Kettin, a large modular protein in the Z-disc of insect muscles

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Z-discs of insect flight muscle contain a large protein of 500–700 kDa. Monoclonal antibodies label an epitope in the molecule at the Z-disc in *Drosophila* and *Lethocerus* (waterbug). A partial cDNA of 1.6 kb from the *Drosophila* gene has been cloned and sequenced. The corresponding amino acid sequence has a modular structure composed of four conserved repeats of 95 amino acids homologous to immunoglobulin C2 domains (called class II domains in muscle proteins), separated by less conserved linker sequences of 35 amino acids. An expressed class II domain with flanking linker sequences binds to actin and α -actinin but not to myosin. Single molecules of the protein would be large enough to span the Z-disc. We suggest that the protein acts as scaffolding in the Z-disc and we call the protein kettin. The Ca^{2+} activated protease, calpain, disrupts the Z-disc of striated muscle, releasing α -actinin intact. Calpain digests kettin to a series of peptides of between 30 and 170 kDa which are released from the myofibril. Digestion of kettin may cause disintegration of the Z-disc and α -actinin release which lead to disassembly of the myofibril.

Key words: calpain/*Drosophila*/insect muscle/kettin/Z-disc

Introduction

The Z-disc of striated muscle anchors the ends of thin filaments from neighbouring sarcomeres and has an ordered lattice structure which is hexagonal in insect flight muscle and tetragonal in vertebrates. It has been shown that in the bee Z-disc, thin filaments of opposite polarity from two sarcomeres interdigitate and are linked by connecting structures (Cheng and Deatherage, 1989; Deatherage *et al.*, 1989). The thick filaments of insect flight muscle are also linked to the Z-disc; extensions from the ends of thick filaments (C-filaments) reach the Z-disc but it is not known if they enter the Z-disc lattice (Reedy, 1971; White and Thorson, 1973).

The protein composition of the connecting structures in the bee Z-disc is not known. α -Actinin, which is a bipolar molecule, is a major component of the Z-disc and probably forms part of the structures connecting pairs of filaments of opposite polarity. Higher molecular weight proteins associated with the flight muscle Z-disc are: Z(210) in *Drosophila*, p230 and p700 in *Lethocerus*, Z(175) in bee and Z(400/600) which is at the lateral edges of the Z-disc

in bee and *Drosophila* (Saide *et al.*, 1989, 1990; Lakey *et al.*, 1990; Vigoreaux *et al.*, 1991). p700 in the Z-disc of *Lethocerus* muscle is distinct from p800 (projectin) which is in the C-filaments: antibody to p700 does not cross react with projectin; the isoform of the protein in *Lethocerus* leg myofibrils is smaller (500 kDa) than that in flight myofibrils whereas projectin is the same size in both muscle types; p700 is in the Z-disc of both flight and leg myofibrils while projectin is in the C-filament region of flight myofibrils and in the A-band of leg myofibrils (Lakey *et al.*, 1990).

In this paper we have characterized p700 further by obtaining a partial amino acid sequence of the *Drosophila* protein. The sequence obtained so far consists of a chain of repeating domains separated by short linker sequences. We have therefore called the protein kettin (from the German, 'Kette', meaning chain). Binding of an expressed cDNA fragment to other proteins has been studied to find the likely position of the protein in the Z-disc lattice.

Z-discs are degraded in the early stages of muscle fibre autolysis by calpain, a calcium dependent protease that is distributed throughout the sarcomere of striated muscle fibres but is more concentrated in the Z-disc (Goll *et al.*, 1990). Calpain disrupts the Z-disc in striated muscles of vertebrates and insect flight muscle without affecting the overall structure of the rest of the sarcomere (Reddy *et al.*, 1975; Dayton *et al.*, 1976; Reedy *et al.*, 1981; Bullard *et al.*, 1990). α -Actinin is released from the vertebrate Z-disc without being cleaved, therefore it is likely that calpain digests a protein that anchors α -actinin in the Z-disc (Goll *et al.*, 1991). This protein has not yet been identified. Calpain also releases α -actinin from isolated Z-discs of insect flight muscle. We have investigated the effect of calpain on kettin in *Lethocerus* flight muscle to find out whether this Z-disc protein is released or is cleaved by calpain. The binding properties of the expressed fragment of kettin, together with the effect of calpain on kettin, suggest that the protein is an important structural component of the flight muscle Z-disc.

Results

Identification and localization of *Drosophila* kettin

Drosophila kettin was identified by reaction of the protein with antibody MAC 155 specific to *Lethocerus* kettin (Lakey *et al.*, 1990). On immunoblots of *Drosophila* thoracic proteins a protein of ~500 kDa, significantly smaller than *Lethocerus* p700, was labelled strongly and one of 700 kDa was labelled more weakly (Figure 1A). The thorax contains predominantly flight muscle and lesser amounts of other muscles, therefore the 500 kDa protein is likely to be the flight muscle isoform and the other labelled protein may be in another muscle type. In order to confirm that the antibody to *Lethocerus* kettin reacted with an equivalent protein in *Drosophila*, the antigen was localized in *Drosophila* flight muscles by immunofluorescence. The antibody labelled the Z-discs specifically in both *Drosophila* and *Lethocerus*

Fig. 1. Identification of *Drosophila* myofibrillar protein reacting with anti-kettin and determination of the size of the message encoding the protein. (A) Comparison of *Drosophila* and *Lethocerus*. Lane 1, SDS-polyacrylamide gel (3–15% acrylamide) of *Drosophila* thoraces. Lanes 2 and 3, immunoblots of gradient gels; lane 2, *Drosophila* thoraces; lane 3, *Lethocerus* flight muscle. Immunoblots were incubated with antibody MAC 155 to *Lethocerus* flight muscle kettin. K, kettin; M, myosin; α A, α -actinin. (B) Northern blot of *Drosophila* RNA with the kettin cDNA probe. A sample of *Drosophila* RNA (15 μ g) from whole flies was run on an agarose gel, then transferred to a nylon membrane and hybridized with the kettin cDNA (15 h exposure). Comparison of the labelled band with the molecular marker ladder (Gibco-BRL) shown on the right, suggests that the size of the full length kettin message is $\sim$ 11.5 kb. (C) Immunofluorescence micrograph of *Drosophila* flight myofibril labelled with MAC 155. Phase contrast micrograph is below the fluorescence micrograph. Scale bar 3 μ m.

myofibrils (Figures 1C and 6a). These results show that the *Drosophila* cDNA clone selected with this antibody (see below) encodes a high molecular weight protein in the *Drosophila* Z-disc which is similar to *Lethocerus* kettin. Electron micrographs show that the epitope in *Drosophila* is on the lateral faces of the Z-disc (Figure 7a).

Molecular cloning of kettin from *Drosophila*

In order to isolate cDNA encoding the MAC155 epitope, $\sim$ 400 000 clones of a *Drosophila* cDNA expression library were screened with the antibody. One positive clone was detected, plaque-purified and shown to contain a 1.6 kb cDNA insert. A Northern blot of whole fly RNA was performed using our probe to find out if the isolated cDNA clone was a partial cDNA of the large kettin molecule

(Figure 1B). An abundant message migrating above the 9.3 kb 28S rRNA from *Drosophila* was observed. A size of 11.5 kb was estimated by comparison with a ladder of marker RNAs. This message is large enough to encode a 500 kDa protein, confirming that the isolated clone is a partial cDNA of the large protein recognized by the antibody.

Modular structure of kettin

The entire sequence of the cDNA selected with MAC 155 was determined and the 1570 bp sequence was found to contain one open reading frame (ORF) encoding a 60 kDa peptide. Self-comparison using DOTPLOT showed that this peptide consists of four repeats of 130 amino acids. Searches of the data library showed that these repeats are made up of 95 amino acids which are homologous to class II (immunoglobulin-C2-like) domains separated by insertions of 35 amino acids that are not significantly homologous to any sequence in the SwissProt databank.

An alignment of class II domains in the kettin sequence with class II domains in the partial sequence of *Drosophila* projectin (Fyrberg *et al.*, 1992) is shown in Figure 2. Identical residues in projectin match well with identical residues in kettin: nine positions in kettin and projectin have identical or highly conserved residues. There is more homology between class II domains of the kettin sequence (27 identical residues) than there is between class II domains of projectin (12 identical residues) which suggests there are greater functional constraints on the kettin class II sequence. The linker sequences between class II domains in kettin are homologous to each other with seven identical residues.

Interactions of kettin domains with other muscle proteins

In order to investigate the interactions of kettin domains with other muscle proteins, a 160 amino acid fragment (the fourth class II domain in Figure 2 with flanking linker sequences) was isolated by PCR and expressed in *Escherichia coli*. After purification, interactions with myosin, actin and α -actinin were measured by a solid phase binding assay. The fragment bound with high affinity to actin and α -actinin but not significantly to myosin (Figure 3). As a control, binding of an expressed titin class II domain was also measured; this showed weak binding to myosin but none to actin and α -actinin (not shown).

Binding of the expressed kettin fragment to filaments of actin and myosin was also estimated by sedimenting the filaments in the presence of the fragment. The expressed fragment clearly co-sedimented with actin filaments (Figure 4A). Although actin and the fragment were in about equimolar proportions, some of the fragment was not sedimented. Circular dichroism measurements on the expressed kettin fragment showed that the protein was incompletely folded; thus the fraction which does not bind to actin may be unfolded. Similar experiments with myosin filaments showed that the fragment did not bind appreciably to myosin (Figure 4B).

The expressed fragment reacted with the monoclonal antibody MAC 155 on immunoblots (not shown), therefore the epitope is within the sequence of this fragment.

The effect of calpain on Z-disc proteins

Kettin was not extracted from flight muscle myofibrils using solutions containing 2 M NaCl or 0.5 M $MgCl_2$ or 1 M KI, conditions that extracted projectin. Also, the protein was not

Fig. 2. Comparison of class II domains in *Drosophila* projectin and kettin. The upper block shows projectin class II domains from Fyrberg *et al.* (1992). The partial amino acid sequence of kettin is in the middle and lower blocks; each row in the middle block is continued in the lower block. Kettin sequence in the middle block is aligned with class II domains of projectin in the upper block. Conserved amino acids are shown below each block.

extracted by low ionic strength solutions containing deoxycholate or CHAPS detergent.

An attempt was therefore made to release kettin from the Z-disc by digesting myofibrils with calpain. Myofibrils were incubated with m-calpain (which is active at millimolar Ca^{2+} concentration) and the proteins remaining in the myofibril and those released were investigated; other myofibrils were incubated in calpain activating solution alone to check if endogenous calpain was activated. Most of the myofibrillar proteins were unaffected by calpain (Figure 5A, panel a); as observed previously (Bullard *et al.*, 1988), troponin H and troponin T were digested. The zeelins were not digested because the myofibrils had been washed with Triton which prevents their digestion (Bullard *et al.*, 1990). α -Actinin and a protein, or proteolytic peptide, of ~200 kDa were released from myofibrils by calpain. Immunoblots, which were more sensitive than stained gels, showed that some α -actinin was released in calpain activating solution alone but much more was released by added calpain (Figure 5B, panel a).

The major high molecular weight proteins of *Lethocerus* flight muscle are projectin (800 kDa), kettin (700 kDa) and the M-line protein p400 (Lakey *et al.*, 1990). All these proteins are substrates for calpain but projectin is digested less than kettin and p400. The proteins and their proteolytic products were identified on immunoblots with specific antibodies. Projectin was digested by calpain to give a 700 kDa peptide [Figure 5A(b) and B(b)]. No 800 kDa projectin remained after digesting with m-calpain. Some 700 kDa peptide stayed in the myofibril and some was released but no smaller peptides were detected on immunoblots. Myofibrils incubated in activating solution alone had 800 kDa projectin and a small amount of 700 kDa peptide; on immunoblots some released 700 kDa peptide was detected (Figure 5B, panel b).

In contrast, kettin was extensively digested by calpain and no protein of 700 kDa reacting with anti-kettin was detected on immunoblots after digestion (Figure 5B, panel c). Kettin peptides of 29–166 kDa were released from the myofibril (Figure 5B, panel d). The molecular weights estimated for these peptides are approximate multiples of 15 kDa, which

Fig. 3. Binding of kettin fragment to actin, α -actinin and myosin. Wells of microtitre plates were coated with actin, α -actinin or myosin and incubated with serial dilutions of biotinylated kettin fragment (class II domain flanked by linker sequences). Values are corrected for BSA background.

is the molecular weight of a class II domain with the linker sequence. Therefore, calpain may cleave a specific site in this repeating unit. We have only detected cleavage products that contain the epitope recognized by the antibody. If there is only one epitope per molecule, there will be other peptides not detected here, which may similarly have molecular weights that are multiples of 15 kDa. To confirm that all the kettin is digested, myofibrils from *Lethocerus* leg muscle were incubated with calpain. Kettin in leg muscle has a

Fig. 4. Sedimentation of kettin fragment with actin and myosin. Kettin fragment (class II domain flanked by linker sequences) was incubated with actin or myosin filaments, the mixture centrifuged and samples of pellets (P) and supernatants (S) run on SDS-polyacrylamide gels. (A) Actin filaments mixed with fragment. Lanes 1 and 4, actin with fragment; lanes 2 and 5, actin alone; lanes 3 and 6, fragment alone. The fragment sedimented with actin and a much smaller amount in the absence of actin. The fragment that was not sedimented with actin and remained in the supernatant may be partially unfolded protein (see text). (B) Myosin filaments mixed with fragment. Lanes 1 and 4, myosin with fragment; lanes 2 and 5, myosin alone; lanes 3 and 6, fragment alone. A small amount of fragment was sedimented with myosin which was approximately equal to the amount sedimented in the absence of myosin. Ar, arthrin; Ac, actin; MHC, myosin heavy chain; MLC, myosin light chain; K, kettin fragment which is ~20 kDa.

molecular weight of 500 kDa and is easily distinguished from the 700 kDa proteolytic peptide of projectin (Lakey *et al.*, 1990). The 500 kDa leg kettin was not present in myofibrils digested by calpain (Figure 5A, panel c). Projectin was digested to give a 700 kDa peptide, as in flight muscle, and some was released from the myofibril.

The p400 M-line protein in flight myofibrils was extensively digested by calpain and none remained in digested myofibrils (Figure 5A, panel b). Flight muscle myofibrils were also incubated with μ -calpain in 10 μ M

Ca^{2+} : under these conditions, not all the projectin was digested to the 700 kDa peptide, however, kettin and p400 were fully digested as with m-calpain (not shown). It may be possible to find conditions under which kettin and p400 are digested by μ -calpain while projectin remains intact. Two proteins associated with the Z-disc, projectin and kettin, are substrates for calpain and it is likely that the release of α -actinin from the Z-disc results from the extensive digestion of kettin with the release of soluble kettin fragments from the myofibril.

Structure of myofibrils treated with calpain

The digestion of kettin by calpain was also observed directly in myofibrils by fluorescence microscopy. Antibody to kettin labelled the Z-disc of *Lethocerus* myofibrils (Figure 6a). After treatment with calpain the dense Z-disc was absent and anti-kettin gave very weak fluorescence in the light region where the Z-disc had been (Figure 6b). Digested myofibrils were shorter than normal myofibrils and the break was usually at the position of the Z-disc.

Anti-projectin labelled myofibrils on either side of the Z-disc (Figure 6c). After calpain treatment, labelling by anti-projectin appeared to be as strong as in untreated myofibrils (Figure 6d). These results support the conclusion that calpain digests kettin extensively but only cleaves the end of projectin. The 700 kDa projectin peptide remaining in the myofibril is probably bound to thick filaments.

The effect of calpain on the Z-disc in *Lethocerus* flight muscle myofibrils is shown at higher resolution in electron micrographs (Figure 7b and c). An anti-kettin antibody labelled the Z-disc of *Lethocerus* myofibrils more heavily than in *Drosophila* and in some regions the label appeared to be concentrated towards the edges of the Z-disc. After digesting with calpain, the density of the Z-disc was reduced and there was no anti-kettin label on the Z-disc.

Discussion

We have shown that the Z-discs of insect flight muscle contain a large protein made up of repeating motifs that bind actin and α -actinin and that the protein is extensively digested by calpain. There have been reports of high molecular weight proteins in the vertebrate Z-disc but kettin differs from these. Z-nin is a 300–400 kDa protein which, unlike kettin, is released intact from the Z-disc by calpain (Suzuki *et al.*, 1981). Zeugmatin is a protein of over 500 kDa, thought to be on the lateral faces of the Z-disc, which is probably digested *in vivo* by calpain (Maher *et al.*, 1985). The amino acid composition of zeugmatin differs from that of the sequence we have obtained so far for kettin and anti-zeugmatin does not cross-react with kettin (A.Lakey and B.Bullard, unpublished result); however, zeugmatin may be a modular protein of the vertebrate Z-disc similar to kettin. The molecular weight of flight muscle kettin differs in the two insect species used here and in two muscle types; it is possible that the pair of proteins of 400 and 600 kDa at the borders of the Z-disc in bee and *Drosophila* flight muscle (Saide *et al.*, 1989, 1990; Vigoreaux *et al.*, 1991) are kettin isoforms.

Localization of extended molecules in the sarcomere by monoclonal antibodies is limited to determining the position of particular epitopes. In *Drosophila* and probably also in *Lethocerus*, the kettin epitope recognized by the antibody

Fig. 5. Digestion of *Lethocerus* flight and leg myofibrils with calpain. Myofibrils in calpain activating buffer were incubated with or without m-calpain. Myofibrils were then sedimented, the supernatants decanted and the pellets resuspended in a volume equal to the volume of the supernatants. Samples of pellets (lanes 1 and 3) and supernatants (lanes 2 and 4) from incubations in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of calpain were electrophoresed on SDS-polyacrylamide gels, (A) Coomassie blue stained gels of equal volumes (3 μ l per lane) of resuspended pellets and supernatants; (a) and (b) flight myofibrils electrophoresed on 12% and 4% polyacrylamide gels respectively, (c) leg myofibrils on a 4% polyacrylamide gel. (B) Immunoblots of SDS-polyacrylamide gels of pellets (3 μ l per lane) and supernatants (20 μ l per lane); (a) flight myofibrils were electrophoresed on a 12% polyacrylamide gel and immunoblots were incubated in anti- α -actinin, (b) flight myofibrils were electrophoresed on a 4% polyacrylamide gel and immunoblots were incubated in anti-projectin, (c) and (d) flight myofibrils were electrophoresed on 4% and 8.5% polyacrylamide gels respectively and immunoblots incubated in anti-kettin. The supernatant of calpain treated myofibrils in (d) was concentrated 10 times; the supernatant of untreated myofibrils (10 times concentrated) had no labelled bands (not shown). The protein of 500 kDa in (c) reacting with antibody is probably contaminating leg kettin. Molecular weights (kDa) are shown for 12, 4 and 8.5% gels; the molecular weights of the kettin fragments in (d) were estimated from standards. T, protein in flight and leg myofibrils with the same mobility as vertebrate titin; P, projectin; K, kettin; M, myosin; p400, M-line protein; α A, α -actinin; TnH, troponin H; TnT, troponin T; A, actin; Z₁ and Z₂, zeelins.

used here is on the lateral faces of the Z-disc. It seems likely that in both species, kettin extends across the width of the Z-disc and forms part of the Z-disc lattice with actin and α -actinin. Kettin molecules are probably arranged in an anti-parallel fashion in the Z-disc and molecules of one polarity may be associated with actin filaments of a particular polarity. The symmetrical position of the epitope on kettin at the faces of the *Drosophila* Z-disc would arise from an anti-parallel arrangement of molecules in the Z-disc.

Several proteins associated with the thick filament have

recently been found to have repeating motifs of two types: class I (related to fibronectin type-III domains) and class II (related to immunoglobulin C2 domains) (Benian *et al.*, 1989; Einheber and Fischman, 1990; Labeit *et al.*, 1990; Lakey *et al.*, 1990; Olson *et al.*, 1990; Shoemaker *et al.*, 1990; Noguchi *et al.*, 1992). The sequence of kettin obtained so far has class II domains only, which may be an adaptation to binding actin and α -actinin rather than myosin. The length of a class II domain in muscle proteins has been estimated by electron microscopy to be 4 nm (Trinick *et al.*, 1984).

Fig. 6. The effect of calpain on kettin and projectin in *Lethocerus* myofibrils. (a and b) Myofibrils labelled with anti-kettin (MAC 155); (c and d), myofibrils labelled with anti-projectin (MAC 150). The myofibrils in (a) and (c) were untreated and those in (b) and (d) were digested with m-calpain. The labelling of anti-projectin on either side of the Z-disc cannot be resolved in these micrographs. Phase contrast micrographs are below the fluorescent micrographs. Scale bar 5 μ m.

In the sequence obtained here, there is one class II domain in each 15 kDa. Therefore, the 500 kDa kettin in the flight muscle of *Drosophila* could be at least 33×4 nm (~ 130 nm) long when extended, if the entire molecule has the same domain structure. The insect flight muscle Z-disc is up to ~ 120 nm thick (Deatherage *et al.*, 1989) and would accommodate ~ 30 class II domains of kettin. If the 35 amino acid linker sequence contributes to the length, *Drosophila* kettin could be ~ 180 nm and *Lethocerus* kettin (700 kDa) could be ~ 260 nm, which is the length of isolated projectin (Nave and Weber, 1990). The molecule could span the Z-disc, winding along actin filaments, or could extend somewhat from the Z-disc into the I-band.

There are five types of connecting structure in the bee Z-disc: four link pairs of filaments of opposite polarity and

Fig. 7. The position of kettin in *Drosophila* myofibrils and the effect of calpain on the Z-disc in *Lethocerus* myofibrils. Electron micrographs of flight muscle myofibrils. (a) *Drosophila* myofibril labelled with anti-kettin. (b) *Lethocerus* myofibril labelled with anti-kettin. (c) *Lethocerus* myofibril digested with m-calpain and then labelled with anti-kettin. In the undigested myofibrils of *Drosophila* and *Lethocerus* the label appears to be concentrated at the edges of the Z-disc. Calpain removes the density of the Z-disc which is no longer labelled by anti-kettin. The scattered labelling on the A-band of the digested myofibril is probably due to fragments of kettin. Scale bar 250 nm.

a fifth links the ends of three filaments of the same polarity from the same sarcomere (Cheng and Deatherage, 1989). The connecting structures between filaments of opposite polarity are oriented axially along the thin filaments as well as spanning the gap between filaments and it has been suggested that these structures contain α -actinin molecules arranged obliquely, since the α -actinin molecule (a 40 nm rod) is longer than the distance between actin filaments in the Z-disc (17–24 nm) (Cheng and Deatherage, 1989; Deatherage *et al.*, 1989). The expressed fragment of kettin binds to both actin and α -actinin and a possible arrangement is one in which kettin runs along the actin filament, zigzagging from one filament to another via the connecting structures. The linker sequences might provide the flexibility needed for this arrangement.

An alternative function for kettin might be to join C-filaments to the Z-disc. The connecting structure that links the ends of three actin filaments of the same polarity is in a plane near the side of the Z-disc and is centred on the position at which an extension of the thick filament would

enter the lattice. Kettin might be a component of this threefold connecting structure and might extend from the hub at the centre of the structure to form part of the C-filaments.

Calpain cleaves the two high molecular weight proteins associated with the Z-disc and this is likely to cause the breakdown of the Z-disc that occurs in the early stages of muscle fibre autolysis. Projectin, which links thick filaments to the Z-disc, has a domain structure similar to that of titin (Lakey *et al.*, 1990; Ayme-Southgate *et al.*, 1991; Fyrberg *et al.*, 1992). By analogy with titin (Fürst *et al.*, 1988; Labeit *et al.*, 1992) it is likely that the NH₂ end of projectin is in the Z-disc and that calpain cleaves the molecule towards the NH₂ end, severing the 700 kDa peptide from the Z-disc. Kimura *et al.* (1992) have recently shown that m-calpain cleaves α -connectin (titin I) to β -connectin (titin II). Kettin, which is probably a scaffolding protein of the Z-disc, is difficult to extract from flight muscle myofibrils, either due to insolubility or to interactions with other proteins, and this may account for the resistance of Z-discs to extraction under non-denaturing conditions. However, the Z-disc is very susceptible to digestion by calpain. The selective digestion of the kettin molecule to soluble peptides that are released from the Z-disc is probably the cause of α -actinin release and Z-disc disintegration. Digestion of the M-line protein, p400, would further loosen the sarcomere structure. The action of calpain on these large modular proteins dismantles the myofibril without affecting the main proteins of thick and thin filaments, a process which is likely to be important in the turnover of myofibrils. Kettin and p400 are extensively digested by μ -calpain in 10 μ M Ca²⁺ and it is likely they would be at least partially digested by endogenous calpain at normal intracellular Ca²⁺ concentrations (up to 1.2 μ M). However, μ -calpain is probably under additional control *in vivo* (Cong *et al.*, 1989).

It is not known if there is a kettin-like protein in vertebrates. So far, cross-hybridization experiments using the kettin cDNA probe and Western blots with antibodies have failed to detect a vertebrate gene product homologous to kettin. In *Drosophila*, the study of kettin by molecular genetics should reveal how this gene is involved in the assembly of the Z-disc.

Materials and methods

Electrophoresis and immunoblotting

Lethocerus indicus were obtained from Thailand. Myofibrils were prepared separately from the indirect flight and thoracic leg muscles and washed and stored in rigor solution with 50% glycerol at -20°C (Bullard *et al.*, 1985). Thoraces were dissected from *Drosophila melanogaster* and, after the wings and legs had been removed, they were dropped into liquid nitrogen. Thoraces were homogenized in rigor solution (0.1 M NaCl, 0.02 M potassium phosphate, pH 6.8, 5 mM MgCl₂, 5 mM EGTA, 5 mM NaN₃) with 0.1% Triton X-100 (Serva 37238) and 0.5 mM *p*-methylsulphonyl fluoride, then washed four times in the same solution, three times in rigor solution and twice in 10 mM Tris, pH 7.0. The thoraces were homogenized in Laemmli SDS sample buffer (Laemmli, 1970) containing 6 M urea and centrifuged to remove cuticle. Samples of *Lethocerus* myofibrils or *Drosophila* thoraces were heated in the SDS sample buffer to 50°C (not boiled) and proteins electrophoresed in SDS-polyacrylamide gels containing 2.5 M urea and 4, 8.5 or 12% or a gradient of 3–15% acrylamide. Gels were immunoblotted and the blots incubated in the monoclonal antibodies MAC 155 (an IgG against *Lethocerus* p700; 0.19 mg/ml), MAC 150 (an IgG against *Lethocerus* p800; 0.16 mg/ml) or MAC 276 (a hybridoma cell supernatant against *Lethocerus* α -actinin) (Lakey *et al.*, 1990). IgG was prepared from the MAC 155 hybridoma cell supernatant using an affinity column of anti-rat IgG (kindly provided by Dr G. Butcher) and from MAC 150 ascites using a DEAE column (Newman *et al.*, 1992).

Molecular cloning and sequencing of kettin

The monoclonal antibody MAC155 was used to screen a λ ZAP adult *Drosophila* cDNA library (Stratagene no. 936601) as described by Mierendorf *et al.* (1987). One positive clone was identified and its cDNA insert was isolated by PCR (Saiki *et al.*, 1985) using primers flanking the *Eco*RI cloning site. A 1.6 kb fragment was isolated, subcloned into M13mp 18/mp19 (Yanisch-Perron *et al.*, 1985) and the sequence determined (Sanger *et al.*, 1977). The nucleotide sequence has been submitted to the EMBL data library (accession number X72709).

Sequence editing, fragment assembly, prediction of ORFs and DOTPLOT self-comparison were performed with the UWGCG software package (Devereux *et al.*, 1984). The predicted peptide encoded by the single ORF in the kettin cDNA sequence was used to search the SwissProt data library (release 19) with Wordsearch (UWGCG).

Northern blot analysis

Adult flies were homogenized in guanidinium isothiocyanate lysis solution using a Polytron tissue homogenizer. RNA was prepared (Chirgwin *et al.*, 1979) and separated on agarose-formaldehyde gels (Maniatis *et al.*, 1982). After transfer of RNA onto Genescreen nylon membranes (Dupont), filters were hybridized with the randomly labelled kettin cDNA probe (Feinberg and Vogelstein, 1984).

Protein expression and purification

A subfragment from the kettin cDNA consisting of the fourth class II domain with flanking linker sequences shown in Figure 2 from LDLD to RTEV was isolated by PCR (Saiki *et al.*, 1985). The subfragment was subcloned into the pET8c vector (Studier *et al.*, 1990) and fused N-terminally with an oligonucleotide linker encoding a His₆ tag sequence. After induction of BL21 cells (Studier and Moffat, 1991) with 0.5 mM IPTG for 4 h, harvested cell pellets were sonicated in 50 mM sodium phosphate pH 8.0, 0.5 M NaCl, 0.2% Tween 20. After centrifuging at 25 000 g, soluble expressed product in the supernatant was purified by metal chelate affinity chromatography on Ni²⁺-NTA agarose (Qiagen) as described by LeGrice and Grueninger-Leitch (1990).

Protein binding assays

Lethocerus actin and myosin were prepared as previously described (Hammond and Goll, 1975; Bullard *et al.*, 1985); the actin preparation contained arthrin (a ubiquitinated actin; Ball *et al.*, 1987) as well as actin. α -Actinin was prepared from rabbit skeletal muscle (Arakawa *et al.*, 1970). Binding of expressed kettin fragment to these proteins was quantified by reacting purified biotinylated fragment with proteins covalently linked to Covalink plates (Nunc) using the bifunctional crosslinker disuccinimidylsuberate (Staros *et al.*, 1986). Covalink plates were coated with 100 μ l of the proteins (10 μ M in 20 mM Na₂CO₃, pH 9), then blocked with milk buffer containing 0.1% fish skin gelatin. Dilutions of kettin fragment in binding buffer (0.2 M KCl, 0.02% Tween 20, 50 mM histidine pH 8.0, 0.1% fish skin gelatin) were added to the plates. After 10 h incubation at 4°C, unbound protein was washed off with TBS-1% Tween 20 and bound protein was detected by a standard ELISA assay with streptavidin-peroxidase conjugate and 1,4-diaminobenzene as substrate. Absorption at 492 nm was measured using a Titerect ELISA plate photometer. Control wells were coated with 1% BSA.

Binding of kettin fragment to actin and myosin

Binding of expressed kettin fragment to actin was estimated by sedimenting actin in the presence of the fragment. The kettin fragment (13 μ M) in F-buffer (50 mM NaCl, 5 mM MOPS pH 7.0, 0.5 mM MgCl₂, 2 mM NaN₃) was centrifuged in a Beckman Airfuge at 150 000 g for 30 min to remove aggregates. *Lethocerus* actin (10 μ M) in F-buffer was mixed with the centrifuged kettin fragment and incubated for 1 h at 25°C, then the mixture was centrifuged at 150 000 g for 30 min in the Airfuge. Control samples with actin only and kettin fragment only were also centrifuged. Pellets and samples of the supernatants were run on SDS-polyacrylamide gels.

Filaments of *Lethocerus* myosin were made by dialysing the myosin into 0.1 M NaCl, 0.02 M potassium phosphate pH 7.0, 1 mM NaN₃. Myosin filaments (10 μ M) were mixed with kettin fragment (13 μ M) in filament buffer, centrifuged in the Airfuge and pellets and supernatants run on SDS-polyacrylamide gels as for the actin binding experiment.

Digestion of myofibrils with calpain

Glycerinated *Lethocerus* myofibrils (1–2 mg) were washed twice in calpain activating solution. Activating solution for m-calpain was 0.1 M NaCl,

20 mM Tris-Cl, pH 7.0, 2 mM MgCl₂, 10 mM β -mercaptoethanol, 2 mM Na₂S₂O₃ with 5 mM CaCl₂ and for μ -calpain was the same solution with 2 mM Ca-EGTA; the Ca-EGTA buffer had 10 μ M free Ca²⁺ (Perrin and Sayce, 1967). Myofibrils were resuspended in 400 μ l of activating solution and incubated with or without calpain (myofibrils:calpain ratio, 1200:1 w/w) for 45 min at 25°C. m-Calpain from turkey gizzard and μ -calpain from bovine skeletal muscle were a gift from Dr D.E.Goll. Myofibrils were centrifuged at 15 000 g for 10 min. Supernatants were added to 200 μ l of SDS sample buffer and the pellets, after rinsing with activating buffer, were resuspended in 600 μ l of SDS sample buffer. In some experiments, myofibrils were centrifuged at 100 000 g for 30 min and supernatants were concentrated 10 times by precipitating with 9% trichloroacetic acid before taking up in SDS sample buffer. Supernatants and pellets were examined on SDS-polyacrylamide gels and by immunoblotting. Protein concentration was determined by the Bio-Rad method.

Immunofluorescence microscopy

Dorsal longitudinal muscles were dissected from *Drosophila* thoraces that had been stored at -20°C in rigor solution with 50% glycerol for 7 days and myofibrils were prepared by homogenizing and washing, as above. Control and m-calpain treated *Lethocerus* myofibrils in rigor solution with 1% BSA (blocking solution) were incubated on a slide with MAC 155 or MAC 150 hybridoma cell supernatants diluted 1:5 (v/v) with blocking solution, followed by fluorescein isothiocyanate conjugated to anti-rat IgG and examined by immunofluorescence microscopy (Lakey et al., 1990).

Electron microscopy

Half thoraces of *Drosophila* were lightly fixed in 2% paraformaldehyde and cryosectioned as described by Lakey et al. (1990). The thorax was removed from a freshly killed *Lethocerus* and the dorsal longitudinal muscles were exposed. The fibres were skinned by incubating the thorax at 4°C in several changes of rigor solution with 1% Triton for 18 h then in several changes of rigor solution for 12 h. Bundles of about five fibres were dissected and rinsed in calpain activating solution three times by transferring between wells of a multiwell plate (Nunc) on ice. They were then transferred to a well containing 200 μ l of m-calpain (2.5 μ g/ml) in activating solution and incubated on ice for 4 h. Digestion was stopped by transferring to three changes of calpain deactivating solution (0.1 M NaCl, 20 mM Tris-Cl, pH 7.0, 2 mM MgCl₂, 10 mM β -mercaptoethanol, 2 mM Na₂S₂O₃, 6 mM EGTA) for 5 min each. Digestion products were washed out of the fibres by incubating in three changes of rigor solution and rocking at 10°C for 3 h. Control fibres were incubated in activating solution without calpain. Unfixed fibres were cryosectioned. Sections of *Drosophila* thoraces or *Lethocerus* fibres were labelled as described previously (Lakey et al., 1990) except that blocking and antibody solution (MAC 155 IgG, 63 μ g/ml) were in rigor buffer with 0.1% Triton X-100 which increased labelling. Protein A-gold was from Dr J.Slot (Utrecht, The Netherlands). Grids were examined in a Philips 400T electron microscope.

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