Molecular Genetics of Drosophila Alpha-Actinin: Mutant Alleles Disrupt Z Disc Integrity and Muscle Insertions

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Abstract. We have isolated a Drosophila melanogaster alpha-actinin gene and partially characterized several mutant alleles. The Drosophila protein sequence is very similar (68% identity) to those of chicken alpha-actinin isoforms, but less closely related (30% identity) to Dictyostelium alpha-actinin. The gene is within subdivision 2C of the X chromosome, coincident with 15 lethal (l)2Cb mutations. At least four alleles, l(1)2Cb l, l(1)2Cb 2, l(1)2Cb 4, and l(1)2Cb 5 are interrupted by rearrangement breakpoints and must be null. In all four cases, hemizygous mutants complete embryogenesis and do not die until the second day of larval growth, signifying that either the role of alpha-actinin in nonmuscle cells is redundant or that a distinct and only distantly related gene encodes the nonmuscle isoform. Allelic but less severely affected ilia mutants are apparently due to point mutations, and develop into adults having thoracic muscle abnormalities. EM of mutant muscles reveals that Z discs and myofibrillar attachments are disrupted, whereas epithelial "tendon" cells are less affected. We discuss these phenotypes in the light of presumed in vivo alpha-actinin functions.

To better understand muscle physiology and mechanical properties of the cytoskeleton, it is essential to learn in more detail how actin filaments are organized and anchored. The observation that distinct muscle types have rather differently arranged Z discs suggests that thin filament organization is a critical determinant of contractile properties (Auber and Couteaux, 1963; Goldstein et al., 1982; Yamaguchi et al., 1985). Likewise, considerable evidence suggests that proteins that anchor or cross-link actin filaments profoundly affect cytoskeletal functions (Smith, 1988).

Probably the best characterized actin filament cross-linking protein is alpha-actinin. The 100,000-mol wt polypeptide occurs as antiparallel homodimers, which approximate rods 7 nm wide by 48 nm long (Imamura et al., 1988). Both muscle and nonmuscle isoforms of alpha-actinin have been described (Endo and Masaki, 1982; Duhaiman and Bamburg, 1984). Skeletal muscle alpha-actinin is stably localized within Z discs (Masaki et al., 1967), whereas the distribution of nonmuscle isoforms is dynamic and complex, generally paralleling that of microfilaments. In avian fibroblasts, for example, alpha-actinin antibodies stain stress fibers in a periodic pattern and also decorate less orderly cortical actin filament arrays, especially in the vicinity of where cells are bound to the substrate (Lazarides and Berridge, 1975; Tokuyasu et al., 1981).

Recent sequence analyses have generated a hypothesis for alpha-actinin structure and function (refer to the review of Blanchard et al., 1989). Alpha-actinins include three distinct domains, an NH2-terminal actin binding domain of ~220 residues, four 122-amino acid central repeats, and a carboxy-terminal region containing two EF-hand-like sequences. The actin-binding regions, which interact most closely with residues 1-12 and 86-123 of actin, would be located at the ends of the rod-shaped antiparallel dimer (Mimura and Asano, 1987). The central repeats are thought to engender electrostatic interactions essential for formation of antiparallel dimers (Imamura et al., 1988). Finally, the EF handlike sequences probably determine whether actin binding of a particular isoform is calcium dependent. For some time it has been known that actin binding of cytoplasmic alpha-actinin isoforms is calcium sensitive, whereas that of muscle isoforms is not (Berridge and Feramisco, 1981). This functional distinction appears to be conferred by sequence differences in the EF hand domains (Arimura et al., 1988).

Immunolocalization studies and structural analyses have partially defined the in vivo roles of alpha-actinin. Within Z discs of skeletal muscle the protein anchors parallel and antiparallel thin filament arrays via several morphologically distinct types of cross-links recently visualized in image reconstructions of electron micrographs (Cheng and Deatherage, 1989; Deatherage et al., 1989). Sato et al. (1987) have suggested that alpha-actinin of nonmuscle cells may dynamically crosslink actin filament networks within the cortical cytoplasm and thus regulate their rates of deformation. However, there is as yet no evidence to suggest that alpha-actinin...
engenders comparable changes in microfilament networks in vivo. Immunolocalization experiments have demonstrated that alpha-actinin, vinculin, and talin are closely associated with microfilaments in the vicinity of focal adhesion plaques. This observation implies that alpha-actinin participates in linking microfilament networks to plasma membranes, although probably not directly (refer to Geiger et al., 1979; Chen and Singer, 1982).

The indirect flight muscles of *Drosophila* are an excellent system for examining functions of alpha-actinin in both muscle and nonmuscle cells. The versatility of *Drosophila* molecular genetic techniques allows one to perturb structures of contractile and cytoskeletal proteins within these fibers, then evaluate the functional consequences. Another attractive feature of flight muscles is the juxtaposition and well-defined relationship of muscle and nonmuscle cells. In contrast to the extracellular collagenous tendon used by vertebrates, in the *Drosophila* thorax specialized epithelial cells serve as "cellular tendons" connecting flight muscle fibers to cuticle (Auber, 1963). Within these specialized nonmuscle cells, microtubule bundles and cytoskeletal elements transmit force from muscle cell to cuticle. Ultrastructural examination of these cuticular insertions therefore allows both sarcomeric and cytoplasmic functions of alpha-actinins to be explored in a single system.

Here we describe isolation of a gene which encodes sarcomeric *Drosophila* alpha-actinin, document several mutant alleles, and illustrate some of the associated muscle abnormalities. Several apparent missense alleles perturb Z discs and severely disrupt myofibrillar insertions into muscle cell membranes. Surprisingly, null alleles do not affect embryonic development, suggesting that either cytoskeletal roles of alpha-actinin are largely redundant, or that cytoskeletal alpha-actinin of *Drosophila* is encoded by a different gene. We discuss these results in light of the proposed functions of alpha-actinin in muscle and nonmuscle cells.

**Materials and Methods**

**Antibody Screening of Lambda gt10 Expression Libraries.** A library of *Drosophila* head-specific cDNAs cloned in the lambda gt1 vector (Young and Davis, 1983) was obtained from Dr. Paul Salvaterra (City of Hope Medical Center, Duarte, CA). Phage were plated on *E. coli* strain Y1090 at a density of 50,000 plaques per 150-ml plate in soft agar on L-broth plates containing ampicillin. Plaques were allowed to form at 42°C for 3–4 h. Subsequently plates were removed to 37°C and fusion protein synthesis induced by covering with nitrocellulose filters that had been soaked for 1 h in 10 mM isopropyl-p-D-thiogalactopyranoside. Filters were removed after 3–4 h, washed with Tris-buffered saline containing 0.05% Tween 20, and incubated in hybridoma cell supernatant containing antibody MAC 274 (provided by Dr. Belinda Bullard and Geoff Butcher) directed against alpha-actinin of *Drosophila* and *Lethocerus* alpha-actinin. The antibody had been shown to bind specifically to Z discs of both *Lethocerus* and *Drosophila* muscles and to recognize a 98-kD protein on immunoblots (Bullard, 1981). In preliminary work the antibody had been shown to bind specifically to Z discs of both *Lethocerus* and *Drosophila* muscles and to recognize a 98-kD protein on immunoblots (Bullard, B., personal communication). The antibody recognizes a polypeptide of the same molecular weight in immunoblots of *Drosophila* thoracic proteins (data not shown). On the basis of the Z disc staining pattern and the molecular weights of the reactive proteins, we presumed that the antibody recognizes *Lethocerus* and *Drosophila* alpha-actinins. From a screen of about 1 x 10⁶ recombinants we isolated four partial clones which collectively represented the entire *Drosophila* alpha-actinin cDNA sequence. All cDNAs were cloned in the pUC19 vector and sequenced using the dideoxy method of Sanger et al. (1977), or the chemical degradation method of Maxam and Gilbert (1980).

**In Situ Hybridization to Polyteny Chromosomes.** Polytene chromo-

somes from larval salivary glands of wild-type (Canton-S strain) and *tt(1)2C2* heterozygotes were isolated and spread using the technique of Gall and Purdie (1971). Tritium-labeled cRNA was prepared from alpha-actinin gene containing recombinant lambda phage templates as described by Wensink et al. (1974). Denaturation and hybridization were as described by Karlak et al. (1984).

**Hybridization to Southern-blotted DNA.** Hybridizations were performed at 42°C in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 50 mM sodium phosphate (pH 6.8) containing 40 μg/ml of denatured calf thymus DNA. Filters were washed two times at room temperature and three times at 68°C in 2 x SSC, 0.1% sodium pyrophosphate, 0.1% SDS. Hybridizing fragments were visualized by exposure to x-ray film. In an attempt to detect additional alpha-actinin genes we performed some genomic blots at lower stringency. In these experiments formamide concentration was reduced to 30%, and blots were washed only at room temperature.

**EM of Indirect Flight Muscles.** Thoraces were surgically hemisedected in the midsagittal plane so as to expose the dorsal longitudinal flight muscles without disturbing fibers or insertions. Hemisedected thoraces were fixed in 3% glutaraldehyde, 0.2% tannic acid in buffered Ringer's solution (110 mM NaCl, 2 mM KCl, 5 mM MgCl₂, 20 mM K-MOPS, pH 6.8) at 4°C for 2 h, rinsed three times in buffered Ringer's solution, then twice in 0.2 M NaPO₄, pH 6.0. The hemithoraces were postfixed in 1% OsO₄, 0.2 M PO₄, 10 mM MgCl₂, pH 6.0 for 1 h on ice, rinsed in H₂O three times, block stained in 2% aqueous uranyl acetate for 1 h on ice, rinsed in H₂O, and dehydrated through an ethanol series. Hemithoraces were infiltrated with Araldite 506 mixture (Reedy, 1968; Reedy and Reedy, 1985) at which time further dissection into individual fibers or groups of fibers and their insertions were performed. Individual fibers or oriented fiber bundles with insertions were arranged on polyethylene sheets, and blocks were propylene-merized at 65°C. BEEM capsules with fresh resin were inverted over the oriented fibers and polymerized at 85°C for 48 h. 25–30-nm longitudinal and cross-sections were cut with a Diatome diamond knife on a Reichert OMU3 ultramicrotome and picked up on thin carbon grids on 200-mesh copper grids. Sections were stained with potassium permanganate followed by Sato lead stain, according to Reedy and Reedy (1985) and photographed with a Siemens 101 electron microscope.

**Results**

To isolate *Drosophila* alpha-actinin genes, we screened a cDNA expression library with an mAb that recognizes waterbug alpha-actinin. The *Drosophila* library (provided by Dr. Paul Salvaterra) was constructed by inserting cDNAs representing mRNA of adult heads into the lambda gt1 vector of Young and Davis (1983). Because large muscles and extensive neuronal arrays are found within *Drosophila* heads, we expected that most cDNAs encoding muscle or cytoskeletal proteins would be represented. We screened the library with antibody MAC 274 (provided by Drs. Belinda Bullard and Geoff Butcher) directed against alpha-actinin of *Lethocerus griseus* and *Lethocerus uhleri* flight muscles. In preliminary work the antibody had been shown to bind specifically to Z discs of both *Lethocerus* and *Drosophila* muscles and to recognize a 98-kD protein on immunoblots (Bullard, B., personal communication). The antibody recognizes a polypeptide of the same molecular weight in immunoblots of *Drosophila* thoracic proteins (data not shown). On the basis of the Z disc staining pattern and the molecular weights of the reactive proteins, we presumed that the antibody recognizes *Lethocerus* and *Drosophila* alpha-actinins. From a screen of about 1 x 10⁶ plaques we recovered one, MK384, in which the fusion protein reacted consistently with the antibody. The insert was subcloned into plasmid pUC19 and analyzed further.

We sequenced portions of cDNA MK384 and found that the encoded protein sequence was very similar to those of chicken alpha-actinin isoforms (Baron et al., 1987a,b; Aimurata et al., 1988). To obtain a series of overlapping
cDNAs we used the original gt1 isolate to screen a library of midpupal stage Drosophila cDNAs cloned in the lambda phage vector gt10. One clone that extended to the initiation codon, MKQ4, and three that included 3' ends of the cDNA were thus isolated, and we determined their complete sequences. The encoded protein sequence, as well as those of chicken skeletal and smooth muscle alpha-actinins, are displayed in Fig. 1. Alignment of these sequences reveals extensive similarity. Drosophila and chicken isoforms, including a chicken fibroblast isoform not shown, have an average 68% amino acid identity. Independent comparisons of the Drosophila sequence with that encoded by the single Dictyostelium alpha-actinin gene revealed considerably more divergence, with only 266 identical residues (Noegel et al., 1986, 1987).

Comparisons of the Drosophila alpha-actinin sequence to those of chicken and Dictyostelium generally support prior proposals for alpha-actinin structure–function relationships (refer to our introduction and the review by Blanchard et al., 1989). The presumed actin binding region of the chicken smooth muscle sequence, including residues 25–244, is 86% identical to that of Drosophila. This high degree of sequence conservation is expected in light of the very slow evolution of actin. Note, however, that there is only poor conservation between the NH2 termini of chicken and Drosophila isoforms, suggesting that this region is not directly involved in actin binding. Central repeats are also highly conserved. Repeats 1, 2, 3, and 4 of the chicken sequence (residues 248–366, 367–481, 482–602, and 603–715, respectively) have 70, 65, 59, and 46% amino acid identity with their counterparts in the chicken smooth muscle isoform. One remaining highly conserved sequence at the COOH terminus, including residues 851–862, has an unknown function.

In light of the import of establishing whether actin binding by particular alpha-actinin isoforms is calcium sensitive, we have compared sequences of both EF-hand-like domains (residues 752–821 of the encoded Drosophila alpha-actinin) with those present within other alpha-actinin isoforms. EF-handlike sequences of the five isoforms so far characterized are aligned with the chicken smooth muscle sequence. The consensus sequence is very divergent from those of chicken and Drosophila isoforms, including Drosophila ef1 and ef4, and chick-ene muscle (CSK) sequences. At the bottom of each line is the consensus (con) sequence. The Drosophila sequence is very similar to both chicken sequences, with 608 (skeletal) or 609 (smooth) of 896 residues identical. These sequence data are available from EMBL/Genbank/DDBJ under accession number X51753.
have suggested that the critical determinant of calcium sensitivity is whether the residue at the Y position of the first EF hand is able to coordinate calcium binding. Finally, note that the Drosophila sequence has five extra residues between EF hands, and in this respect more resembles the chicken nonmuscle isoform than either of the two chicken muscle isoforms. Collectively, these observations demonstrate that both EF handlike sequences of Drosophila alpha-actinin are theoretically capable of binding calcium, and that this portion of the Drosophila protein is more similar to nonmuscle isoforms of chicken and alpha-actinin of Dicyostelium than to vertebrate sarcomeric and smooth muscle isoforms.

To delineate the chromosomal arrangement of the Drosophila alpha-actinin gene we isolated an overlapping series of phages containing the entire gene by screening the Drosophila cDNA clone library of Maniatis et al. (1979) with our pool of cDNA clones and sequenced all hybridizing fragments. The protein coding region defined as such is organized as seven exons, none of which obviously corresponds to any of the hypothetical protein domains (refer to Table 1). The only irregularity of the sequence arrangement is that the last 36 codons of exon six (which specify residues 370–405) are duplicated within the intronic sequence just preceding exon seven (data not shown). This duplication is very unusual in that all 108 nucleotides are identical. The intronic duplicate must not be alternatively utilized, however, as it is not preceded by an acceptable splice acceptor sequence. Finally, note that there must be at least one additional exon encoding the loop (refer to Fig. 3 B), the expected result if portions of the distal portion of polytene subdivision 2C (refer to Fig. 3 A). This particular chromosome region has been well characterized genetically, as Perrimon et al. (1985) previously determined the number and order of all complementation groups within subdivisions 2C and 2D. One of the loci thus defined, l(1)2Cb, which is associated with an X chromosome inversion encompassing bands 2C3 to 7B1. In an initial experiment we hybridized DNA which included the entire alpha-actinin gene to polytene chromosomes of l(1)2Cb heterozygotes. In these heterozygotes the inverted and normal X chromosomes were separated during their preparation, we again saw two foci of hybridization on the inverted chromosome (refer to Fig. 3 C), and in this case they were clearly at opposite ends of the inverted region. These observations confirmed the chromosomal location of the cloned sequences and established that one of the inversion breakpoints divides the sarcomeric alpha-actinin gene into two portions.

We analyzed chromosomal DNA of several l(1)2Cb heterozygote strains by hybridization to cloned portions of the Drosophila alpha-actinin gene. Results of experiments are summarized in Fig. 4. The figure illustrates the organization of the protein-encoding exons, locates restriction sites within chromosomal DNA, and delineates the approximate locations of chromosome rearrangements associated with particular mutants. These breakpoint locations were deduced by a series of Southern blots, in which DNA of mutant heterozygotes was probed with various portions of the alpha-
Figure 3. In situ localization of the Drosophila alpha-actinin gene. A illustrates hybridization of cRNA representing the alpha-actinin gene to an overstretched X chromosome. Brackets denote the locations of the 2B, 2C, and 2D subdivisions. A cluster of grains is seen within the distal portion of subdivision 2C. B illustrates hybridization to the synapsed X chromosomes of a l(1)2Cb+/+ heterozygote. This mutant allele is associated with an inversion of the 2C3-7B portion of the X chromosome, which forms a loop when paired with an uninverted chromosome. When the same probe is hybridized, we observe grains at the base of the loop, where the opposite ends of the inversion are juxtaposed. C illustrates a heterozygote mutant chromosome wherein the inverted and uninverted (not visible in photo) X chromosomes have been separated during preparation. The probe hybridizes to both 2C and 7B chromosome regions, demonstrating that the inversion is within the alpha-actinin gene sequences used as probes. Bar, 20 μm.

actinin gene (data not shown). We found that at least four alleles, l(l)2Cb+2+ and 3 have breakpoints within the 5' half of the alpha-actinin gene. These rearranged alleles may be capable of synthesizing portions of the alpha-actinin amino terminus, but such truncated peptides are seldom stable, and all four are almost certainly null. Substantiating this hypothesis is the fact that hemizygotes for all four mutants are lethal, and all die within precisely the same period of Drosophila larval development, the expected consequence if the four mutations eliminate, rather than alter, the encoded protein.
Because \( \text{fliA} \) mutants display less severe phenotypic consequences than do the allelic \( l(l)2Cb \) mutants, it seemed logical that they would have milder lesions at the DNA level. When we compared patterns of hybridizing bands in Bam HI-digested DNA derived from four homozygous \( \text{fliA} \) mutants to that of Canton-S, no differences in band sizes or intensities were observed (data not shown), suggesting that in all four instances muscle abnormalities are due to point mutations which perturb alpha-actinin structure and function, rather than to mutations which abolish protein accumulation as do \( l(l)2Cb \) alleles. Preliminary immunoblotting experiments further substantiate this notion. We find levels of alpha-actinin within thoraces comparable to those seen in wild type in \( \text{fliA}^1 \), and \( ^j \), while \( \text{fliA}^4 \) has an \( \sim 50\% \) reduction of steady-state alpha-actinin level (data not shown). Thus, all four \( \text{fliA} \) mutant homozygotes accumulate high levels of alpha-actinin.

We examined normal and mutant flight muscles using EM to visualize effects of mutant alpha-actinins on muscle structure and function. The most conveniently analyzed mutant

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**Figure 4.** Localization of chromosomal breakpoints associated with particular \( l(l)2Cb \) alleles. The top portion of the diagram depicts the chromosomal arrangement of the *Drosophila* alpha-actinin gene. Lines immediately below illustrate the relative positions of Eco RI, Hind III, and Bam HI restriction sites. Sizes of restriction fragments, in kilobase pairs, are indicated by numbers. Hatched areas delimit the chromosome regions within which we have localized rearrangement breakpoints associated with particular \( l(l)2Cb \) alleles.

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**Figure 5.** Longitudinal sections of wild-type and \( \text{fliA}^4 \) flight muscles. *A* shows a low magnification view of a longitudinal section of wild type *Drosophila* flight muscle, including its cuticular insertion. The layered cuticle (C) and apposed epithelial tendon cells (ETC) can be seen at the bottom of the micrograph. The ETC is flattened against the cuticle except where it forms junctions with the muscle cell. Within the junction electron-dense faces of muscle and tendon cell membranes interdigitate to form a zigzag pattern. On the muscle side of the junction a modified Z disc forms a terminal feltwork (TF) at the end of the terminal sarcomere (TS). The light and dark variably spaced stripes (moiré) marking the myofibrils in these and following micrographs result when the 25-nm section alternately samples regions containing thick filaments and those that contain only thin filaments. *B* shows a comparable section of one mutant \( \text{fliA}^4 \), illustrating the disruption of both Z discs and muscle insertions. Note that disruption of the filament lattice can involve only the terminal sarcomere, as in the myofibril marked by the arrowhead, or can involve the terminal several sarcomeres, as in other myofibrils. Bar, 1.0 \( \mu \)m.
Figure 6. Higher magnification view of a junctional area of a wild-type myofibril illustrating the fascia adherens. Note the modified terminal Z disc (terminal feltwork, TF) of the terminal sarcomere (TS) and the epithelial tendon cell (ETC). Also note microtubule bundles (MT) crossing the tendon cell, and the dense tonofibrillae (gf) extending into the cuticle. Bar, 0.1 μm.

Phenotypes are those engendered by the \textit{fliA} alleles, in part because \textit{fliA} homozygotes survive to adulthood, and in part because these mutations are known to affect indirect flight muscles of the thorax (Homyk and Emerson, 1988), which have been previously examined in detail using EM (Beall et al., 1989; Reedy et al., 1989). Fig. 5A displays a longitudinal section of a \textit{Drosophila} flight muscle and its cuticular insertion, whereas Fig. 6 illustrates the insertion of one myofibril at higher magnification. At low magnification, the most striking feature of wild type muscles is the regularity of both Z disc structure and myofibrillar insertions. Cuticular attachments of \textit{Drosophila} flight muscles appear similar to those described for \textit{Calliphora} (Auber, 1963; for descriptions of similar muscle attachments see Lai-Fook, 1967;
Figure 7. Higher magnification view of the junctional area of \( \text{flia}^2 \) homozygotes. The section should be compared to the wild-type insertion illustrated in Fig. 6. Note that only vestiges of terminal feltwork (TF) are apparent and that thick and thin filaments in the terminal sarcomere (TS) are misoriented and disrupted. This general appearance is typical of many \( \text{flia}^2 \) insertions, and is not due to any fixation artifact. Despite these muscle cell abnormalities microtubules (MT) nevertheless extend from the junctional area to the cuticular surface and tonofilaments penetrate the cuticle. The overall morphology of the tendon cell appears almost normal. Bar, 1.0 \( \mu \text{m} \).
The flight muscles of fliA mutants appeared unusual even in the course of preparing them for microscopy. We noted that muscles of all four fliA strains were easily detached from cuticle and readily dissociated into single fibers. This contrasts to wild-type flight muscles which are fused together and difficult to dislodge from the cuticle. Consistent with the observed mechanical weakness, EM revealed that flight muscle insertions of all fliA mutants are disrupted (refer to Figs. 5 B and 7), and that myofibrils, especially those closest to the sarcolemma, have structural defects.

Fig. 8 illustrates the myofibrillar abnormalities in more detail. fliA2 homozygotes appear most disrupted (Fig. 8 A).
Z-discs are discontinuous and nonplanar, and sarcomeres are irregular. The muscle side of myofibrillar insertions are completely disrupted, as can be appreciated by inspection of Fig. 7. In the terminal sarcomeres, thick and thin filaments are not held in a lattice and only a small remnant of the terminal feltwork is apparent. filA⁴ myofibrils (Fig. 8 B) have a rather different phenotype. Z discs appear to be planar arrays of punctate densities, and the myofibrillar lattice exhibits few discontinuities. However, the terminal sarcomeres are very disrupted (Fig. 5 B); the thick and thin filaments lose the hexagonal packing characterizing the rest of the fibril and the terminal feltwork is abbreviated and misshapen. filA⁴ flight muscles are the least disrupted of the homozygote mutants examined (Fig. 8 C). The principal defect is that a few peripheral filaments bypass the Z disc in most myofibrils. Again, despite these comparatively minor defects, insertions are disrupted; the terminal feltwork is irregularly shaped, and thick and thin filaments are misoriented (not illustrated).

We also examined flight muscles of heterozygotes (null/+ of the lethal l(1)2Cb⁴ and l(1)2Cb⁵ alleles. Heterozygotes are capable of flight, but we wished to directly ascertain whether flight muscles have completely wild-type structure. We found that in both mutants, the distinction between internal sarcomeres and those near insertions was striking. Sarcomeres throughout the myofibril appear normal, while the terminal sarcomeres are variably disordered. In l(1)2Cb⁴ heterozygotes, illustrated in Fig. 9, the terminal four or five sarcomeres weave in and out of the section plane and myofilaments in the ultimate and penultimate sarcomeres spread out as if released from constraints of a Z band. The terminal feltwork varies in size and shape in both mutants. However, this perturbation of the insertions in heterozygotes is not sufficient to prevent flight.

In general, epithelial tendon cell morphology does not appear to be as disturbed as muscle structure in any of the mutants (Figs. 5 B, 7, and 9). One unusual feature observed in mutants was the abundance of fine, short filaments along the membrane and microtubules, which frequently give a "feathered" appearance to the microtubules, particularly in filA mutants. However, the dense junctional membranes of the "tendon" and muscle cells remain folded into a zigzag pattern, even in the absence of an ordered terminal sarcomere or a terminal feltwork. Microtubules cross the "tendon" cell opposite a myofibril insertion even when the terminal sarcomere is disrupted. The density of the junctional membrane areas does not appear reduced in any of the mutants, despite the disruption or reduced density of Z discs and the apparently weakened connection of the myofibrils to the sarcolemma. Apparently the epithelial tendon cell can develop almost normally even when insertions of myofibrils into the sarcolemma are very abnormal.

Discussion

Alpha-actinin is an important component of both myofibrils and cytoskeleton. In both contexts, its role is probably to cross-link actin filaments. However, the in vivo consequences of these cross-links or the means by which they are quantitatively and qualitatively regulated are not well understood. A more comprehensive view of cytomechanics therefore necessitates a more detailed knowledge of the structure and function of alpha-actinin and related microfilament cross-linking proteins.

Molecular genetic techniques used in conjunction with ultrastructural methods offer a means by which to perturb and assess in vivo function of alpha-actinin and other contractile and cytoskeletal proteins. The combined approach offers sufficient resolution to refine our understanding of the cellular roles of the various proteins, and in addition may help to delineate functions of particular polypeptide domains. As a first step toward developing such an approach we have reported here the isolation of a gene which encodes alpha-actinin of Drosophila, and partial characterization of several mutant alleles.

The sequence encoded by the Drosophila alpha-actinin gene is very similar to chicken smooth muscle, skeletal muscle, and fibroblast isoforms, having roughly 68% residue identity with each. The proposed actin binding sequence, spanning residues 25-244 of the chicken smooth muscle sequence, is very highly conserved (~86% residue identity) between Drosophila and the chicken isoforms, and highly conserved (60% residue identity) even when compared with the primitive eukaryote, Dictyostelium. This degree of sequence similarity is expected in light of the slow evolution of actin. Significant sequence similarities are also seen in central repeats and EF handlike sequences. In the case of central repeats the observed levels of sequence similarity (repeats 1, 2, 3, and 4 are 70, 65, 59, and 46% conserved, respectively, between Drosophila and the chicken smooth muscle isoform), as well as the observation that alpha-actinin central repeats are related to those of spectrin and dystrophin (see Blanchard et al., 1989) may imply that these repeats interact with cellular macromolecules that, like actin, are slowly evolving. It is also clear from the collective data that individual repeats, and in particular repeat 4, are under significantly different functional constraints, as they are evolving at different rates.

Regarding the EF handlike sequences, one difference between Drosophila and chicken muscle specific isoforms is that both EF handlike sequences of Drosophila could theoretically be functional. Inspection reveals that the amino-terminal Drosophila EF hand, unlike its chicken smooth and skeletal muscle counterparts, could coordinate calcium binding at the calcium-liganding Y position. Both Drosophila sequences also meet the requirements for functional EF hands outlined by Krebsinger (1980), whereas in both chicken muscle isoforms one of the EF-hand-like pairs fails this same test. This result suggests that alpha-actinin encoded by this Drosophila gene could function in nonmuscle cells, where actin binding is typically calcium sensitive. Conversely, it is not clear why calcium sensitive actin binding would be tolerated in a sarcomeric context, where alpha-actinin is thought to stably cross-link actin filament arrays. Direct testing of the ability of the Drosophila sequences to bind calcium will be required to substantiate whether or not the Drosophila EF handlike sequences are indeed functional.

That the l(1)2Cb mutants are due to chromosome rearrangements within the sarcomeric Drosophila alpha-actinin gene is clear from our described Southern blotting experiments. We conclude that l(1)2Cb hemizygotes produce no alpha-actinin, as rearrangement breakpoints associated with
all four mutants analyzed are located within 5' portions of the genes, and all $l(l)2Cb$ mutants have identical lethal phases. One unexpected finding which will require further investigation concerns the effects of these null alleles on development. Perrimon et al. (1985) observed that $l(l)2Cb$ hemizygotes complete embryogenesis and die as second instar larvae even when maternal RNA transcripts of the gene are eliminated from oocytes. That embryonic development is unaffected demonstrates that alpha-actinin encoded by the herein described gene is not required for the differentiation of larval tissues, including muscles, or for initial larval movements requisite for hatching from the embryonic eggshell. We have noted, however, that these larvae become progressively paralyzed within 24 h and die soon thereafter.
Why do these null mutations not impede embryogenesis or initial muscle function? There are two obvious explanations, and choosing between them will necessitate further studies of the accumulation and utilization of Drosophila alpha-actinin and related proteins. On the one hand, it is possible that nonmuscle alpha-actinin is encoded by a distinct gene. If this is the case, the encoded alpha-actinin must have a rather different sequence than that described here, because Southern blots performed at low stringency have not revealed evidence for any additional closely related genes. On the other hand, it is possible that demands for alpha-actinin function during embryogenesis can be met by other proteins having similar roles. Only after embryogenesis would a sarcromeric form of alpha-actinin become absolutely essential, perhaps because the amounts or specific functions of alpha-actinin required for skeletal muscle assembly could not be met by other functionally overlapping or redundant proteins.

With respect to this hypothesis it is interesting to note that comparisons to all available polypeptide sequences revealed that alpha-actinin is related to spectrin and dystrophin (Byers et al., 1989). All three proteins may cross-link microfilaments in submembranous meshworks, opening the possibility that they may be able to functionally complement one another. Indeed, prior work has suggested that Dictostelium mutants lacking alpha-actinin are not functionally impaired (Walraff et al., 1986; Schleicher et al., 1988; Noegel and Witke, 1988), and certain muscular dystrophy patients lacking most of dystrophin sequence manifest a relatively mild Becker syndrome (Koenig et al., 1988). A straightforward explanation for both observations is functional complementation of one member of the protein superfamily by another.

Electron micrographs of flight muscles in fliA hemizygotes and l(1)2Cb heterozygotes complement one another in highlighting the differential effects of the mutations on central versus terminal Z discs. Each hemizygous fliA mutant is associated with both disrupted myofibrillar attachments and distinctive Z disc defects. The distinctive phenotypes conferred by particular fliA alleles are consistent with our tentative conclusion that they are point mutations within the structural gene encoding sarcromeric alpha-actinin. It will be of interest to further investigate these phenotypes and correlate them with the amino acid replacements conferred by each, especially if the different mutants are located within distinct alpha-actinin domains.

EM of the l(1)2Cb null allele heterozygotes reveals that only the muscle insertions are affected. This may be due to lack of proper (stoichiometric) levels of alpha-actinin in heterozygotes, similar to effects observed in actin and myosin heavy chain heterozygotes (Beall and Fyrberg, 1989). However, in light of models for myofibril formation in which new sarcomeres are assembled at the ends of the muscle fiber (Goldspink, 1972; Periitanis and Gregory, 1971), the preferential disruption of terminal sarcomeres might reflect a more dynamic and biochemically distinct situation, perhaps constant incorporation of new alpha-actinin subunits, or requirements for interactions of proteins not present elsewhere in the myofibril.

Despite the described defects in flight muscle fibers, the epithelial tendon cell appears much less affected in the alpha-actinin mutants (Figs. 5, 7, and 9). The persistence of both dense junctional membrane folds and oriented microtubules, even in the absence of the modified terminal Z disc, suggest that the effects on the tendon cell are secondary to the disruption of muscle insertions by mutations within the described alpha-actinin gene.

The cloned Drosophila alpha-actinin gene and its mutant alleles represent an excellent model system for future studies of alpha-actinin in both muscle and nonmuscle cells. The muscle phenotypes associated with extant alleles can be evaluated and correlated with particular amino acid replacement determined by sequencing mutant alleles. In addition, it is now possible to manufacture mutant alleles using site-directed mutagenesis. These can be introduced into the null alpha-actinin background using P element-mediated transformation. Effects on assembly and function of site-directed mutations within the actin-binding, spectrin repeat, and EF-hand-like domains can be systematically evaluated in both muscle and nonmuscle cells.

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Note Added in Proof. We have recently discovered that an intron of 75 nucleotides resides between codons 843 and 844 of the Drosophila alpha-actinin gene.

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