Kettin, a major source of myofibrillar stiffness in Drosophila indirect flight muscle

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Kettin is a high molecular mass protein of insect muscle that in the sarcomeres binds to actin and α-actinin. To investigate kettin’s functional role, we combined immunolabeling experiments with mechanical and biochemical studies on indirect flight muscle (IFM) myofibrils of Drosophila melanogaster. Micrographs of stretched IFM sarcomeres labeled with kettin antibodies revealed staining of the Z-disc periphery. After extraction of the kettin-associated actin, the A-band edges were also stained. In contrast, the staining pattern of projectin, another IFM–I-band protein, was not altered by actin removal. Force measurements were performed on single IFM myofibrils to establish the passive length-tension relationship and record passive stiffness. Stiffness decreased within seconds during gelsolin incubation and to a similar degree upon kettin digestion with μ-calpain. Immunoblotting demonstrated the presence of kettin isoforms in normal Drosophila IFM myofibrils and in myofibrils from an actin-null mutant. Dot blot analysis revealed binding of COOH-terminal kettin domains to myosin. We conclude that kettin is attached not only to actin but also to the end of the thick filament. Kettin along with projectin may constitute the elastic filament system of insect IFM and determine the muscle’s high stiffness necessary for stretch activation. Possibly, the two proteins modulate myofibrillar stiffness by expressing different size isoforms.

Introduction

Many insects use a special type of muscle, the indirect flight muscle (IFM),* which is characterized by high resting (or passive) stiffness and stretch activation, a delayed active force response to stretch (Pringle, 1967; Rüegg, 1968; Maughan and Vigoreaux, 1999; Josephson et al., 2000). The stiffness of nonactivated IFM resides in the contractile units, the sarcomeres, and is thought to arise mainly from connecting filaments linking thick filaments to Z-discs (Garamvölgyi, 1966; Trombitas and Tigi-Sebes, 1974; White, 1983). More recently, two proteins present in the actin-myosin overlap zone, flightin (Vigoreaux et al., 1993; Reedy et al., 2000) and troponin H (Reedy et al., 1994; Clayton et al., 1998), have also been suggested to potentially contribute to passive stiffness. During active contraction of IFM, passive stiffness persists in parallel with actomyosin crossbridge stiffness (White, 1983). Therefore, it is conceivable that strain on the connecting filament affects crossbridge activity and thus enhances the muscle’s mechanical performance (Vigoreaux et al., 2000). The nature of connecting filaments is not entirely clear yet, although a main constituent is projectin, an ~900-kD polypeptide of the titin-like protein family (Bullard et al., 1977; Saide, 1981; Ayme-Southgate et al., 1991; Fyrberg et al., 1992; Benian et al., 1999). In the IFM of Drosophila, projectin was shown to be anchored at the Z-disc and associate with myosin at the A-band edge (Saide et al., 1989).

Another high molecular weight protein in the I-band of Drosophila and Lethocerus sarcomeres is kettin (Lakey et al., 1990, 1993). The kettin sequence predicts a modular structure, with 35 repeating Ig domains separated by short linker sequences (Hakeda et al., 2000; Kolmerer et al., 2000). In contrast to other titin-like proteins, the published kettin sequence (which shows an ~500-kD polypeptide) contains no fibronectin-like modules, kinase, or PEVK domains. Kettin binds to actin (and α-actinin) with high affinity and a stoichiometry of one Ig linker module to one actin protomer, suggesting that the protein contributes to thin filament...
stability and anchorage in the Z-disc (van Straaten et al., 1999). Kettin may have an essential function for sarcomere formation because adult fruit flies heterozygous for a kettin mutation cannot fly (Hakeda et al., 2000). Although many members of the titin-like protein family, including projectin and titin, are known for their elasticity, kettin’s tight association with actin and lack of myosin-binding propensity, according to an early report (Lakey et al., 1993), made it seem less likely that this protein is also functionally elastic. The work reported here now suggests that kettin does function as an elastic filament in addition to the projectin filaments.

_Drosophila_ kettin could be part of a larger protein, D-titin (Machado et al., 1998; Zhang et al., 2000). The D-titin gene encodes a 1.8-MD polypeptide with homology to the NH$_2$-terminal half of vertebrate titin (Labeit and Kolmerer, 1995). The kettin and D-titin genes are located at the same chromosome position, and the 5′ end of the D-titin genomic sequence contains the sequence of _Drosophila_ kettin (Machado and Andrew, 2000). Thus, the possibility exists that kettin is a cleavage product of D-titin. However, kettin is expressed in several isoforms of different molecular mass. An abundant ∼500-kD variant and a rare ∼700-kD isoform are found in _Drosophila_ IFM (Lakey et al., 1993). Although the precise relationship between kettin and D-titin needs to be established, it is likely that kettin derives from an alternative splice form of D-titin. Alternative splicing has been confirmed for _Drosophila_ projectin (Daley et al., 1998) and vertebrate titin (Labeit and Kolmerer, 1995). In mammals, different titin isoforms are expressed in a muscle type-specific manner. Vertebrate cardiac titin occurs in many size variants that coexist in the same cell (Freiburg et al., 2000). The functional relevance of the titin isoform diversity may lie in a modulation of myofibrillar passive stiffness.

If kettin exists in different isoforms, it could assume roles extending beyond those ascribed to the protein so far. For instance, if kettin connected actin and myosin, just as titin does in vertebrate-muscle sarcomeres, it could act as an elastic filament and contribute to passive stiffness. In the present study, we combined cell biological and biochemical approaches with single myofibril mechanics to elucidate the molecular basis of _Drosophila_ IFM stiffness. The results show that kettin indeed provides a link between thin and thick filaments. A model emerges in which both kettin and projectin are responsible for the high stiffness of relaxed insect IFM necessary to develop stretch activation.

**Results**

**Passive tension of single _Drosophila_ IFM myofibrils**

To characterize the sarcomere length (SL)-tension relationship of nonactivated _Drosophila_ IFM, force measurements were performed on single myofibrils (Fig. 1). The force of three to four myofibrils of similar length was recorded in identical step-stretch protocols, and median-filtered force traces were superimposed to obtain clearer signals (Fig. 1 B). A summary of stress-strain curves obtained from 24 myofibrils is shown in Fig. 1 C. Because stretched myofibrils showed inhomogeneous SLs, Fig. 1 C depicts passive tension related either to the length of the longest sarcomere (solid line) or to mean SL (dotted line). At larger stretches, the difference between the two curves was significant. Passive tension rose steeply on low stretch and reached a first plateau after ∼5% extension. Additional stretch further increased force until at ∼12% extension a second plateau phase (or some force decline) commenced. Higher stretches frequently led to myofibril breakage. Upon release of myofibrils from the stretched SL, large force hysteresis was seen.

The passive SL-tension curve of single _Drosophila_ IFM myofibrils was compared with that of vertebrate cardiac and
skeletal myofibrils established previously (Linke, 2000). Fig. 1 C, inset, demonstrates that Drosophila IFM has by far the steepest stress-strain curve; the stiffness (approximated linear slope at modest extension) is about one order of magnitude higher than in rabbit cardiac myofibrils. Much lower stiffness is found in rat psoas myofibrils.

Stretch-dependent behavior of I-band proteins in normal IFM myofibrils

Kettin. The position of kettin in relaxed Drosophila IFM sarcomeres was analyzed by immunofluorescence (IF) and immunoelectron microscopy (IEM). Two different antibodies were used, one against the central part (Ig16) and the other against the COOH terminus (Ig34/35) of the published kettin sequence (Fig. 2 A). By IF, kettin epitopes appeared as a single stripe in the Z-disc region of sarcomeres independent of the stretch state (Fig. 2 B and C). IEM showed that α-Ig34/35 labels both nonstretched and modestly stretched sarcomeres at the periphery of the Z-disc (Fig. 2 D). In extremely stretched myofibrils, some rearrangement of kettin could be seen: IF with α-Ig16 or α-Ig34/35 revealed a “smear” near some Z-discs (Fig. 2, B and C, arrows).

Projectin. We also investigated the staining pattern of antibodies to projectin (Ig18). Antiprojectin labeled the Z-discs in short sarcomeres but fuzzy bands at the A-band edges in stretched sarcomeres (Fig. 2 E).

PEVK-sequence. Antibodies (9D10) to the PEVK region, a unique titin sequence of obscure secondary/tertiary struc-
ture, did not stain unstretched myofibrils but labeled the Z-disc region in modestly stretched sarcomeres and two distinct epitopes near the Z-disc in long sarcomeres (Fig. 2 F). Analysis of the intensity profiles (Linke et al., 1999) revealed that in stretched myofibrils the PEVK epitopes are located in the I-band.

Damage to Drosophila IFM sarcomeres upon extension

Low extensibility and stretch-induced high SL inhomogeneity were consistently observed in relaxed Drosophila IFM myofibrils. Somewhat surprisingly, when nonactivated myofibrils were stretched by ~10% from slack SL and stained with rhodamine-phalloidin, actin always appeared to be broken at the Z-disc (Fig. 3 A); it seemed impossible to pull the actin filaments out of the A-band. Myofilament breakage at the Z-disc could be observed also on electron micrographs of stretched IFM sarcomeres (Fig. 3 B). In addition, actin-filament breakage was seen in stretched myofibrils stained with α-actin antibodies (unpublished data). A possible explanation of this result is that some actin-myosin connections remained in the A-band, despite the presence of 20 mM BDM (an active force-suppressing agent) in the relaxing buffer. Alternatively, some additional structure(s) could link actin and myosin filaments thereby stiffening the sarcomere.

Proteins potentially involved in the passive stiffening were extracted from IFM myofibrils by various procedures. Actin was removed with a Ca$^{2+}$-independent gelsolin fragment, which extracted all but the Z-disc actin when used at concentrations of 0.2–0.3 mg/ml (Fig. 3 D, top). At concentrations >0.3 mg/ml, Z-disc actin was removed also (Fig. 3 D,
bottom). Even after complete actin extraction, myofibrils did not disintegrate; rather, they could be stretched highly while showing reasonable SL homogeneity (Fig. 3 D and Fig. 4). Removal of actin allowed the myofibrils to be extended reversibly over a wider SL range.

Myosin was extracted by application of a buffer containing 400 mM KCl. Within seconds of application, thick filaments became depolymerized and sarcomeres broke apart at the M-line (unpublished data). The remaining myofibril fragments (sometimes only one or a few half sarcomeres long) were so fragile that they could not be used for stretch experiments.

A Ca\textsuperscript{2+}-dependent protease, \(\mu\)-calpain (3 \(\mu\)g/ml), selectively digests kettin in \textit{Drosophila} IFM sarcomeres (Fig. 5, D and E). After treatment of IFM myofibrils with \(\mu\)-calpain, SL homogeneity upon stretch in relaxing buffer was much improved compared with control myofibrils (Fig. 3 C). More importantly, rhodamine-phalloidin staining revealed that the actin filaments could now be pulled out of the A-bands. Only rarely did actin still break at the Z-disc (Fig. 3 C, arrow); usually, the actin filaments of two adjacent half sarcomeres remained connected at the Z-band (Fig. 3 C, arrowhead at bottom). Thus, cleavage of kettin removed the elements that normally prevent \textit{Drosophila} IFM sarcomeres from tolerating larger stretches. The leftover sarcomere structure might be held together by the remaining projectin molecules. (Projectin is cleaved only slightly at the \(\mu\)-calpain concentration of 3 \(\mu\)g/ml chosen [Fig. 5 D].)

**Figure 5.** Effect of treatment with \(\mu\)-calpain (A–E) and Igase (F–H) on \textit{Drosophila} IFM myofibrils. (A) IF of single myofibril stained with \(\alpha\)-kettin Ig16 after calpain treatment and stretch; the A-band edge is labeled (arrowheads), and the Z-disc is labeled also in some sarcomeres (arrow). (B) \(\alpha\)-Projectin staining of stretched sarcomeres showed a fuzzy epitope at the A-band edge (arrowheads) and also Z-disc labeling (arrow). (C) PEVK sequence was not stained by 9D10 antibody in calpain-treated, stretched, myofibrils. (D) Low percentage SDS-gel to show the effect of \(\mu\)-calpain (3 \(\mu\)g/ml, 25°C, 45 min) on high molecular weight proteins. Only kettin (K) isoforms are substantially digested, whereas projectin (P) and M-line protein (M) remain largely intact. Nebulin (N) and titin (T) from rabbit soleus muscle are used as standards. (E) Lower molecular weight proteins are not affected by \(\mu\)-calpain treatment as detectable on 10–18% SDS-gradient gels. (F) Igase-mediated digestion of tropomin H effectively decreased the staining intensity of \(\alpha\)-TnH34 antibodies (MAC 143) on myofibrils. (G) 12% SDS-gel electrophoresis of washed IFM myofibrils to separate TnH33 and TnH34 isoforms. Igase treatment eliminated the TnH34 isoform. (H) Western blot with antibodies to either TnH33 or TnH34 confirms that Igase treatment removed TnH34 but left TnH33 intact.

**Stretch-dependent kettin epitope position in actin-extracted IFM myofibrils**

The epitope positions of kettin antibodies were investigated in single \textit{Drosophila} IFM myofibrils from which actin was completely extracted (Fig. 4). Actin removal was confirmed at the end of each experiment by rhodamine-phalloidin staining. Interestingly, actin-free sarcomeres showed a kettin labeling pattern much different from that of nonextracted sarcomeres (Fig. 2, B–D). Whereas \(\alpha\)-kettin Ig16 was still seen by IF to stain the Z-disc region (Fig. 4 A, arrow), stretched myofibrils exhibited two additional stripes per sarcomere (Fig. 4 A, arrowheads). These stripes were located on either side of the Z-disc and separated during myofibril stretch but moved toward one another (un-
published data) during release. The segment flanking the Z-disc and the Ig16 kettin epitope thus showed reversible extensibility.

The stretch-dependent position of kettin Ig16 epitopes was measured from intensity profiles recorded along the myofibril axis (Fig. 4 B, bottom trace) by using a custom-written peak detection program (Linke et al., 1999). Also, the A-band edges were detected on corresponding phase images (Fig. 4 B, top trace) to obtain the position of “out-of-Z-disc” kettin epitopes in the sarcomere. The graph in Fig. 4 B shows the SL-dependent separation of Ig16 kettin epitopes compared with that of A-band edges measured across the M-line (top) or the Z-disc (bottom). The graph (a) confirms that the Ig16 kettin position varies with the stretch state, (b) indicates that the length of the A-band (normally ~3.0 μm) increases upon extreme stretch by ~0.15 μm (Trombitas and Tigyi-Sebes, 1977), and (c) demonstrates that the Ig16 kettin epitopes are positioned somewhat inside the A-band.

A similar staining pattern was seen with γ-kettin Ig34/35. An example is shown in Fig. 4 C. This actin-extracted stretched myofibril initially exhibited strong Z-disc staining but faint labeling of the A-band edges. However, when the myofibril (held at constant SL) was exposed to small amplitude sinusoidal length oscillations (20 Hz), the fluorescence intensity of the Z-disc epitopes decreased with time (Fig. 4 C, arrows), whereas that of Ig34/35 epitopes at the A-band edge increased strongly (Fig. 4 C, arrowheads). These findings clearly show that the Ig34/35 kettin epitopes are connected to both the Z-disc and the A-band edge.

IEM was employed to investigate Ig34/35 kettin staining in actin-extracted Drosophila IFM fibers. Fig. 4 D demonstrates that in contrast to nonextracted fibers (unpublished
data) sarcomeres could be readily stretched to reveal a wide I-band region. After gelsolin treatment and high stretch, the specimens showed lower structural preservation than normal IFM sarcomeres (Fig. 2 D). The IEM measurements on actin-extracted fibers again showed that α-Ig34/35 labels both the periphery of the Z-disc (Fig. 4 D, arrows) and the A-band edges (Fig. 4 D, arrowheads). Additional confirmation comes from a histogram showing the distribution of nanogold particles (indicating α-Ig34/35 binding) in the I-Z-I region of ~3.8-μm-long sarcomeres (Fig. 4 D, graph).

For comparison, IF images were recorded from Drosophila IFM myofibrils that had been actin extracted, stretched, and then stained with either α-projectin (Ig18) or α-PEVK (9D10) antibodies. The projectin staining pattern was similar to that of nonextracted sarcomeres (Fig. 2 E); fuzzy epitopes appeared near the A-band edge and faintly at the Z-disc (Fig. 4 E). In contrast, α-PEVK 9D10 failed to label myofibrils even at high stretch (Fig. 4 F). Apparently, some structure connecting the PEVK site to the Z-disc had been damaged or removed by the actin extraction procedure.

Digestion of sarcomere proteins: effect on staining pattern and myofibril stiffness

Exposure of IFM myofibrils to μ-calpain (3 μg/ml) digested both the 500 and 700 kD kettin isoforms to a large degree (Fig. 5 D). Projectin was barely affected, whereas no effect was seen on low molecular weight sarcomere proteins (Fig. 5 E). Single Drosophila IFM myofibrils were treated with 3 μg/ml μ-calpain, stretched, and investigated by IF for their reactivity to α-kettin Ig16, α-projectin Ig18, and α-PEVK 9D10 antibodies (Fig. 5, A–C). Antikettin Ig16 stained the Z-disc in a few sarcomeres but a distinct stripe at the A-band edge in all sarcomeres (Fig. 5 A). Antiprojectin Ig18 antibody stained broader stripes at the A-band edge and the Z-disc (Fig. 5 B). Thus, projectin labeling was not much different than in normal or actin-extracted myofibrils. AntipeVK 9D10 antibody did not label stretched myofibrils in which kettin had been cleaved (Fig. 5 C), resembling the result obtained after actin removal.

To investigate how μ-calpain and gelsolin may affect passive stiffness, we measured the force response of single nonactivated Drosophila IFM myofibrils to sinusoidal motor movement (Fig. 6 A). Calpain-treated myofibrils were stretched in relaxing buffer to 3.7 μm SL, and stiffness was recorded immediately after the stretch and after a waiting period of 20 and 30 min, respectively (Fig. 6 B). Fig. 6 C shows that stiffness of μ-calpain-treated myofibrils dropped by 50–60% within 20–30 min, whereas that of control specimens decreased only by ~20% (normal stress relaxation). In contrast to controls, digested myofibrils exhibited no stiffness increase upon further stretch. Thus, cleavage of kettin significantly depresses myofibril stiffness. Whereas passive stiffness was recorded after μ-calpain digestion (because digestion requires Ca²⁺), it was measured during treatment with gelsolin fragment in relaxing buffer (Fig. 6 D, bottom panels). As shown in Fig. 6 E (black solid curve), stiffness decreased greatly within the first minute of gelsolin application. This initial stiffness drop could be as large as 50%. Then, stiffness decreased only a little and after 20–30 min reached values comparable to those measured in calpain-treated myofibrils (Fig. 6, C and E). Further stretch of actin-extracted specimens failed to increase stiffness appreciably.

Part of the IFM stiffness could potentially be due to cross-linking of actin and myosin by a thin filament-associated protein, troponin H, which exists in two isoforms, TnH33 and TnH34. To test whether some stiffening can be ascribed to TnH, we tried to digest the protein with Igase (Clayton et al., 1998). Digestion was monitored by IF in myofibrils stained with α-TnH34 antibody on SDS-gels and Western blots (Fig. 5, F–H). The fluorescence intensity of α-TnH34–stained myofibrils disappeared almost entirely upon Igase treatment (Fig. 5 F). Western blots revealed that TnH33 was little affected by Igase, whereas TnH34 was digested completely (Fig. 5 H). Since both isoforms are normally present in more or less equal amounts (Fig. 5 G), we assumed that digestion of one isoform should significantly decrease stiffness if these proteins played some role for myofibril stiffening. However, Fig. 6 E shows that stiffness recorded over a 2-h period of Igase treatment (gray solid curve) remained at a high level not significantly different from that of control myofibrils (dotted curve). Thus, TnH34 is unlikely to contribute much to the passive stiffness of Drosophila IFM.

Protein composition of insect muscles: in search of large proteins

In a more systematic effort to detect high molecular mass proteins, 2% SDS-PAGE was carried out on myofibrils from both Drosophila and Lethocerus IFM and Lethocerus leg muscle (Fig. 7 A). Vertebrate titin and nebulin served as size markers. The analysis confirmed that all insect muscle types contain various large proteins (Fig. 7 A, left). In Drosophila IFM, stronger protein bands likely correspond to 400-kD M-line protein, 500-kD kettin, and ~900-kD projectin (Fig. 7 A, lane 3). A faint band appearing at ~700 kDa may correspond to another kettin isoform (see next paragraph).

The possibility exists that larger titin-like proteins were not visualized because of degradation during myofibril preparation. (The ORFs from the genome sequence of D-titin predict that the maximum size of the protein is 1.8 MD [Machado and Andrew, 2000; Zhang et al., 2000].) Therefore, Drosophila thoraces were frozen in liquid nitrogen immediately after dissection and were also tested for their protein composition (Fig. 7 A, right). No protein larger than ~1,100–1,200 kDa was detectable, even on SDS-gels stained with silver to improve sensitivity. Thus, Drosophila flight muscles, which make up a majority of the thoracic muscles, may not express a protein of 1.8-MD size corresponding to a full-length D-titin. Lethocerus leg muscle has a protein of high molecular mass not present in the IFM.

Kettin binds to thick filaments

If kettin were important for myofibril stiffness, the protein should bind not only to thin filaments (van Straaten et al., 1999) but also directly or indirectly to thick filaments. We therefore probed kettin for a possible interaction with thick filaments. Immunoblots with α-kettin Ig16 on Drosophila IFM myofibrils or whole thoraces demonstrated the presence of two major isoforms of 500- and 700-kD size (Fig. 7 B, lanes 1–3). In addition, two lesser species estimated to be
and 1,000 kD were identified (Fig. 7 B, lane 1). We then reasoned that myofibrils from a Drosophila actin-null mutant, Act88F KM88, which contains neither thin filaments nor Z-discs in IFM, should not react with kettin antibodies unless kettin is associated with the thick filaments. (Thin filament proteins, for example, troponin and tropomyosin, are not accumulated in actin-null myofibrils [Clayton et al., 1998].) Fig. 7 B, lane 4, demonstrates that -kettin Ig16 did label the actin-null mutant, detecting the abundant 500-kD form and the 700- and the 1,000-kD variants. Further, isoform composition is altered in the actin-null species: KM88 IFM has less 700-kD and more 1,000-kD isoform compared with wild-type IFM (Fig. 7 B, lanes 2 and 4). The presence of kettin in actin-null mutant is consistent with attachment of kettin to thick filaments.

Western blots with normal IFM myofibrils were also prepared using -kettin Ig34/35 and -PEVK antibodies. -kettin Ig34/35 detected the 500-kD isoform but failed to react with the 700-kD variant; instead, an ~800-kD isoform was labeled (Fig. 7 B, lane 5). The 800-kD isoform was detected by -PEVK, but this antibody did not react with the 500- and 700-kD variants (Fig. 7 B, lane 6). These findings strongly support the idea that Drosophila IFM myofibrils contain several kettin isoforms arising from alternative splicing.

To more directly test for kettin-myosin binding, a dotblot method was used (Fig. 7 C). When IFM myosin was incubated with kettin fragment Ig34/35 (followed by anti-His and secondary antibody), a signal was detected whose intensity depended on the myosin concentration. Incubation with only anti-His and secondary antibody revealed no signal. Thus, the COOH terminus of the published kettin sequence may interact with myosin filaments.

Discussion

Connecting filaments were clearly identified in insect flight muscles in the late 1960s and early 1970s (Garamvölgyi, 1966; Pringle, 1967; Reedy, 1971; Trombitas and Tigy-Sébes, 1974; White, 1983), but the protein(s) constituting these structures at first remained unknown. With the discovery of projectin, a large polypeptide in the I-band of IFM sarcomeres (Bullard et al., 1977; Saide, 1981; Ayme-Southgate et al., 1991), the protein making up part or all of the connecting filament structure was found. In this study, we provide evidence that the elastic filament system of Drosophila IFM sarcomeres also contains kettin as a major constituent.

Partial sequence analysis showed that kettin is composed of Ig domains interspersed with short unique sequences...
Kettin was found to bind to both α-actinin and actin in the center and at the periphery of the Z-disc (Lakey et al., 1993; van Straaten et al., 1999). Association with thick filaments initially was considered unlikely because an Ig domain from the middle part of kettin did not interact with myosin in vitro (Lakey et al., 1993). When more sequence information on kettin became available (Hakeda et al., 2000; Kolmerer et al., 2000), the presence of additional Ig domains at the protein’s COOH terminus was revealed. Currently, it is unresolved whether the published kettin sequence could be part of a larger protein, D-titin, in Drosophila muscles (Hakeda et al., 2000; Kolmerer et al., 2000; Machado and Andrew, 2000; Zhang et al., 2000). Possibly, kettin is a splice form of D-titin. We showed that, at least in Drosophila thoraces D-titin is not expressed as a 1,800-kD protein, the maximum size expected from the genome sequence data (Fig. 7 A). Support for the idea that kettin exists in isoforms of ≤1,000 kD now comes from our results on the reactivity of kettin-specific antibodies with different molecular size bands on Western blots (Fig. 7 B).

With kettin’s NH₂ terminus known to be positioned within the Z-disc (Kolmerer et al., 2000), we hypothesized that the protein may span the half I-band of an IFM sarcomere and at the COOH terminus be attached to thick filaments. If kettin indeed connected Z-discs and thick filament ends, it could act in parallel with projectin to contribute to passive stiffness. Therefore, we investigated whether stiffness is affected by removal of one anchorage point of kettin, the actin filaments near the Z-disc. We also studied the effect of proteolytic digestion of kettin on myofibrillar stiffness. Along the way, we established the passive SL-tension relationship of single Drosophila IFM myofibrils. The combined results of mechanical, IF, and IEM measurements, using antibodies to kettin, projectin, and PEVK-titin, led us to propose a model as to how kettin may function in the I-band under the different experimental conditions. This model, shown in Fig. 8, also considers the presence of different size kettin isoforms.

The scheme of Fig. 8 A depicts kettin in normal unstretched sarcomeres where the protein may provide a short link from the actin filaments over to the A-band edge. The kettin thick filament interaction involves myosin (Fig. 7 C) but could include other thick filament-associated proteins also. For example, one could speculate that some kettin domains also bind to projectin at the A-band edge. Such ternary complexes might have their structural counterparts in the blob-like structures just outside the A-bands frequently seen on high resolution electron micrographs of insect IFM (Reedy et al., 1988; Trombitas, 2000). At this point, it is unclear whether all kettin isoforms can interact with the thick filament. Since we demonstrated myosin binding for the COOH-terminal Ig domains of the published kettin sequence (Ig34/35), association with thick filaments is likely for the 500- and 800-kD isoforms; both variants are recognized by α-kettin Ig34/35 (Fig. 7, B and C). However, this does not exclude that 700-kD kettin can also interact with A-band proteins, perhaps via other Ig domains. What is clear is that even the shortest of these kettin isoforms (500 kD) should be able to attach to the Z-disc and still reach over to the A-band edge, because at physiological SLs the I-band of Drosophila IFM sarcomeres is very short (Kolmerer et al., 2000).

Fig. 8 B shows the situation in a highly stretched sarcomere. Although under these conditions, no clearly defined kettin epitopes appeared outside the Z-disc, a “smear” in the I-band region seen on IF images indicated that at least some kettin had moved toward the A-band (Fig. 2, B and C). Moreover, only in stretched (nonextracted) sarcomeres did we observe PEVK staining, most likely because the PEVK epitope was pulled out of its A-band location. This scenario implies that the PEVK epitope is connected to some structure located upstream (toward the Z-disc). Then, if kettin isoforms are considered alternatively spliced forms of D-titin whose genomic sequence encodes two major PEVK regions (Machado and Andrew, 2000; Zhang et al., 2000) some kettin variants, such as the 800-kD isofrom, may in fact contain PEVK domain(s). This conclusion is supported by the results of immunoblot analysis with α-PEVK antibody, which labels 800-kD kettin; the same isoform is recognized by kettin antibody Ig34/35 (Fig. 7 B). A striking observation on relaxed stretched sarcomeres was that the actin filaments appeared to be broken at the Z-line (Fig. 3), for example, in the region of actin-kettin interaction. In Fig. 8 B, we suggest that the actin breakage may also cause damage to kettin. Possibly, it is this kind of damage which causes the first plateau in the passive SL-tension curve of single Drosophila

Figure 8. Model of the arrangement of kettin (red), projectin (black), actin (blue), and myosin (green) in Drosophila IFM sarcomeres. The model attempts to explain this study’s results obtained under the respective experimental conditions indicated above each panel. Kettin is drawn in different length variants to take into account the presence of 500-, 700-, and 800-kD isoforms (from top to bottom in each panel). Yellow color indicates PEVK domain (position of PEVK domain was tentatively induced from the genome sequence of D-titin; Machado and Andrew, 2000; Zhang et al., 2000). Gray bars indicate Z-disk. For further explanation, see text.
IFM myofibrils already after 5% stretch (Fig. 1 C). However, such a degree of extension may not be reached in situ because the length change of *Drosophila* IFM fibers in vivo is estimated to be 3.5% (Chan and Dickinson, 1996). In any case, we showed that *Drosophila* IFM myofibrils are at least one order of magnitude stiffer than vertebrate myofibrils (Fig. 1 C). In comparison, *Lethocerus* IFM fibers are also relatively stiff and reach an elastic limit after 6–7% stretch (Granzer and Wang, 1993). In contrast, bee flight muscle appears to be more extensible (Trombitas and Tиги-Sebes, 1977); it can be stretched highly while retaining a greater SL homogeneity than *Drosophila* IFM fibers. In this context, we note that the great fragility of single *Drosophila* IFM myofibrils should be considered when such preparations want to be probed for their stretch activation characteristics (Maughan and Vigoreaux, 1999).

Treatment of single IFM myofibrils with gelsolin fragment immediately reduced passive stiffness and effectively extracted the actin filaments. These results are similar to those reported by Granzer and Wang (1993) on *Lethocerus* IFM fibers. Fig. 8 C schematically shows part of an actin-extracted stretched sarcomere by taking into account the results of Fig. 4. Projectin did not seem to be much affected by the actin extraction procedure, which is not unexpected because the protein does not bind to actin (Bullard et al., 2000). In contrast, gelsolin treatment strongly influenced the position of kettin, which was freed from its association with actin. Some kettin filaments apparently still remained attached to the Z-disc, probably via interaction with α-actinin. However, many kettin molecules moved to the edge of the A-band, indicating their connection to thick filaments. IF and IEM images suggested that the thick filament interaction is just inside the A-band. We speculate that the 800-kD kettin isoform, which might contain a PEVK domain (Fig. 7 B, lanes 5 and 6), may also lose connection to the Z-disc upon actin extraction. Sarcomere stretch then fails to pull out the PEVK epitope from its A-band location thus explaining the observed lack of PEVK staining.

Fig. 8 D depicts the myofilament arrangement in stretched sarcomeres after treatment with μ-calpain to cleave mainly kettin and to a much lesser degree projectin (Fig. 5 D). Remarkably, the digestion made it possible to pull the actin filaments out of their overlap with myosin (Fig. 3 C). We propose that this reflects a loosening up of the tight connection between actin filaments and the structural elements of the I-band that link actin and myosin. Because projectin does not interact with actin (Bullard et al., 2000), kettin may be the component responsible for the linkage. This conclusion is supported twofold. First, projectin staining in calpain-treated specimens was not significantly different from that in normal myofibrils, whereas kettin labeling was altered with distinct epitopes now located mainly at the A-band edge (Fig. 5, A and B). Second, after μ-calpain treatment the PEVK epitope was not stained even at high stretch (Fig. 5 C), suggesting damage to the connection upstream, which might be a kettin isoform. Moreover, myofibrillar stiffness dropped by a similar amount in calpain-digested and gelsolin-treated specimens (Fig. 6), which demonstrates that the passive stiffness decrease on actin extraction is not primarily due to the removal of weakly bound actin-myosin crossbridges in the overlap region as suggested earlier (Granzer and Wang, 1993).

Although the main conclusions of this study are based on results obtained with IFM myofibrils of *Drosophila*, they may bear more general significance. IFM sarcomeres are more extensible (Lakey et al., 1990) and somewhat less stiff (unpublished data) in *Lethocerus* than in *Drosophila*. Consistent with this is that in *Lethocerus* IFM the major kettin isoform is 700 kD, whereas in *Drosophila* it is 500 kD (Lakey et al., 1993; van Straaten et al., 1999; Fig. 7 A). Because kettin is present in the I-band region of both flight and leg muscle, some kettin isoforms may also be constituents of the elastic filaments of the latter muscle type. Possibly, larger isoforms from the D-titin gene are expressed in leg muscle where the I-bands are longer than in IFM. Consistent with this is the presence of higher molecular mass bands in gels and on the Western blots of wild-type *Drosophila* thoraces (which contain both IFM and leg muscle) compared with those of IFMs. Further, the other I-band protein contributing to IFM stiffness, projectin, is larger in *Lethocerus* than in *Drosophila* IFM (Fig. 7 A). Projectin of IFM is somewhat smaller than that of other muscle types (Vigoreaux et al., 1991). Interestingly, whereas projectin spans the I-band of insect IFM sarcomeres, the protein is found in the A-band of leg muscle sarcomeres (Hu et al., 1990; Lakey et al., 1990; Nave and Weber, 1990; Vigoreaux et al., 1991). Consistent with this is the observation that resting stiffness is high in IFM but lower in the leg muscle of *Drosophila* (Peckham et al., 1990). It remains to be seen whether in insect leg muscle the protein determining passive sarcomere stiffness could indeed be kettin. Taken together, we suggest that the stiffness of insect myofibrils may be modulated by the particular length isoforms of projectin and kettin expressed. This concept will need further proof, but it is similar to the concept proposed for vertebrate titin where differential expression of I-band segments underlies myofibrillar elastic diversity (Frieburg et al., 2000).

These results suggest that kettin and projectin together determine most of the high passive stiffness of insect IFM necessary for stretch activation. However, it is possible that other proteins are also involved in the stiffening. A protein from the actin-myosin overlap zone, troponin H, hypothesized to participate in the stiffening (Clayton et al., 1998), was shown here to play no significant role for passive stiffness. The *Drosophila* A-band–associated protein, flightin (Vigoreaux et al., 1993; Reedy et al., 2000), may contribute to the passive mechanical properties to some degree. However, flightin is cleaved by Igase, like troponin H (Clayton et al., 1998), which suggests it is also unlikely to contribute to passive stiffness. Finally, a novel member of the titin/myosin light chain kinase (MLCK) family has been described recently in *Drosophila* and was named stretchin-MLCK (Champagne et al., 2000). Its genomic sequence predicts a nearly 1-MD size polypeptide containing a kinase domain (like projectin but unlike D-titin/kettin), 32 Ig domains, two fibronectin type 3 modules, and two highly repetitive PEVK domains. The function of stretchin-MLCK in the sarcomere is unclear; expression at the protein level has not yet been studied. Hence, we conclude that, according to current knowledge, mainly projectin and kettin in the I-band...
Experiments were performed at room temperature. tested for their effect on stiffness. The average force amplitude (mean time intervals before, during, and after application of various substances. Force was sampled at 1 kHz. The motor sweeps were imposed at variable to bursts of sinusoidal length oscillations (20 Hz; 20 nm/half-sarcomere). during the first stretch-release cycle were used for data analysis. Myofibrils were released in steps back to slack SL. Only forces recorded specimen was held at a constant SL for 10 s to wait for stress relaxation. The stretch amplitude per step usually was 0.05 stretch protocols with relaxed single myofibrils extended step-wise from column (Amersham Pharmacia Biotech) (van Straaten et al., 1999).

M9 minimal medium, diluted fourfold in SB medium, and induced with 0.2 sert was confirmed and the expression vector transformed into His6tag at the NH2 terminus (Studier et al., 1990). The sequence of the in-EMBL/DDBJ under accession no. AJ245406) coding for Ig domains 34 and

A Ca2+-independent gelsolin fragment was prepared by proteolytic diges- tion of pig stomach smooth muscle gelsolin with thermolysin (Hellweg et al., 1993). After purification by anion exchange chromatography, small units were stored at −80°C and thawed just before use. Rhodamine-philaloidin (Sigma-Aldrich) was used to visualize actin in myofibrils and to rou- tinely test for actin extraction.

A protease, µ-calpain from bovine skeletal muscle, digests kettin in IFM myofibrils (Lakey et al., 1993). Other myofibrillar proteins are not affected, except projectin, which is degraded only slightly (Fig. 5; projectin is more strongly digested at higher calpain concentrations (Lakey et al., 1993). Here, myofibrils were incubated with low µ-calpain concentrations (3 μg/ml) in a buffer containing 10 μM free Ca2+ for up to 1 h at 25°C. Digest- tion was stopped by inhibusing relaxing buffer containing 15 mM EGTA. µ-Calpain was a gift from Dr. D.E. Goll (University of Arizona, Tucson, AZ). TnH proteolysis was induced by incubating myofibrils for 2 h at 25°C in relaxing buffer containing 20 μg/ml Igase (MobiTec), a protease specific to a Pro-Pro-Y-Pro peptide sequence present in TnH (Clayton et al., 1998).

SDS-gel electrophoresis and immunoblotting

Large proteins of Drosophila and Lethocerus IFM and leg myofibrils, Drosophila thoraces, and for comparison rat heart and rabbit psoas myo-fibrils were analyzed by 2 or 2.6% SDS-PAGE (Linke et al., 1997). Whole Drosophila thoraces were frozen in liquid N2 immediately after dissection to avoid proteolysis. If not indicated otherwise, gels were stained with Coomassie brilliant blue R. Proteins in whole Drosophila thoraces or washed IFM myofibrils were separated on 2.5–7.5% polyacrylamide gradient gels and transferred to nitrocellulose by electroeloresis at 900 mA for 3 h in a buffer containing 0.1% SDS (Lakey et al., 1990). Immunoblots were incubated with α-kettin IgG or α-lg34/35 or 9D10. Kettin-Ig16 antibody was also used on IFM myofibrils from a Drosophila actin-null mutant, Act88Fex. Antibody binding was detected by incubating in peroxidase-conjugated secondary antibody (Sigma-Aldrich) followed by ECL chemiluminescent reagents (Amersham Pharmacia Biotech). Variable exposure times were selected to compare relative amounts of kettin in wild-type and actin-null samples. For analyzing TnH digests, Act88F- or Act88Fex-myofibrils were sonicated on 12% gels and immunoblotted (Clayton et al., 1998).

Binding of kettin fragment Ig34/35 to myosin was estimated by dotblot. Lethocerus myosin (2.0–0.05 μg) was spotted onto nitrocellulose mem- brane, and after blocking in milk buffer (Harlow and Lane, 1988) strips were incubated in recombinant Ig34/35 (0.1 mg/ml) then in anti-His tag antibody (Dianova) diluted 1:500 followed by peroxidase-conjugated sec- ond antibody (Sigma-Aldrich). Antibody binding was detected by chemilu- minescence as above.

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