclear. *Drosophila* residue Ala-116 corresponds to a conserved alanine at position 25 in the vertebrate skeletal muscle Tn I sequence in an N-terminal α-helix that makes hydrophobic

contacts with Cys-98, Ile-101, and Phe-102 of the Tn C ''E helix'' (Vassylyev et al., 1998; Tanaka et al., 2003). The subtle increase in residue size may alter the Tn I-C interface and destabilize the Tn I association with actin and tropomyosin and, therefore, steric regulation. As most *Mhc* mutations suppress the hdp^2 phenotype, any weakening of acto-myosin interactions might diminish hypercontraction and thus the accompanying muscle destruction (Kronert et al., 1999; Nongthomba et al., 2003). Further interpretation awaits an atomic structure of Ca^{2+}-free troponin and a detailed model of its interactions with tropomyosin and actin (cf. Tanaka et al., 2003). In contrast, models of actin-tropomyosin interaction are available (Phillips et al., 1986; Brown et al., 2001), and the ability to dissect the impact of the hdp^2 mutation on tropomyosin position both genetically and structurally validates our approach. Thus, it should be possible to analyze other previously described or newly designed *Drosophila* mutants to determine structural effects on the actin monomer, F-actin helical symmetry, and steric regulation.

Tn C and Ca^{2+}-induced tropomyosin movements

Although the mechanism of steric regulation in insect and vertebrate muscles is comparable, the mass of troponin in insects, and in arthropods in general, differs from that of troponin in vertebrates. A-IFM Tn I and T, for example, contain protein extensions not present in vertebrate troponin (Bullard et al., 1988; Barbas et al., 1993). The functional significance of these variations and of unique thin-filament components in some insect muscles is not known (cf. Bernstein et al., 1993). In addition, Tn C, the most conserved of the troponin subunits, also differs in the A-IFM. Amino acid substitutions in three of the four EF hands in DmTnC4 (Qiu et al., 2003), the major A-IFM Tn C isoform, leave only one metal-binding motif to regulate troponin-tropomyosin interactions. (In the A-IFM system, a minor Tn C isoform (DmTnC1) present in ~10% of the total troponin, binds two Ca^{2+}; that isoform and DmTnC4 apparently are randomly distributed among troponin complexes along thin filaments (Qiu et al., 2003). The Tn C in molluscs, like DmTnC4, also binds Ca^{2+} only at site IV (Lehman et al., 1980; Ojima et al., 2000).) In contrast, vertebrate skeletal muscle Tn C contains four active EF hands, consisting of two low-affinity Ca^{2+}-specific binding sites (I and II) located at the N-terminal lobe of the dumbbell-like protein, and two high-affinity Ca^{2+}/Mg^{2+}-exchange binding sites (III and IV) at the C-terminal lobe. (Tn C in vertebrate cardiac muscle contains three active EF hands, consisting of one low-affinity site (II), and two high-affinity sites (III and IV) (Tobacman, 1996).) Only the low-affinity sites I and II bind Ca^{2+} rapidly enough to control skeletal and cardiac muscle. In vertebrates, sites III and IV are thought to be purely structural, not regulatory (reviewed in Tobacman, 1996). Interestingly, in DmTnC4, only the C-terminal lobe EF hand corresponding to the type IV site is capable of binding Ca^{2+} (Qiu et al., 2003). This is surprising since engineered vertebrate Tn C mutants that lack active sites I and II (but retain sites III and IV) do not activate myosin ATPase or support force in skinned fibers, because Tn I-T inhibition cannot be relieved by Ca^{2+} (Morris et al., 2001). Reconstruction of filaments reconstituted with this mutant vertebrate ''CBMII'' TnC showed that in this system neither site III nor site IV is sufficient for Ca^{2+}-dependent tropomyosin shifts, even when given time to bind Ca^{2+}. Tropomyosin in these mutant vertebrate filaments, in fact, was in the blocked state both in the presence and absence of Ca^{2+} (Figs. 2 and 3). Thus, in the vertebrate system, Ca^{2+} binding to low-affinity binding sites (and not to high-affinity ones) is necessary for the tropomyosin movement. This observation highlights the differences between *Drosophila* and vertebrates in the function of Tn C EF hands at site IV. Unlike that in vertebrates, site IV in *Drosophila* is likely to be regulatory, and by binding Ca^{2+} responsible for steric regulation by tropomyosin. Despite the possibility that insect Tn C may display fairly tight Ca^{2+} binding (Qiu et al., 2003), the relative affinities of Ca^{2+} and Mg^{2+} presumably combine to give an apparent Ca^{2+} affinity at site IV that accounts for a Ca^{2+}-activation of A-IFM myofibrillar ATPase comparable to that in vertebrate striated muscle (Marston and Tregear, 1974). It is intriguing that the hdp^2 mutation involves part of Tn I that interacts with the C-lobe of Tn C, not with the N-lobe, and that the hdp^2 mutation leads to defective steric regulation. This adds credence to the premise that in *Drosophila* and possibly other invertebrates the C-lobe may be regulatory.

Our studies have specifically addressed the structural mechanism of troponin-tropomyosin-linked Ca^{2+} regulation in the A-IFM. It is well known that the rhythmic contraction of A-IFM used for insect flight is not based on cyclic alternation in Ca^{2+} levels but is dependent instead on alternation between stretch-activation and release-deactivation (Pringle, 1978; Tregear et al., 1998). How a troponin-tropomyosin-linked steric blocking mechanism can be designed to be partly dependent on stretch-activation is an unsolved but exciting question for future investigation. The effect of Ca^{2+} binding to troponin-tropomyosin explored here, and necessary in vertebrate and invertebrate synchronous muscles, must be more complex in A-IFM, particularly since these muscles are likely to be additionally modulated by myosin-phosphorylation.

We have demonstrated that *Drosophila* A-IFM thin filaments can be easily isolated from myosinless flies and then studied structurally by electron microscopy and 3D reconstruction. Overall our results show that thin filament regulation in the vertebrate and insect systems is very similar, but that much remains to be learned from the A-IFM model by the comparative genetic and structural approach taken.

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