Chapter 17

Some Functions of Proteins from the *Drosophila Sallimus* (*sls*) Gene

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Summary

Insect flight muscles contract at high frequencies and are activated by periodically stretching the muscles. For the stretch to have an effect, the muscles must be stiff. Two elastic proteins, projectin and kettin, are responsible for a large part of the muscle stiffness. Thin filaments containing actin emerge from Z-discs, which occur at periodic intervals along the myofibril, and thick filaments containing myosin interdigitate with the thin filaments. Both projectin and kettin form a mechanical link between the Z-discs and the ends of thick filaments. Kettin is made up of immunoglobulin-like (Ig) modules separated by linker sequences, and is associated with actin in the region of the Z-disc. The protein is an isoform derived from the *Drosophila sallimus* (*sls*) gene. Longer isoforms from the *sls* gene have additional, more extensible, sequence and these are found in non-flight muscles that are less stiff. Isoforms of the protein Sls have several different functions. Kettin causes thin filaments to align side-by-side in an anti-parallel fashion, which could nucleate Z-disc formation in developing myofibrils. Kettin is in the enlarged Z-discs close to the site of attachment of myofibrils to the cuticle, and may reinforce actin filaments in this region, giving the structure the required stiffness.

Sls appears early in development of the *Drosophila* embryo and is needed for fusion of myoblasts to form myotubes which will become muscle fibres. Sls is associated with the membrane at the site of myoblast fusion, together with other proteins (Duf and Rols) that are needed for fusion.

The elastic properties of single molecules of kettin have been measured using optical tweezers. The Ig domains unfold at relatively low stretching forces and refold at high forces. This suggests that kettin could be a folding-based spring, which may be relevant to its function in early muscle development, as well as in the adult myofibril.

Introduction

All striated muscles have large modular proteins that determine the elastic properties of the sarcomere. The *Drosophila* thorax contains muscles that vary widely in function, and the ultrastructure of the sarcomere is correspondingly varied. The structure of the indirect flight muscles (IFMs) is characteristic of asynchronous muscle. In these muscles, oscillatory contractions are produced by a delayed response to stretch, combined with the resonant properties of the thorax and wings. The high frequency (200 Hz) of oscillations in *Drosophila* IFM is possible because the muscles themselves are stiff; they have short I-bands and the sarcomere length changes very little during the contractile cycle. The relative inextensibility of IFM is due to connecting filaments extending from the Z-disc to the end of the thick filaments.[1] These filaments have two components: kettin and projectin, both of which contribute to the high resting tension.[2]

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Other muscles in the *Drosophila* thorax have wider I-bands; their function would not require them to be unusually stiff and they have longer connecting filaments.

Kettin and larger isoforms from the *sls* gene have two distinct functions. The first is the regulation of muscle development in the early embryo and the second is in determining the elastic properties of different muscle types. We have recently reviewed the function of elastic proteins in the mechanics of IFM,[3] and here we will concentrate mainly on other functions of kettin and Sls. We also discuss some properties of single kettin molecules.

sls, the Gene

When a large modular protein containing immunoglobulin (Ig) domains is identified in the muscles of any animal, it is often called titin (or connectin). Invertebrate species have several proteins that have some similarity to vertebrate titin and a more specific nomenclature is needed. Vertebrate titin has a characteristic pattern of modules along the molecule, interspersed with domains of unique sequence: a region of tandem Igs, an elastic PEVK domain, more tandem Igs, and a region with a repeating pattern of fibronectin (Fn) and Ig domains, followed by a kinase domain and more Igs. We suggest that if a protein is called titin it should have these defining features, otherwise it should be given a different name.

The first gene coding for a modular elastic protein to be identified in *Drosophila* was the projectin gene.[4,5] Projectin has the pattern of domains characteristic of titin,[6] although the protein (1000 kDa) is about a third the size of titin. Kettin (from the German kette = chain) is a second modular protein in *Drosophila*[7-9] and it has been mapped to chromosome position 62C1-3. The complete cDNA has been sequenced and corresponds to a protein of 540 kDa, largely made up of Ig domains separated by linker sequences of 35 residues; at each end of the molecule there are tandem Ig domains and regions of unique sequence (Fig. 1). Andrew and colleagues[10] obtained partial cDNA sequences from a gene coding for a large protein (larger

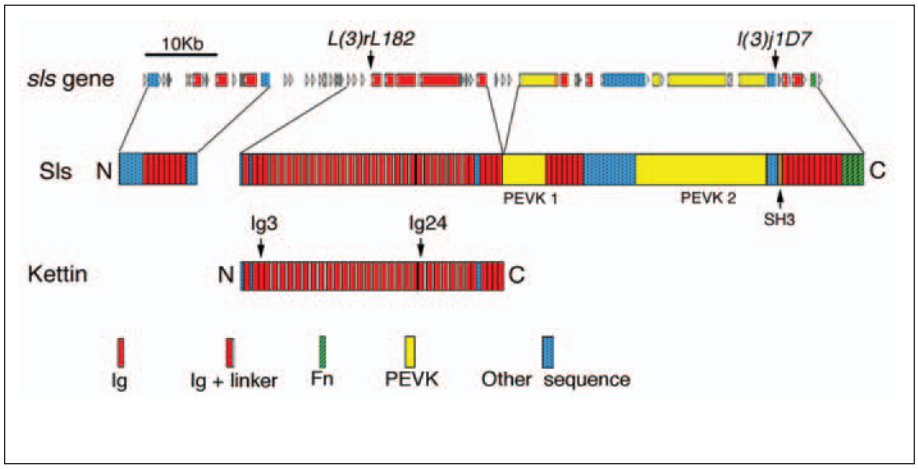

Figure 1. The *sls* gene and Sls protein. The map of open reading frames in the gene is taken from the sequence in FlyBase (http://flybase.bio.indiana.edu); the small triangles in the gene map are short ORFs. The positions of P-element insertions *l(3)rL182* and *l(3)jlD7* in the gene are shown. Domains in the largest protein that is predicted to be encoded by the gene are shown below the gene. The block of sequence at the N-terminus is expressed as an isoform, or spliced to different parts of the sequence downstream (Burkart et al, unpublished result). The domain structure of kettin is shown below the whole predicted Sls protein. Variable splicing pathways produce other isoforms. SH3 is a src homology domain. The positions of the epitopes in kettin (Ig3 and Ig24), reacting with the two antibodies used in stretching experiments are shown.

than kettin) with Ig and PEVK sequence, that is expressed during *Drosophila* embryogenesis; this they called *D-titin*. The gene is at the same chromosomal position as *kettin* and genetic mapping showed that known mutations in the *kettin* region, for example the P-element insertion *l(3)rL182*, failed to complement another P-element insertion, *l(3)j1D7*, in the region of unique sequence near the end of the gene (Fig. 1). This was evidence that the gene extended beyond *kettin*.[9,11,12] Since the *Drosophila* genome has been sequenced, the entire ~100 kb sequence of the gene is known. The gene is called *sallimus* (*sls*) (from the Finnish = fate) or *ket* in FlyBase and we have used *sls* as an alternative to *D-titin.*[3] There is no orthologous gene in vertebrates. The largest protein that could be encoded by the gene is predicted to be about 1.9 MDa; the modular structure is shown in Figure 1. The N-terminal region has tandem Ig domains and stretches of unique sequence, and this region is expressed as an isoform of Sls. Kettin is another isoform. Further downstream there are tandem Ig regions and two PEVK domains. At the C-terminus there are four Fn domains but the molecule does not have the super repeats of Ig and Fn domains found in titin, nor does it have a kinase domain.

Sls, the Protein

Details of splicing pathways in the *sls* gene have not yet been described. The predominant isoform of Sls in the adult *Drosophila* thorax (detected by SDS-PAGE) is 540 kDa kettin and there are minor amounts of 700 and 1000 kDa isoforms, both of which contain the entire kettin sequence. The flight muscle has mainly 540 and 700 kDa isoforms, while other thoracic muscles including leg muscles, also have the 1000 kDa isoform. All *Drosophila* muscles have 540 kDa kettin and muscles with long I-bands have larger isoforms in addition. Immuno-electron microscopy has shown that the N-terminus of kettin is in the Z-disc and the C-terminal region of the molecule is some way out from the Z-disc.[13] Kettin is associated with actin, and the stoichiometry, determined by binding assays with kettin from the giant waterbug *Lethocerus*, suggests each Ig domain binds to an actin monomer; the linker sequence between Ig domains would space the domains to coincide with helically arranged actin monomers. The cartoon in Figure 2a illustrates the second of the alternative models suggested by van Straaten et al.[13] Recent immunolabelling results, using antibodies to different regions of kettin, favour a model in which kettin crosses the whole width of the Z-disc and follows the long-pitch helix of actin. Labelling with antibody to Ig24 is shown in Figure 2b. Tandem Ig domains in the N-and C-terminal regions of the molecule probably would not bind to actin because there are no linker sequences. The effect of kettin binding to actin is to reinforce the thin filament near the Z-disc. Kettin inhibits the binding of tropomyosin to actin, and the region of the thin filament close to the Z-disc that is occupied by kettin does not have tropomyosin.[13]

There is no protein in *Drosophila* with the domain structure of nebulin in vertebrate skeletal muscle. Kettin has some features in common with nebulin. Both proteins are anchored in the Z-disc, and regularly spaced modules bind to actin monomers; however, kettin, unlike nebulin, could not determine the length of thin filaments because it is not long enough. Sls, like nebulin, has a src homology (SH3) domain near the C-terminus (Fig. 1). Nebulin is oriented with the C-terminus in the Z-disc so that this domain is close to the Z-disc, whereas in Sls, which has the opposite polarity, the SH3 domain is separated from the Z-disc by almost the whole length of the molecule. The vertebrate nebulin SH3 domain binds the signalling molecule, myopalladin, which is essential for myofibril assembly,[14] and the SH3 domain in Sls may also have a signalling function.

In IFM, the region of the thin filament with bound kettin crosses the short I-band; and the C-terminus of kettin is attached to the end of the thick filament, either directly or through association with projectin. This link is estimated to be responsible for 70% of the passive stiffness of IFM.[2] In other thoracic muscles, the longer I-bands are spanned by Sls isoforms that include varying lengths of sequence downstream of kettin, including the two elastic PEVK domains.[15] It is likely that the Fn domains at the C-terminus of the gene are spliced onto the

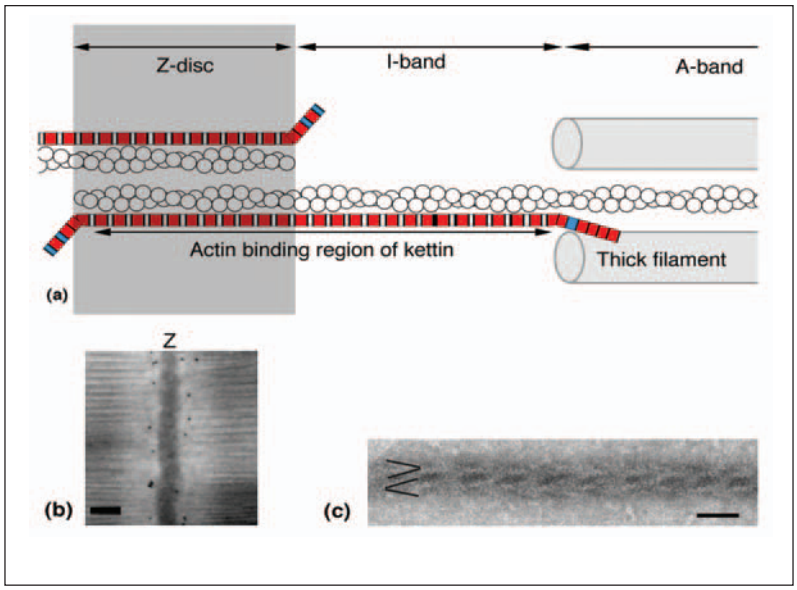

Figure 2. (a) A cartoon showing the layout of kettin in the IFM sarcomere near the Z-disc. Each Ig domain in the Ig-linker region of kettin binds to an actin monomer. The model shows kettin crossing the Z-disc and shows that a kettin molecule following the long-pitch actin helix would extend over about 5 half-repeats of the actin helix. Ig domains at the ends of kettin are not separated by linker sequences and are not expected to bind to actin. Regions of unique sequence may be extensible. The C-terminal region is associated with the thick filament. The model is based on the positions of Ig3, Ig16, Ig24 and Ig35 determined by labelling cryosections with antibodies. (b) A cryosection of the Z-disc region of a sarcomere labelled with antibody to Ig 24 and protein A-gold. (c) Negatively stained paired actin filaments decorated with myosin S1 showing anti-parallel association of filaments. Arrowheads show the polarity of the S1 on actin. Scale bars are 100 nm for (b) and 40 nm for (c).

several longer isoforms of Sls, and that these domains link the ends of the molecules to the thick filaments.

Kettin may be responsible for the formation of the Z-disc at an early stage in the development of the sarcomere. Thin filaments from neighbouring sarcomeres interdigitate in the Z-disc and adjacent filaments with an anti-parallel orientation are crosslinked by α-actinin. When the 540 kDa isoform of kettin is added to isolated actin filaments, the filaments associate with each other laterally; the orientation of the filaments is shown by decorating them with myosin S1. The arrowheads formed by S1 face in different directions in the filaments of a pair, which suggests that kettin molecules in one filament interact with oppositely oriented ones in another filament[13] (Fig. 2c). Thus actin filaments with associated kettin molecules could form a lattice of interdigitating filaments of alternating polarity in the Z-disc. The requirement for interacting kettin molecules to be anti-parallel would prevent cross-linking of thin filaments in the I-band, where neighbouring filaments have the same orientation.

The IFMs in *Drosophila* are attached to the cuticle by a modified Z-disc at the end of the myofibril. The terminal Z-disc is linked to an epithelial tendon cell and bundles of microtubules in the tendon cell join spike-like tonofibrillae that are inserted into the cuticle. The terminal Z-disc is modified into a wide region of high density.[16] The whole terminal Z-disc is labelled by antibody to kettin, suggesting the structure is composed of actin filaments reinforced by kettin (Fig. 3). No regular lattice has been observed in this terminal Z-disc, even in high quality electron micrographs.[16] This may be because all the thin filaments are likely to

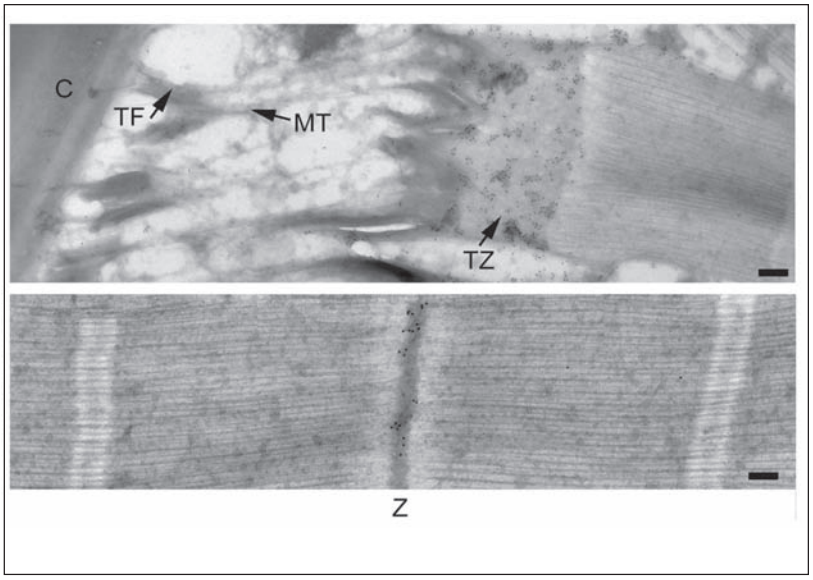

Figure 3. The position of kettin in the terminal Z-disc of *Drosophila* flight muscle. Cryosections are labelled with monoclonal antibody to an epitope in the kettin sequence and protein A-gold. The top panel shows the end of a myofibril and the connection to the cuticle. A modified terminal Z-disc is linked to the cuticle by bundles of microtubules that are attached to tonofibrillae inserted into the cuticle. Kettin is present across the whole of the terminal Z-disc. The lower panel shows kettin in a normal Z-disc in a myofibril, labelled with the same antibody. TZ, terminal Z-disc; MT, microtubule bundle; TF, tonofibillus; C, cuticle; Z, Z-disc. Scale bar 100 nm.

have the same polarity and formation of a regular lattice by cross-linking oppositely oriented filaments with α-actinin would not be possible. α-Actinin also binds to kettin[7] and both Sls and α-actinin are essential for the formation of the terminal Z-disc. The structure is particularly sensitive to mutations in these genes. Homozygous *sls* mutations are lethal at stages that vary from embryonic to pupal, but heterozygotes develop into viable adults.[8,9,11,12] Hakeda et al[8] describe a null mutant (*ket*^{14}) with a deletion in the N-terminal region of the kettin sequence. Electron micrographs of flight muscle in adult flies that are heterozygous for this mutation have much reduced terminal Z-discs, although Z-discs in the rest of the sarcomere appear normal, at least before the flight muscle contracts at eclosion. Similarly, electron micrographs of heterozygotes of the α-actinin null allele *l(1)2Cb* show terminal Z-discs that are reduced in size, resulting in disrupted muscle insertions, while the structure of the rest of the myofibril is unaffected.[17] Homozygotes of the *fliA* series with point mutations in the α-actinin gene also have reduced or absent terminal Z-discs, but in this case, the rest of the myofibril is abnormal too.

The requirement that IFM is resistant to stretch means that the connection of the myofibrils to the cuticle must be fairly rigid. The large terminal Z-disc in which actin is reinforced with kettin may provide the correct compliance. Kettin molecules are likely to be arranged end-to-end along actin in the structure; it is also possible that there are longer isoforms of Sls in this part of the sarcomere and that these include PEVK sequence to give just the right stiffness to the connection between muscle fibre and cuticle.

Sls in the *Drosophila* Embryo

Sls appears at an early stage of embryogenesis. The mRNA and protein are detected in the embryo at early stage 11 (5:20 to 7:20 h after egg laying at 25°C), mainly in the gut region. Later during stage 11, the mRNA and protein are seen in the precursors of somatic and visceral muscles and in the head.[8,9,11,12] Sls is detected several hours before myoblast fusion, which starts at stage 12.[18] It is not clear which isoforms of Sls are expressed in the embryo. In all the studies quoted, antibody to the kettin region of the molecule was used to detect the protein in whole mount embryos. However, Machado et al[10] found that antibodies both to kettin and to PEVK-2 sequence labelled a megadalton protein on immunoblots of embryos before and after nuclei differentiation. In addition, cDNA probes from different regions of the *sls* gene detected the same expression pattern throughout embryogenesis[11] (Fig. 4). Therefore, the whole 1.9 MDa protein is probably expressed in the embryo, though there may be other isoforms as well. Myofibrils are formed at stage 16[19] and by this stage, Sls RNA and protein are concentrated at muscle attachment sites. This is seen clearly at the ends of body wall muscles where they are attached to the epidermis.[8,11,12]

Sls and Myoblast Fusion

The importance of Sls in myoblast fusion is shown by the effect of mutants on the fusion process. Unfused myoblasts are seen in stage 16 embryos of homozygous *sls* mutants stained with antibody to kettin; body wall muscles are disorganized and attachment sites are absent, even though many of the mutants express normal levels of protein.[8,11,12] Some idea of the function of Sls in myoblast fusion can be gained by examining the relationship of *sls* to other genes involved in the fusion process. The events leading to myoblast fusion are characterised by the effects of a series of mutants.[20] The body wall muscles of the *Drosophila* embryo are derived from two types of myoblasts originating from the somatic mesoderm. Founder cells determine the characteristics of particular muscles, and these combine with fusion competent myoblasts (FCMs) to form multinucleated myotubes of the type specified by the founder cells.[21] Most genes involved in the fusion process, including *sls*, are expressed in both types of myoblast, but a few are expressed either in founder cells or in FCMs. Two proteins associated with the membrane are present only in founder cells, dumbfounded (Duf), or only in FCMs, sticks and stones (Sns).[22,23] Duf attracts FCMs to the founder cells before fusion. Both Duf and Sns are predicted to be transmembrane proteins with extracellular Ig domains and the two may interact during fusion.[24,25] Changes both at the cell surface and inside the cell, are necessary for successful fusion and rolling pebbles (Rols), another protein specific to founder cells, is thought to link processes occurring in the two regions.[26] The interest of Rols here is that it is needed for migration of Sls to the site of membrane fusion between muscle precursors and FCMs.[26] Rols accumulation at the membrane coincides with Duf, and in *duf* mutants, Rols is not membrane-associated and Sls remain cytosolic. However, Sls is also concentrated in the mem-

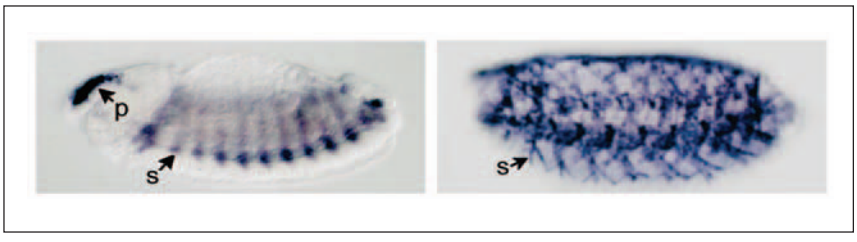

Figure 4. Expression of Sls RNA in *Drosophila* embryos. RNA was detected by in situ hybridization with cDNA coding for part of the sequence in the PEVK 2 domain of Sls. Whole mount embryos are seen in lateral view with the anterior end to the left: The left panel shows a stage 13 embryo and the right panel shows a stage 16 embryo. *Sls* transcripts are seen in pharyngeal and somatic muscles. p, pharyngeal muscle, s, somatic muscle. The figure is modified from Machado C, Andrew DJ. J Cell Biol. 2000;151(3):639-652.

brane of FCMs at the site of cell contact with myotubes, even though FCMs do not express Rols; some other unknown mechanism is thought to produce this localisation in FCMs.[26] In general, the expression of genes needed for fusion is transitory and stops once fusion is complete and myotubes have their full complement of nuclei. The localizationof Sls once Rols expression has expression has decreased is not known; Sls may no longer be associated with the myotube membrane at sites of fusion and could take up its second function in the mature muscle sarcomere. Again, an antibody specific to the kettin region of Sls was the only one used to localize the protein in fusing myoblasts. Sls isoforms vary in size from 200 kDa to 1.9 MDa and not all isoforms have the kettin sequence. The use of antibodies recognizing different regions of the molecule would show which isoforms are important in the fusion process.

The early appearance of *sls* (*kettin*) RNA was detected in screens for genes that are expressed in the mesoderm.[27] The effect of mutations that reduce or increase the amount of mesoderm, on gene expression were compared up to stage 12. There was a clear difference in the effect of these mutations on the expression of *sls* and *bent* (the projectin gene). In mutants with less mesoderm, *sls* expression is reduced at stage 11-12 compared with expression in wild type embryos, and in those with more mesoderm, *sls* expression is increased from stage 9-11; but *bent* is hardly affected because very little is expressed at these early stages. Thus Sls has a function in the embryo that projectin does not, although both are large modular proteins with Ig domains that have some functions in common in the adult fly. A similar screen using mutant embryos in which either founder cells or FCMs were enriched, showed there is significantly more *sls* expression in FCMs than in founder cells.[28] It is possible that several different isoforms of Sls that will be needed in the final muscle are produced in FCMs.

Kettin as a Spring

The kettin sequence in Sls is highly conserved in different invertebrates, probably because the molecule is associated with actin in the same way in all muscles.[3] As mentioned above, the predominant isoform in *Drosophila* IFM is 540 kDa. Since the function of kettin in determining the stiffness of muscle fibres is rather different from the function of vertebrate titin, it is of interest to compare the elastic properties of the two molecules. The properties of single molecules of 540 kDa kettin isolated from *Lethocerus,*[13] have been determined by mechanical stretch experiments using optical tweezers. Antibodies to two regions of the sequence that are 19 complete Ig-linker modules apart (Fig. 1) were bound to microspheres held in independent optical traps (Fig. 5a,b).[29] Molecules of kettin were held between the microspheres by attachment to the antibodies, and were stretched and released at different rates by changing the separation of the microspheres; force was measured during stretches and releases (Fig. 5c). The force-extension traces showed hysteresis due to step-like events during both stretch and release (Fig. 5d). The calculated change in contour length, using a freely-jointed chain model[30] corresponding to these steps was about 30 nm, which is consistent with unfolding and refolding of single Ig domains. The range of lengths of the stretched region predicted from the sequence[8,9] was larger than that modelled from polymer fits to the curves. This suggested that the inter-Ig linkers act as more condensed structures than a simple string of independent residues, with the long-range order of each extending over at least two neighbouring residues.

Kinetic analysis of the forces at which the steps occurred suggested the spontaneous rate of Ig domain unfolding was high (~5 x 10^{-3} s^{-1}). This is two orders of magnitude higher than the spontaneous rate of unfolding of the more stable Ig domains of titin; although it is comparable to the rate of unfolding of some less stable titin Igs that are thought to unfold under physiological conditions.[31] Kettin Ig domains can refold at significantly higher forces than found for titin Ig domains at equivalent rates of loading; this is borne out by the fact that refolding events occur at forces in excess of ~10 pN (Fig. 5d). In the IFM myofibril, most of the kettin sequence is bound to actin, which would mean Ig domains would not be subjected to the high forces experienced by titin. However, the C-terminal tandem Ig domains extend towards the myosin filaments and would be subjected to periodic stretching forces during oscillatory contraction.

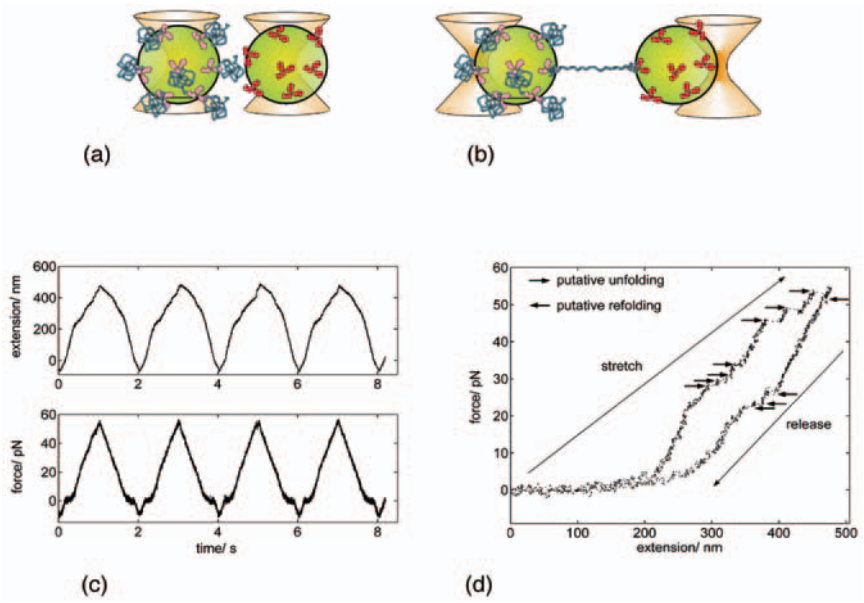

Figure 5. Unfolding of Ig domains during extension of kettin. (a) Schematic representation of double laser optical tweezers with two microspheres trapped at the cross over of the laser beam. Single kettin molecules are bound between the microspheres by two antibodies (Fig. 1). (b) Beads are subjected to cycles of stretch and release by movement of the right hand laser trap. The bead on the left is held stationary by a feedback system controlling the position of the laser trap. As domains unfold, the elastic force drops and the bead on the right moves towards the centre of its trap. (c) Typical data for extension (upper trace) and force (lower trace). Some step events can be made out by eye. (d) Example of a stretch-release cycle showing hysteresis. Arrows mark the position of positive (stress-relaxation) and negative (recovery) steps attributed to unfolding and refolding of Ig domains. Changes of slope in the lower part of the curves were not detected as steps. Ig domains unfold at moderate forces and refold at significantly higher forces than observed for vertebrate titin. The figure is modified from Leake MC, Wilson D, Bullard B et al. FEBS Lett. 2003;535(1-3):55-60.

These Igs may act as shock absorbers; a potentially damaging stretch could result in limited unfolding of Ig domains, and refolding at relatively high forces would not require the sarcomere to be slack before normal operation could be resumed. The ease with which kettin Ig domains refold suggests that kettin may act as an efficient folding-based spring. This may be relevant to the function of Sls in embryogenesis (see below).

Concluding Remarks

Sls is an elastic molecule that would vary in mechanical properties depending on which modules a particular isoform contained. This means the protein can have more than one function. The *sls* gene has a conserved region coding for kettin. Kettin binds strongly to actin and reinforces the attachment of actin to the Z-disc. In IFM, the C-terminus binds to the thick filament and this contributes to the high stiffness of IFM needed for oscillatory contraction. Alternative splicing of kettin to different ORFs downstream would produce isoforms differing in size and extensibility, which could cater for the differing requirements for the elasticity of different muscle types. When the modular composition and position of minor isoforms of Sls in the IFM sarcomere has been determined, it will be possible to make a more accurate predic-

tion of the contribution of the sum of the isoforms to the passive elasticity of the muscle. The relative ease of unfolding and refolding of kettin Igs is not typical of Ig domains. Measurement of the elastic properties of recombinant peptides from different regions of Sls, using optical tweezers or atomic force microscopy, will also be necessary before the contribution of isoforms to passive tension can be determined; as has been shown for vertebrate titin.[31,32]

The need for Sls in myoblast fusion is unexpected because it is not clear what the function of the defined number of actin binding modules would be, or how they might attach Sls to the region of fusion between myoblast membranes. Nor is it known what the function of long elastic PEVK regions might be in fusing myoblasts. Recently, it has been shown that mechanical stresses are sensed by cells in the developing *Drosophila* embryo.[33,34] As the cells in the embryo change shape and migrate, forces are produced by movement relative to the extracellular matrix. These forces affect the patterning of the embryo by changing gene expression. Sls, as an elastic molecule, is expected to respond to stress. A possible function of Sls in embryogenesis might be as an intermediary between a mechanical change and signalling to the nucleus. The kettin region of Sls may function as a folding based spring during embryogenesis, before it has any function in Z-disc nucleation. More is known about the properties of Sls than any other protein in the cascade involved in fusion. Most are known only as genes. Sls might be the protein to start with in studying the interaction of proteins making up the complex at the site of membrane fusion.

References

1. White DC. The elasticity of relaxed insect fibrillar flight muscle. J Physiol 1983; 343:31-57.
2. Kulke M, Neagoe C, Kolmerer B et al. Kettin, a major source of myofibrillar stiffness in *Drosophila* indirect flight muscle. J Cell Biol 2001; 154:1045-1057.
3. Bullard B, Linke WA, Leonard K. Varieties of elastic protein in invertebrate muscles. J Muscle Res Cell Motil 2002; 23:435-447.
4. Ayme-Southgate A, Vigoreaux J, Benian G et al. *Drosophila* has a twitchin/titin-related gene that appears to encode projectin. Proc Natl Acad Sci USA 1991; 88:7973-7977.
5. Fyrberg CC, Labeit S, Bullard B et al. *Drosophila* projectin: Relatedness to titin and twitchin and correlation with *lethal(4)102 CDa* and *bent-dominant* mutants. Proc R Soc Lond B Biol Sci 1992; 249:33-40.
6. Southgate R, Ayme-Southgate A. Alternative splicing of an amino-terminal PEVK-like region generates multiple isoforms of *Drosophila* projectin. J Mol Biol 2001; 313(5):1035-1043.
7. Lakey A, Labeit S, Gautel M et al. Kettin, a large modular protein in the Z-disc of insect muscles. Embo J 1993; 12:2863-2871.
8. Hakeda S, Endo S, Saigo K. Requirements of Kettin, a giant muscle protein highly conserved in overall structure in evolution, for normal muscle function, viability, and flight activity of *Drosophila*. J Cell Biol 2000; 148:101-114.
9. Kolmerer B, Clayton J, Benes V et al. Sequence and expression of the kettin gene in *Drosophila melanogaster* and *Caenorhabditis elegans*. J Mol Biol 2000; 296(2):435-448.
10. Machado C, Sunkel CE, Andrew DJ. Human autoantibodies reveal titin as a chromosomal protein. J Cell Biol 1998; 141:321-333.
11. Machado C, Andrew DJ. D-Titin: A giant protein with dual roles in chromosomes and muscles. J Cell Biol 2000; 151:639-652.
12. Zhang Y, Featherstone D, Davis W et al. *Drosophila* D-titin is required for myoblast fusion and skeletal muscle striation. J Cell Sci 2000; 113 (Pt 17):3103-3115.
13. van Straaten M, Goulding D, Kolmerer B et al. Association of kettin with actin in the Z-disc of insect flight muscle. J Mol Biol 1999; 285:1549-1562.
14. Bang ML, Mudry RE, McElhinny AS et al. Myopalladin, a novel 145-kilodalton sarcomeric protein with multiple roles in Z-disc and I-band protein assemblies. J Cell Biol 2001; 153:413-427.
15. Bullard B, Hååg P, Brendel S et al. Mapping kettin and D-titin in the invertebrate sarcomere. J Mus Res Cell Motil 2002; 22:602.
16. Reedy MC, Beall C. Ultrastructure of developing flight muscle in *Drosophila*. II. Formation of the myotendon junction. Dev Biol 1993; 160(2):466-479.
17. Fyrberg E, Kelly M, Ball E et al. Molecular genetics of *Drosophila* alpha-actinin: Mutant alleles disrupt Z disc integrity and muscle insertions. J Cell Biol. 1990; 110(6):1999-2011.

18. Hartenstein V. Atlas of *Drosophila* development. In: Bate M, Martinez Arias A, eds. The Development of *Drosophila melanogaster* Vol II. Plainview, New York: Cold Spring Harbor Laboratory Press, 1993.
19. Bate M. The mesoderm and its derivatives. In: Bate M, Martinez Arias A, eds. The Development of *Drosophila melanogaster*. Vol II. Plainview, New York: Cold Spring Harbor Laboratory Press, 1993:1013-1090.
20. Taylor MV. Muscle differentiation: How two cells become one. Curr Biol 2002; 12(6):R224-228.
21. Bate M. The embryonic development of larval muscles in *Drosophila*. Development 1990; 110:791-804.
22. Ruiz-Gomez M, Coutts N, Price A et al. *Drosophila* dumbfounded: A myoblast attractant essential for fusion. Cell 2000; 102(2):189-198.
23. Bour BA, Chakravarti M, West JM et al. *Drosophila* SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. Genes Dev 2000; 14(12):1498-1511.
24. Frasch M, Leptin M. Mergers and acquisitions: Unequal partnerships in *Drosophila* myoblast fusion. Cell 2000; 102(2):127-129.
25. Dworak HA, Charles MA, Pellerano LB et al. Characterization of *Drosophila hibris*, a gene related to human *nephrin*. Development 2001; 128(21):4265-4276.
26. Menon SD, Chia W. *Drosophila* Rolling pebbles: A multidomain protein required for myoblast fusion that recruits D-Titin in response to the myoblast attractant Dumbfounded. Dev Cell 2001; 1(5):691-703.
27. Furlong EE, Andersen EC, Null B et al. Patterns of gene expression during *Drosophila* mesoderm development. Science 2001; 293(5535):1629-1633.
28. Artero R, Furlong EE, Beckett K et al. *Notch* and *Ras* signaling pathway effector genes expressed in fusion competent and founder cells during *Drosophila* myogenesis. Development 2003; 130(25):6257-6272.
29. Leake MC, Wilson D, Bullard B et al. The elasticity of single kettin molecules using a two-bead laser-tweezers assay. FEBS Lett 2003; 535(1-3):55-60.
30. Smith SB, Cui Y, Bustamante C. Overstretching B-DNA: The elastic response of individual double-stranded and single-stranded DNA molecules. Science 1996; 271(5250):795-799.
31. Li H, Linke WA, Oberhauser AF et al. Reverse engineering of the giant muscle protein titin. Nature 2002; 418(6901):998-1002.
32. Watanabe K, Mühle-Goll C, Kellermayer MS et al. Different molecular mechanics displayed by titin's constitutively and differentially expressed tandem Ig segments. J Struct Biol 2002; 137(1-2):248-258.
33. Farge E. Mechanical induction of Twist in the *Drosophila* foregut/stomodeal primordium. Curr Biol 2003; 13(16):1365-1377.
34. Scott IC, Stainier DY. Developmental biology: Twisting the body into shape. Nature 2003; 425(6957):461-463.