

Characterization of Components of Z-bands in the Fibrillar Flight Muscle of *Drosophila melanogaster*

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Abstract. Twelve monoclonal antibodies have been raised against proteins in preparations of Z-disks isolated from *Drosophila melanogaster* flight muscle. The monoclonal antibodies that recognized Z-band components were identified by immunofluorescence microscopy of flight muscle myofibrils. These antibodies have identified three Z-disk antigens on immunoblots of myofibrillar proteins. Monoclonal antibodies α :1–4 recognize a 90–100-kD protein which we identify as α -actinin on the basis of cross-reactivity with antibodies raised against honeybee and vertebrate α -actinins. Monoclonal antibodies P:1–4 bind to the high molecular mass protein, projectin, a component of connecting filaments that link the ends of thick filaments to the Z-band in insect asynchronous flight muscles. The anti-projectin antibodies also stain synchronous muscle, but, surprisingly, the epitopes here are within the

A-bands, not between the A- and Z-bands, as in flight muscle. Monoclonal antibodies Z(210):1–4 recognize a 210-kD protein that has not been previously shown to be a Z-band structural component. A fourth antigen, resolved as a doublet (\sim 400/600 kD) on immunoblots of *Drosophila* fibrillar proteins, is detected by a cross reacting antibody, Z(400):2, raised against a protein in isolated honeybee Z-disks. On Lowicryl sections of asynchronous flight muscle, indirect immunogold staining has localized α -actinin and the 210-kD protein throughout the matrix of the Z-band, projectin between the Z- and A-bands, and the 400/600-kD components at the I-band/Z-band junction. *Drosophila* α -actinin, projectin, and the 400/600-kD components share some antigenic determinants with corresponding honeybee proteins, but no honeybee protein interacts with any of the Z(210) antibodies.

THE Z-band is an electron-dense structural component of striated muscle. It serves as an attachment site for thin filaments and transmits tension between neighboring sarcomeres during contraction. Electron micrographs of both vertebrate muscle and insect fibrillar muscle show Z-bands with a highly ordered, almost crystalline, appearance in cross section (for reviews see 1, 4, 12, 40, 42). Several Z-band proteins have been identified from both vertebrate and insect species (2, 3, 6, 7, 18, 20–22, 25, 29, 30, 33–38); however, the manner in which these proteins are organized within the Z-band lattice is poorly understood. Moreover, the developmental programs that lead to the early organization of the Z-band are only beginning to be clarified.

The study of insect Z-bands has been carried out primarily on the flight muscles of the honeybee (*Apis*) and the giant water bug (*Lethocerus*). These insects are particularly favorable for biochemical studies of muscle because their size and the predominance of the flight muscle permit isolation of reasonable amounts of homogeneous muscle tissue. The much smaller size of *Drosophila* presents obstacles for biochemical analyses but facilitates the genetic analyses that are proving to be another useful approach to the study of muscle structure and function. Several flightless *Drosophila* mutants with abnormal Z-bands have been described (8, 14, 16, 23).

These mutants are readily maintained in the laboratory and their analyses should contribute greatly to an understanding of the Z-band in insect muscle. Since many similarities exist between insect and vertebrate muscle, such studies should provide insight into the structure and assembly of the vertebrate Z-band as well.

As a first step toward genetic and developmental studies on Z-bands, we have used monoclonal antibodies to identify and characterize components of the Z-band of *Drosophila* fibrillar flight muscle. Z-disks were isolated from flight muscle myofibrils and used as immunogens to develop antibodies against the structural proteins. The Z-band-specific monoclonal antibodies that we have obtained have allowed us to identify four proteins associated with *Drosophila* flight muscle Z-bands and to determine the anatomical distribution of each of these proteins within the intact myofibril by immunoelectron microscopy.

Materials and Methods

Purification of Myofibrils

Myofibrils were isolated from adult wild-type *Drosophila melanogaster* of the Canton S stock. Approximately 5 g of flies (5,000) were collected in a

plastic bag and immobilized at 2°C. All subsequent procedures were performed at ice-cold temperatures. Flies were suspended in 140 ml of Hodges-EGTA (0.1 M KCl, 0.001 M MgCl₂, 2.5 mM EGTA, pH 7) containing 0.5 mM PMSF, 0.1 mg/ml soybean trypsin inhibitor, 5 µg/ml leupeptin, 2 µg/ml antipain, 0.7 µg/ml pepstatin, and 1 mg/ml α-casein to serve as a competitive substrate for proteases. The flies were homogenized at half-maximal speed for 90 s in an omnimixer (Sorvall Instruments Div., Newtown, CT) and strained through nylon cloth (250 threads/inch) to remove heads, legs, pieces of exoskeleton, and other large debris. The filtrate was layered in six 50-ml tubes above cushions of homogenization buffer containing 0.5 M sucrose and centrifuged at 1,700 g for 35 min at 2°C to sediment fibrils through the sucrose layer and away from soluble proteins in the solution above the interface. Pellets were resuspended in 100 ml of homogenization buffer containing 1.25 M sucrose, and aggregates were dispersed by gentle homogenization. Tubes were centrifuged at 1,200 g for 30 min. This high-viscosity centrifugation removed considerable particulate debris. Fibrils, recovered in the pellets, were collected, blended for 30 s in 35 ml of homogenization buffer, and layered over six exponential gradients (0.83–2.5 M sucrose in Hodges-EGTA). After centrifugation for 60 min at 100,000 g, the lowermost cloudy white band was removed and collected in 35 ml of homogenization buffer. This fraction was reappplied to a second gradient, identical to the first, and centrifuged as above. The fibril layer was removed and washed by centrifugation and resuspension in Hodges-EGTA until the absorbance at 280 nm of the supernatant was zero. Each preparation yielded 10–20 mg of purified myofibrils.

Isolation and Purification of *Drosophila* Z-disks

Z-disks were isolated from purified myofibril preparations in 0.7 M KCl, 0.04 M NaHCO₃, pH 8.5 (2). This solution dissolved thick and thin filaments and, after extraction at 2°C overnight, Z-disks were released and floated freely. The suspension was dispersed by drawing it through a 20-gauge needle, and the Z-disks were separated from particulate contaminants at 1,200 g for 20 min. Most Z-disks remained in the supernatant which was collected, layered over a cushion of extraction solution containing 1.6 M sucrose, and centrifuged 60 min at 75,000 g at 2°C. Z-disks sedimented through the sucrose solution leaving most of the debris floating at the interface. Z-disks were washed by centrifugation (30 min at 75,000 g) followed by resuspension in extraction solution until no protein was detected in the supernatant by absorbance at 280 nm. Although yields were low (100–400 µg from each fibril preparation), these steps yielded highly purified *Drosophila* Z-disks.

Immunization

Preimmune blood was collected from the tail vein of a 7-wk-old BALB/cByJ mouse before immunization with isolated Z-disk proteins. For each injection, a Z-disk preparation was solubilized in 7 M guanidine hydrochloride, 2.5 mM EGTA, 0.1% β-mercaptoethanol, pH 7. This solution was dialyzed first into a solution of 8 M urea, 0.5% β-mercaptoethanol, 0.05 M Tris-sulfate, pH 6.1, and finally into PBS (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4). The suspension was emulsified with an equal volume of Freund's complete adjuvant for the primary injection and with incomplete adjuvant for subsequent booster injections. The animal was immunized intraperitoneally three times over a 10-wk period.

Monoclonal Antibody Production

The technique used for monoclonal antibody preparation was based on that of Oi and Herzenberg (31). 3 d after the final injection, the mouse was etherized, exsanguinated by cardiac puncture, and the spleen was surgically removed. Spleen cells were combined with mouse NS-1 myeloma cells at a ratio of ~1:1 and fused with 40% (vol/vol) polyethylene glycol 1450 (J. T. Baker Chemical Co., Phillipsburg, NJ) in DME containing 9% DMSO. Cells were plated in normal medium at a density of 400,000 per well using mouse thymocytes as feeder cells. 24 h later, selection medium containing hypoxanthine (0.1 mM), thymidine (3.1 × 10⁻⁵ M) and aminopterin (4.5 × 10⁻⁷ M) was added to each well to double its volume.

2 wk after the fusion, supernatants from wells with colonies were screened for antibodies to *Drosophila* Z-disk proteins by immunofluorescence microscopy and immunoblot analysis. Hybridomas of interest were expanded and cloned three times by limiting dilution to insure that cell lines were monoclonal. Ascites tumors were produced in pristane-primed male mice (BALB/cByJ) by intraperitoneal injection of 2–5 × 10⁶ cloned cells. Ascites fluid was clarified by centrifugation at 95,000 g for 60 min, ali-

quoted, and frozen at –20°C. Some aliquots were stored in the freezer in 50% glycerol.

In our laboratories, specific hybridomas are named by the plate number, row, and column of the wells in which they first grew. To simplify communication, in this manuscript we have coded the hybridomas to indicate the antigen identified by the secreted antibody and the order in which the clone was identified (for example, α:3 is the third hybridoma that was found to produce antibody against α-actinin).

Clones producing antibodies against *Drosophila* proteins: α:1–4; Z(210):1–4; P:1–4 refer to hybridomas 2G3, 3A1, 4G6, 5E8, 1D3, 5C9, 6E4, 7G5, 4E2, 4C5, P5:3B3, and 5D8, respectively. Clones producing antibodies against honeybee proteins: α, Z(400):2, and P refer to hybridomas 3B9, 1B8, and 3B11 (35).

With one exception, the antibodies are of the subclass IgG1. Monoclonal antibody α:1, directed against *Drosophila* α-actinin, has an IgG2a isotype.

Electrophoresis and Immunoblot Analysis

SDS-PAGE was performed with the discontinuous buffer system of Neville (27) or Laemmli (19). Isolated Z-disks and fly myofibrils were first dissolved in 7 M guanidine hydrochloride, 0.5% β-mercaptoethanol, 2.5 mM EGTA, pH 7, and then dialyzed into the appropriate sample buffer containing 8 M urea. When it was desirable to resolve very high molecular mass proteins, we used 3.2% gels with the buffer system of Fairbanks et al. (10) as described by Wang (41). Molecular mass standards were purchased from Sigma Chemical Co. (St. Louis, MO). To prepare molecular mass markers for proteins >200 kD we used rabbit myosin, cross-linked according to the methods of Knight and Offer (15).

For immunoblot analysis, proteins were electrophoretically transferred from the SDS-PAGE gels to nitrocellulose sheets (39) (0.2 µm pore size) in buffer (25 mM Trizma base, 80 mM glycine, pH 8.3) using a Trans-Blot cell (Bio-Rad Laboratories, Rockville Centre, NY) overnight at 3 W. Blotted sheets were cut into 3 mm widths and soaked overnight in nonfat dry milk (Carnation Co., Los Angeles, CA). The strips were bathed in 200 µl of an antibody preparation and gently rocked on a shaker for 2 h at room temperature. They were washed by gentle agitation three times for 10 min each in PBS and then treated as above with peroxidase-conjugated goat anti-mouse immunoglobulins (IgG, IgA, and IgM) (Cappel Laboratories, Cochranville, PA) diluted in PBS containing 3% BSA. Strips were further washed in PBS, as above, and developed in 4-chloro-1-naphthol (13). For any given clone, culture supernatant and ascites fluid produced indistinguishable immunoblot patterns.

Two-dimensional gels were run according to the technique of O'Farrell (28) as described by Mogami et al. (24), except for the following modifications. First-dimension IEF gels were 4% acrylamide, 3% cross-linker (bis-acrylamide), and second-dimension SDS gels were 10% acrylamide. These modifications were included to reduce streaking and improve resolution of high molecular mass components. Electrophoresis was done in a two-dimensional cell system (Mini Protean II; Bio-Rad Laboratories) following the manufacturer's instruction manual. Proteins were electrophoretically transferred to nitrocellulose sheets (Hybond C extra; Amersham Corp., Arlington Heights, IL) as described above except that transfer was done for 12 h at 35 V using a Transphor TE42 cell (Hoefer Scientific Instruments, San Francisco, CA). Filters were blocked with 0.5% BSA in TBS-Tween 20 (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature followed by a 1-h incubation in primary antibody culture supernatant diluted in TBS-Tween 20 (α:4, 1:200; honeybee α, 1:10; anti-bovine α-actinin [clone BM-75.2; Sigma Chemical Co.], 1:20). After three washes of 5–10 min each in TBS-Tween 20, filters were incubated for 30 min at room temperature in a 1:6,000 dilution of anti-mouse IgG-alkaline phosphatase conjugate (Promega Biotec, Madison, WI). The filters were washed again three times for 5–10 min each in TBS-Tween 20 and finally incubated in color development substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega Biotec). At this point filters were photographed and then either treated with a different primary antibody or stained with 0.1% amido black (in 45% methanol, 7% acetic acid) to reveal the total protein pattern.

Isolation of α-Actinin cDNA Clones

An adult *Drosophila* head cDNA library (26), in the expression vector λgt11, was provided by P. Salvaterra (City of Hope, Duarte, CA). Two of the anti-α-actinin monoclonal antibodies, α:2 and α:4, were used to probe this library. The screening of the phage library was done by the method of Young and Davis (43) as modified by Goldstein et al. (11).

Indirect Immunofluorescence Microscopy

Purified *Drosophila* myofibrils that had settled onto microscope slides from suspension in Hodges-EGTA were processed for immunofluorescence microscopy as previously described (33).

For studies of larval gut muscle, third instar larvae were dissected in testis isolation buffer (TI buffer: 10 mM Tris-HCl, pH 6.8, 47 mM NaCl, 183 mM KCl) (17). The gut was removed and squashed in TI buffer between a siliconized coverslip and a lysine-subbed slide. (Slides had been dipped into 0.1% gelatin, 0.01% chrome alum plus 1 mg/ml polylysine and dried; this procedure was repeated twice.) The preparation was frozen in liquid nitrogen and the coverslip popped off. The preparation was fixed for 20 min with 10% formaldehyde in *Drosophila* PBS (10 mM Na-phosphate, pH 7.4, 130 mM NaCl). The tissue was permeabilized in *Drosophila* PBS plus 0.1% Triton X-100 for 2 min. Slides were washed twice for 5 min in *Drosophila* PBS and preincubated for 20 min in *Drosophila* PBS plus 10% FCS. The preparation was incubated overnight with primary antibody at 4°C. Slides were washed twice for 15 min in *Drosophila* PBS. Fluorescein-conjugated secondary antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) incubation was carried out at room temperature for 30 min in the dark. Slides were washed twice for 15 min and mounted in 63% glycerol plus *p*-phenylenediamine. Photographs were taken with Tri-X film and developed with Diafine (Acufine, Inc., Chicago, IL).

Electron Microscopy

Isolated Z-disks were applied to carbon-coated Formvar films on 300 mesh copper grids from suspension in extraction solution (see above). Grids were washed with several drops of this concentrated salt solution, then deionized water, and then 1% phosphotungstic acid. They were examined in a microscope (Elmskop 1A; Siemens-Allis Inc., Cherry Hill, NJ) calibrated with a diffraction grating replica.

For immunoelectron microscopy, thoraces were isolated from flies that had been immobilized in the cold. Wings were removed and the exoskeleton was nicked with a sharp blade to insure penetration of fixative. The thoraces were immersed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 2 h at 0°C and then washed in four changes of buffer without fixative for 1 h each. Thoraces were then embedded in Lowicryl K4M by the low temperature method of Carlemalm et al. (5). Polymerization was induced by exposure to ultraviolet light. Thin sections (60–90 nm) were mounted on collodion-coated nickel grids.

An indirect immunogold procedure was used to localize the antigens. Grids were inverted on a droplet of normal goat serum diluted 1:30 with 0.1 M Tris-HCl containing 1% BSA and 0.01% NaN₃ for 30 min. Grids were then transferred to a droplet of culture supernatant and incubated overnight at 4°C in a moist chamber. Specimens were washed under a gentle stream of 0.05 M Tris-HCl buffer, pH 7.4, followed by two 5-min buffer soaks and a final wash in 0.05 M Tris-HCl with 1% BSA, pH 8.3. Grids were immersed in a solution of goat anti-mouse IgG immunogold reagent (Janssen Pharmaceutica, Olen, Belgium) diluted 1:6 in the last wash solution for 1 h at room temperature in a moist chamber. Grids were soaked twice, as above, in 0.05 M Tris-HCl, pH 7.4, followed by a final rinse under a stream of distilled water.

Specimens were stained with uranyl acetate and lead citrate and examined in an electron microscope (300; Philips Electronic Instruments, Inc., Mahwah, NJ).

Results

Drosophila Myofibrils and Isolated Z-disks

Because *Drosophila* are very small insects, it is most practical to use the whole organism for large scale purification of myofibrils. This presents the problem of the release of proteases from the gut during homogenization. Since certain Z-band proteins of the honeybee were found to be highly susceptible to proteolysis (35), we tried to minimize potential protein degradation in the *Drosophila* preparations by working rapidly in the cold, adding a battery of protease inhibitors to our solutions, and separating fibrils from soluble enzymes as quickly as possible after homogenization. These precautions seem to have been relatively successful. At the level of the light microscope, we noted no difference in the appear-

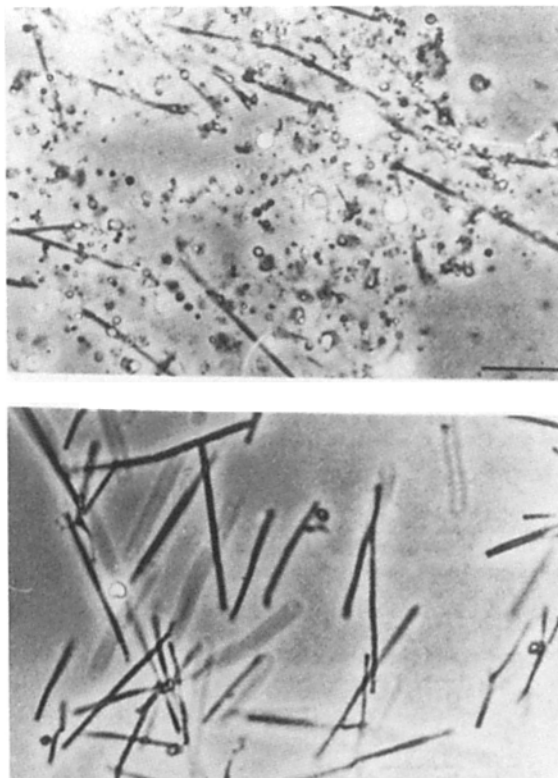


Figure 1. Phase-contrast micrographs of whole fly homogenate (top) and purified myofibrils (bottom). The fibrils are characteristic of those from fibrillar flight muscle. Bar, 20 μ m.

ance of fibrils before and after purification (Fig. 1). In released fibrils, Z-bands were visible, although faint, but no loss of density was apparent during preparative procedures. A much more critical assessment of protein degradation, immunoblot analysis, indicated that proteolysis of Z-band antigens did occur but was not extensive (see below).

The myofibrils that were purified from whole fly homogenates were typical of those found in fibrillar flight muscle. They were discrete, cylindrical, and fairly uniform in diameter. In addition, the A-bands occupied nearly the entire sarcomere, another characteristic of fibrillar muscle. We rarely saw evidence of the larger, more irregular fibril bundles that are derived from other types of insect fibers. In the honeybee, nonfibrillar muscles are very fragile; homogenizations that do not affect the integrity of flight muscle fibrils cause the contractile apparatus in other muscle fiber types, such as those of the leg, to disintegrate. Mogami et al. (24) have noted a similar phenomenon in *Drosophila*. For these reasons we assume that our *Drosophila* fibril preparations and isolated Z-disks are predominantly derived from fibrillar muscles.

Z-disks were readily isolated by dissolving other fibrillar components with concentrated salt solutions. Although the isolated disks were small and pale, we could identify them by phase-contrast microscopy as long as they tumbled, revealing their disk-shaped contours. In the electron microscope, Z-disks resting on Formvar films were electron dense but no structural organization was apparent (Fig. 2). It is likely that the disks were too thick or too disrupted by the preparative procedures to reveal the ordered lattice charac-

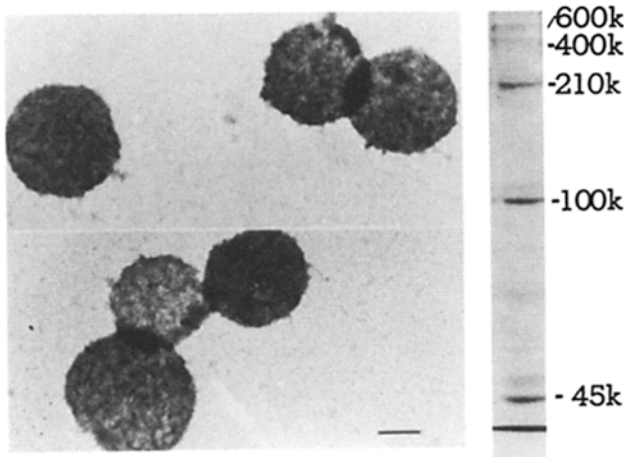


Figure 2. (Left) Electron micrographs of isolated *Drosophila* flight muscle Z-disks on Formvar films stained with 1% phosphotungstic acid. Bar, 0.5 μ m. (Right) SDS (7.5%) polyacrylamide gel (19) displaying proteins from purified *Drosophila* Z-disks. Proteins are detected by Coomassie blue stain. The 45-kD polypeptide is probably actin. Other Z-disk antigens recognized by the monoclonal antibodies are labeled with their apparent molecular masses.

teristic of Z-bands of fibrillar flight muscle. (The lattice can be seen in sections cut through intact *Drosophila* flight muscle; see below.) Although the isolated disks ranged in diameter from 1.2 to 1.6 μ m, most were \sim 1.4 μ m, the estimated diameter of the intact fibrils. In preparations of purified *Drosophila* Z-disks, several high molecular mass proteins have been identified (Fig. 2).

Production of Monoclonal Antibodies

Hybridomas were produced by fusion of NS-1 myeloma cells with spleen cells from a mouse immunized with a mixture of isolated *Drosophila* Z-disk proteins. Culture supernatants were screened both by indirect immunofluorescence microscopy of *Drosophila* myofibrils and by immunoblot analysis of myofibrillar proteins. Twelve hybridomas were selected for expansion and cloning. These hybridomas produced antibodies that provided intense, specific labeling of the Z-band/I-band region of *Drosophila* myofibrils. On the basis of the antigens detected on immunoblots of *Drosophila* myofibrillar proteins, these antibodies were assigned to three groups, corresponding to the three major high molecular mass Z-disk proteins that were recognized. A fourth *Drosophila* Z-band protein was identified by a cross reacting monoclonal antibody directed against a honeybee Z-disk component (35).

Identification of *Drosophila* Z-disk Proteins

Monoclonal Antibodies α :1-4. Four of the monoclonal antibodies that selectively label the Z-band of *Drosophila* myofibrils (Fig. 3) bind to a single band on immunoblots of fly fibrillar proteins separated on SDS-PAGE (Fig. 4). The protein identified by these antibodies is enriched on gels of isolated Z-disks (Fig. 2) and has an apparent molecular mass of 90,000-100,000 D. The 90-100-kD protein has been identified as α -actinin by several criteria. The protein comigrates with honeybee α -actinin (9) and is recognized by both a monoclonal antibody directed against honeybee α -actinin (Fig. 5 and Fig. 6 C) and a monoclonal antibody raised against vertebrate α -actinin (Fig. 6 E).

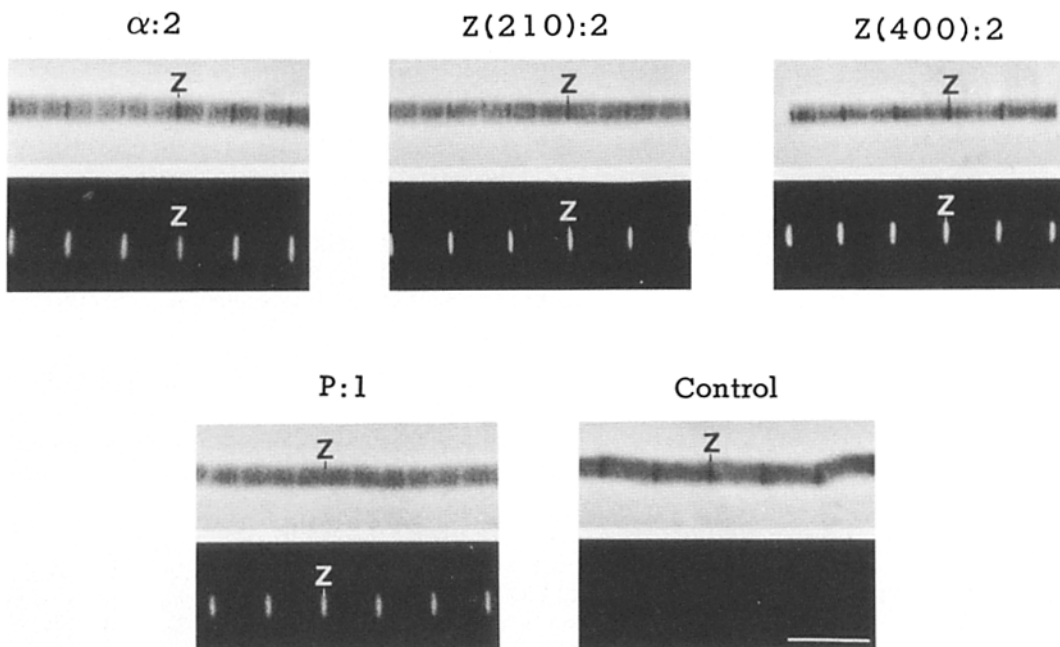


Figure 3. Phase (top) and fluorescence (bottom) micrographs of isolated *Drosophila* myofibrils, showing the localization of each of the proteins identified by our antibodies. Each fibril was treated with ascites fluids (1:200 in Hodge-EGTA) from a representative hybridoma clone (indicated above each pair of images). In the fluorescence micrographs the antibody is detected by fluorescein-labeled anti-mouse immunoglobulin. In most cases the antibody binding enhances the contrast of the Z-band in phase images. Control fibril was treated with ascites fluid (as above) from a clone (2C3) producing an IgG1 lacking specificity for fibrillar proteins. Bar, 5 μ m.

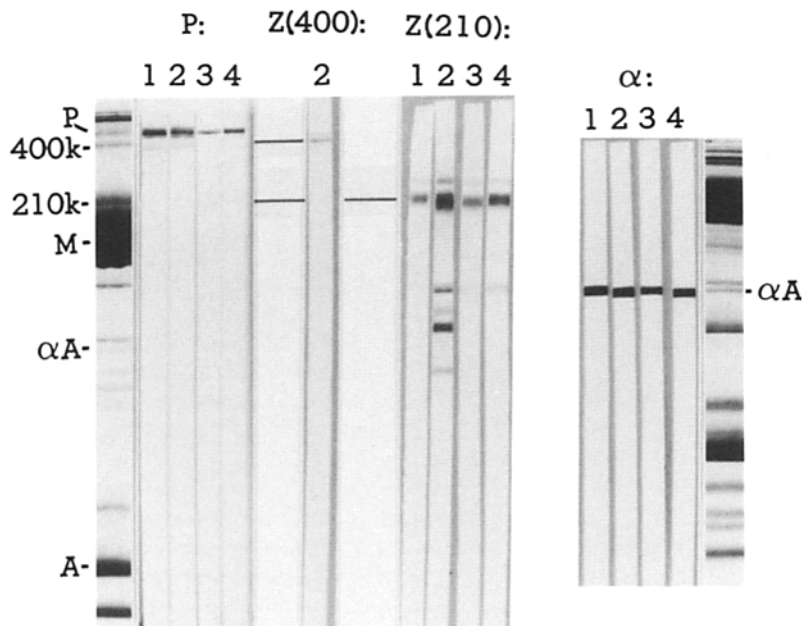


Figure 4. Electrophoretic identification of the four *Drosophila* fibrillar proteins detected by the antibodies described in this study. Proteins were separated by SDS-PAGE, electroblotted to nitrocellulose, and treated with culture supernatants (P:1 and P:2 [1:20] and α :1 and α :4 [undiluted]) or ascites fluids (1:500) from the clones indicated above the nitrocellulose strips. (All dilutions were made in tissue culture media.) On the far right, blots of a 7.5% gel, corresponding to the adjacent Coomassie blue-stained sample, were probed with antibodies to α -actinin. All other strips are immunoblots of a 6% gel corresponding to the stained gel at the far left. P, projectin; M, myosin heavy chain; α A, α -actinin; A, actin. The positions of the 210,000- and 400,000-D proteins are also indicated. Control strips (not shown) treated either with ascites fluid (as above) from a clone (2C3) secreting a nonspecific IgG1 or preimmune serum (1:100 in media) were negative.

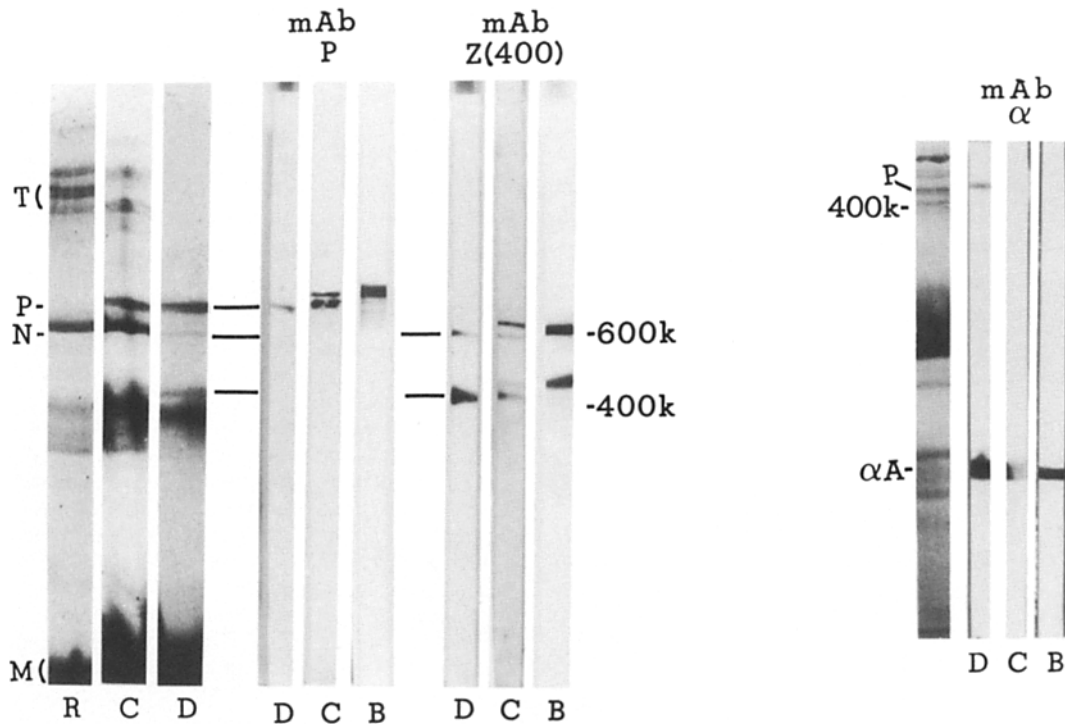


Figure 5. Electrophoretic comparison of the sizes of myofibrillar proteins from *Drosophila*, rabbit, and honeybee. The far left grouping shows Coomassie blue-stained SDS gels (3.2%) of proteins from rabbit psoas muscle (lane R), *Drosophila* flight muscle (lane D), or combined preparations (lane C). Other groupings show immunoblots of fibrillar proteins from *Drosophila* (lane D), honeybee (lane B), or the two preparations combined (lane C). Proteins were separated by SDS-PAGE on 5% gels (right grouping) or 3.2% gels (other groupings). Only the upper part of the 3.2% gels is presented. Blots were probed with monoclonal antibodies raised against the honeybee proteins α -actinin (mAb α [1:500 dilution of ascites fluid in tissue culture medium]), the 400/600-kD proteins (mAb Z[400]:2 [1:50 dilution]), and projectin (mAb P [1:500 and 1:1,000 to probe *Drosophila* and honeybee proteins, respectively]). To clearly resolve the projectin bands in the combined samples, relative loads of the two preparations were optimized, and blots were probed with both monoclonal antibody P (1:1,000) and culture supernatant from P:3, a clone that produces an antibody that, like monoclonal antibody P, recognizes projectin from both insects. A Ponceau red-stained blot of *Drosophila* myofibrillar proteins is presented in the far right grouping. The high molecular mass species that is labeled on the adjacent blot, probed with monoclonal antibody α , is rarely seen on other similar immunoblots. It may represent a cross-linked form of the antigen. T, titin (identified by an antibody generously provided by K. Wang); P, projectin; N, nebulin; M, myosin; α -A, α -actinin. Note that both projectin and the 400/600-kD proteins of *Drosophila* have slightly greater mobilities than the corresponding proteins of the honeybee, but that α -actinins from these two species comigrate on SDS gels.

The *Drosophila* flight muscle proteins that bind the antibodies against α -actinin colocalize with proteins 26 and 27 on the two-dimensional gels of *Drosophila* flight muscle proteins published by Mogami et al. (24). However, when we use the gel system of Mogami et al. (24) the spots 26 and 27 frequently do not resolve completely but form an elongated streak (data not shown). In the gel system that we usually use we can resolve two major spots of anti- α -actinin binding and these spots may be flanked by smaller spots (Fig. 6 A). Thus, it appears that the fibrillar flight muscle fibrils have at least two isoforms of α -actinin. Antibodies against both honeybee and vertebrate α -actinins appear to bind to most, if not all, of the isoforms. In contrast, three of the *Drosophila* monoclonals, α :1, α :2, and α :4, recognize honeybee α -actinin but none cross react with vertebrate proteins. In immunostaining of sectioned *Drosophila*, none of our antibodies show binding to nonmuscle cells.

A third criterion by which we have identified the *Drosophila* α -actinin is by isolation of the cloned gene with antibody binding to the expressed protein. Monoclonal antibodies α :2 and α :4 were used to screen an expression library of recombinant lambda phage carrying *D. melanogaster* cDNA linked in frame behind a bacterial promoter. Such genes cause the bacteria to produce polypeptides encoded in large part by *Drosophila* DNA. The library of recombinant molecules that was screened with the α :2 and α :4 antibodies yielded two clones: one with ~ 0.9 kb of sequence from the *Drosophila* cDNA and another with ~ 1.1 kb of *Drosophila* sequence. Both cloned DNAs directed the synthesis of polypeptides recognized by monoclonal antibodies that were raised against *Drosophila* α -actinin as well as by a monoclonal antibody directed against a vertebrate α -actinin. When hybridized in situ to polytene chromosomes, both of the cloned

cDNA sequences bound to a single site, region 2C near the tip of the X chromosome. This is the same site that E. Fyrberg (Johns Hopkins University, Baltimore, MD) identified as the *Drosophila* α -actinin locus by hybridization with a recombinant DNA molecule that he sequenced (personal communication). Hybridization to restriction enzyme-cleaved genomic DNA indicated that the genome contains a single copy of this gene (data not shown).

Monoclonal Antibodies Z(210):1-4. These four antibodies label the Z-band of *Drosophila* fibrils (Fig. 3) and, on immunoblots, bind to a protein that migrates at the trailing edge of the myosin heavy chain band and is often obscured by it (Fig. 4). This protein has an estimated molecular mass of 210,000 D and, like α -actinin, is enriched on gels of isolated Z-disks (Fig. 2). The monoclonal antibodies directed against this protein have been coded Z(210):1-4. On immunoblots each of these antibodies identifies a broad major band of 210 kD, a trailing component, and a faint lower molecular mass polypeptide that may be a breakdown product. Antibody Z(210):2 recognizes several additional lower molecular mass peptides that we assume to be protein fragments retaining the Z(210):2 epitope (Fig. 4). This 210,000-D protein is a Z-band component that has not been previously identified in insects. We have not detected binding of Z(210) antibodies to any vertebrate muscle protein.

Monoclonal Antibody Z(400):2. A third *Drosophila* Z-band protein has been identified by a cross reacting antibody that is directed against a honeybee Z-disk protein (35). This antibody, designated Z(400):2, selectively labels the Z-line of *Drosophila* myofibrils (Fig. 3) and, on immunoblots, stains a high molecular mass protein, estimated to be $\sim 400,000$ D, as well as a more weakly labeled trailing band with a molecular mass close to 600,000 D (Figs. 4 and 5).

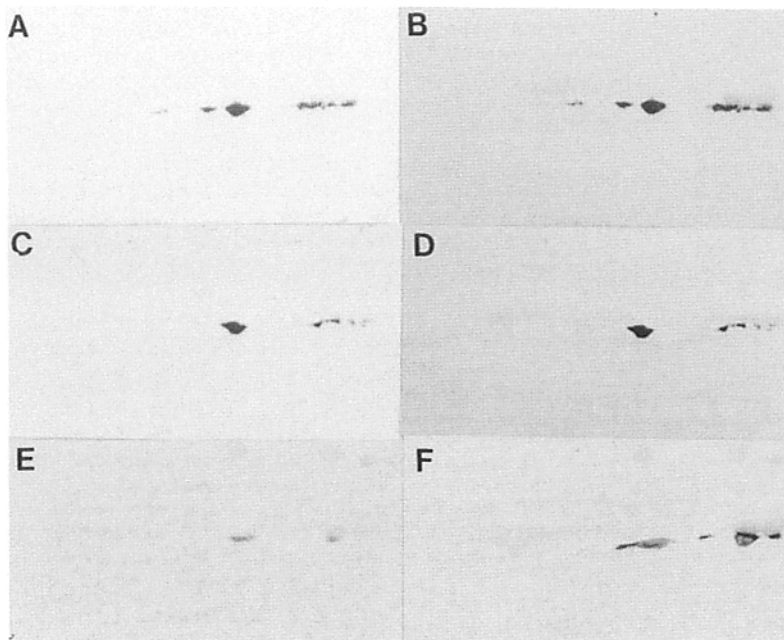


Figure 6. Immunoblots showing cross-reactivity of *Drosophila* muscle α -actinin with antibodies raised against honeybee and bovine α -actinin. Proteins extracted from the whole *Drosophila* thorax were subjected to two-dimensional gel electrophoresis and transferred to nitrocellulose filters as described in Materials and Methods. IEF is from left (cathode) to right (anode) and SDS-PAGE is in a 10% gel is from top to bottom. Filters were treated with monoclonal antibodies followed by alkaline phosphatase-conjugated second antibody to detect binding. Only the region of the antibody staining is shown in each panel. In each case antibodies stain identical proteins. (A) Monoclonal antibody α :4; (B) filter in A, after antibody labeling, was stained with amido black to reveal total protein pattern; (C) monoclonal antibody α directed against honeybee α -actinin; (D) filter in C, originally stained with antibody against honeybee α -actinin, restained with antibody against *Drosophila* α -actinin (α :4) followed by amido black staining; (E) monoclonal antibody against bovine α -actinin; (F) filter in E, originally stained with antibody against bovine α -actinin, restained with antibody against *Drosophila* α -actinin (α :4) followed by amido black staining. The antibodies specific for honeybee and bovine α -actinins both recognize the same spots (or a subset of the same spots) recognized by monoclonal antibody α :4.

On blots of honeybee fibrillar proteins, antibody Z(400):2 binds to two high molecular mass proteins, each with a slightly lower mobility than the corresponding labeled species detected on immunoblots of *Drosophila* muscle proteins (Fig. 5). Although the two bands of the honeybee doublet bind antibody about equally, the lower band of the *Drosophila* doublet is characteristically more strongly labeled than the upper one (Fig. 5). While the two proteins of the doublet may be distinct, it is possible that the smaller one is a breakdown or processing product of the larger.

Monoclonal Antibodies P:1-4. This fourth group of antibodies recognizes a very high molecular mass protein with a mobility slightly greater than that of honeybee projectin, a protein found in connecting filaments that link the thick filaments to the Z-band in insect fibrillar flight muscle (33) (Figs. 4 and 5). Both a monoclonal antibody (Fig. 5) and polyclonal antibodies (data not shown) raised against honeybee projectin cross react with the *Drosophila* protein; however, only one of the *Drosophila* antibodies, P:3, interacts with the corresponding bee polypeptide.

The mass of honeybee projectin was earlier judged to be 360,000 D by extrapolation of log molecular mass vs. mobility plots of proteins separated on 5% SDS gels (33). However, using a gel system that resolves extremely high molecular mass proteins (41), we have found that both the honeybee protein and its counterpart in *Drosophila* migrate behind the vertebrate protein nebulin and have apparent molecular masses >600,000 D.

In indirect immunofluorescence microscopy experiments, each of the antibodies directed against *Drosophila* projectin, P:1-4, appears as a single band of stain at the Z-band of *Drosophila* flight muscle myofibrils (Fig. 3). A cross reacting monoclonal antibody raised against honeybee projectin, monoclonal antibody P, gives identical results (data not shown). Since anti-projectin antibodies bind to honeybee myofibrils on either side of the Z-band and not within it (33), the failure to detect a double band of staining at the *Drosophila* Z-band was unexpected. The apparent contradiction of these results appears to be due to our inability to resolve two closely spaced bands of fluorescence in the *Drosophila* myofibrils with the light microscope. Using indirect immunoelectron microscopy, however, we find that projectin is located in the *Drosophila* fibril in a region corresponding to that in the honeybee, between the ends of the thick filaments and the border of the Z-band (see below).

The location of projectin in nonfibrillar muscle appears to differ from its position in flight muscle myofibrils. In *Drosophila* larval gut muscle, for example, each of the four anti-projectin antibodies bind within the A-band, not between the A-band and the Z-band, as in fibrillar muscle (Fig. 7). These results parallel those found in the honeybee; a monoclonal antibody to honeybee projectin labels nonfibrillar leg muscle only within the A-band (35). The muscle type-specific distribution of this protein provides an additional point of identification between *Drosophila* and honeybee projectin and raises interesting questions about the location and function of this protein in sarcomeres of different insect fiber types.

Immunoelectron Microscopy

The increased resolution of immunoelectron microscopy has

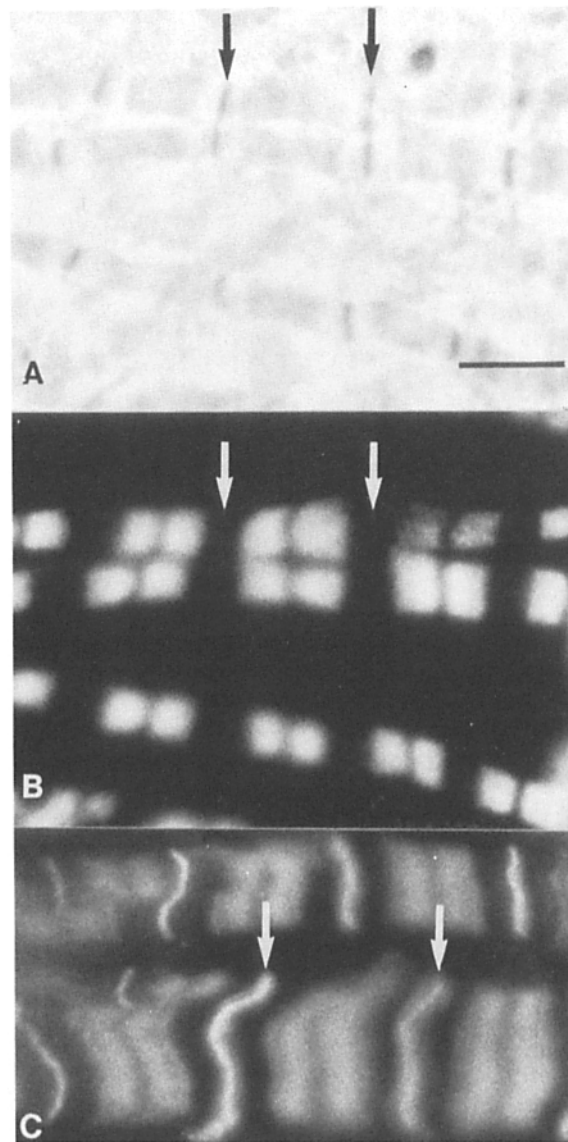
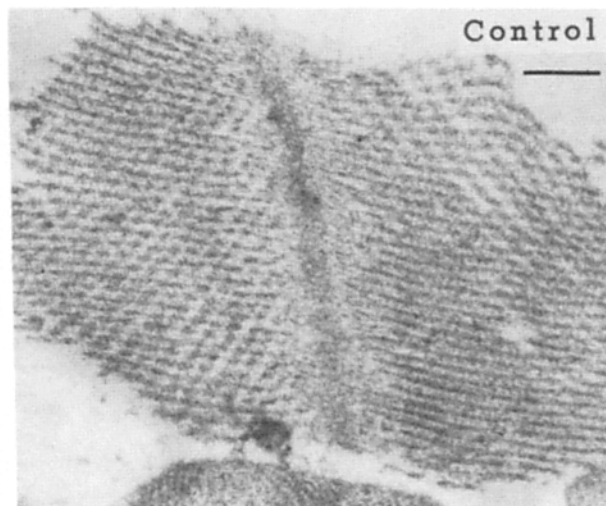
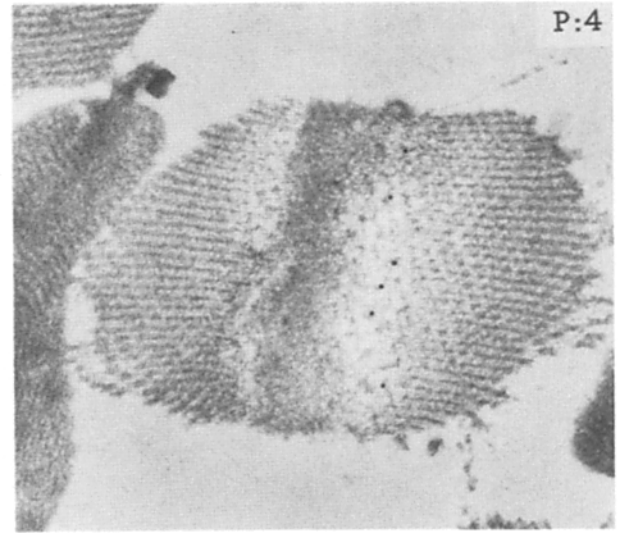
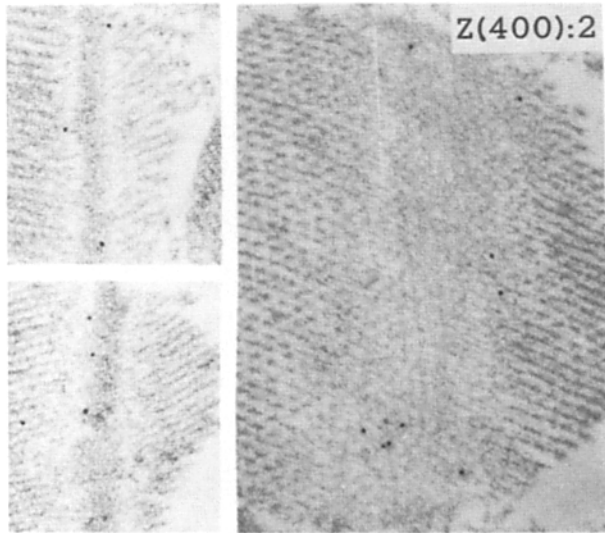
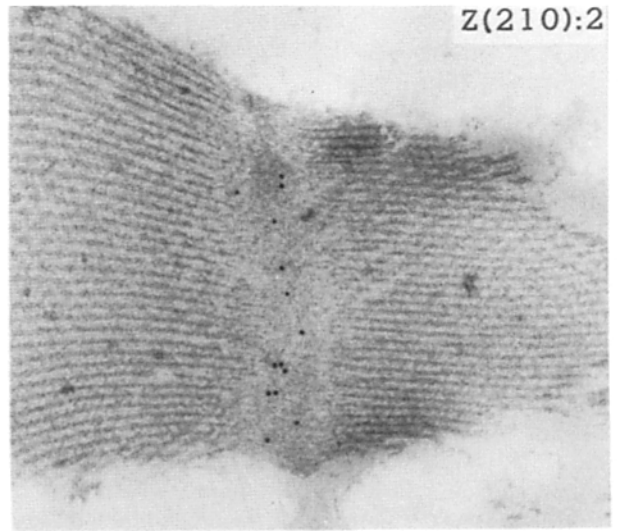
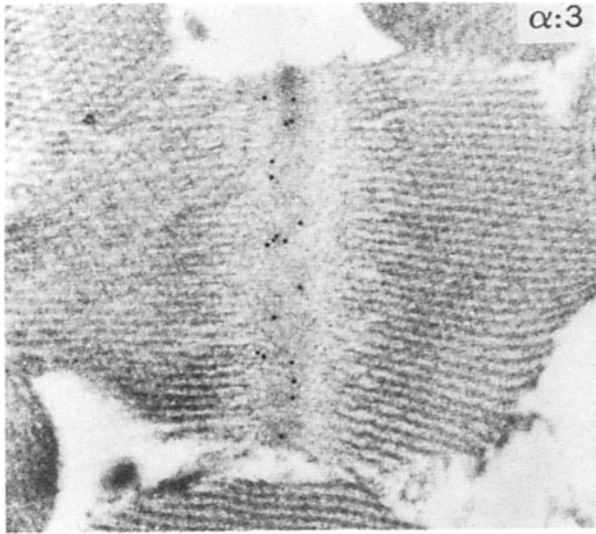


Figure 7. Indirect immunofluorescence micrograph showing localization of projectin antibody within the A-band of larval gut muscle. (A) Phase-contrast micrograph of a segment of larval gut. Part of the meshwork of myofibers that surround the gut can be seen. Although their phase image is partly obscured by underlying parenchymal cells, the striations of the muscle fibers are clearly visible. Arrows indicate two of the Z-bands. (B) Fluorescence image of the segment shown in A. Antibody P:1 detects projectin throughout the A-band regions. Arrows indicate the Z bands marked in A. All antibodies that recognize projectin (four monoclonals and one polyclonal) show the same staining pattern. (C) Fluorescence image of a segment of larval gut doubly stained with antibodies for projectin (P:1) and α -actinin (α :4) that clearly show the separation between the α -actinin staining in the Z-band and the projectin staining in the A-bands. Antibody concentrations were chosen to give more intense staining of the α -actinin regions. Arrows mark two Z-bands. Bar, 5 μ m.

made it possible to define more precisely the location of the antigens which, with the light microscope, are detected in the Z-band/I-band region of *Drosophila* myofibrils.

Sections through *Drosophila* thoraces, mounted in Lowicryl, were incubated with selected monoclonal antibodies



directed against each of the four Z-disk antigens that have been identified. Bound antibody was visualized by subsequent binding of anti-mouse IgG-coated colloidal gold particles (Fig. 8). Since embedding protocols limited our ability to orient specimens, sections through the thorax were random, and fibrillar muscle was most often cut obliquely. Monoclonal antibodies against α -actinin, α :3, and the 210-kD protein, Z(210):2, were both localized within the matrix of the Z-band of the *Drosophila* flight muscle myofibrils. The labeling with both of these antibodies was intense and highly specific (Fig. 8). Gold particles were rarely found outside of the Z-band.

Although all of the antibodies against projectin strongly label unfixed myofibrils in light microscope experiments, the binding of these antibodies to fixed muscle in Lowicryl sections was significantly weaker than that found with monoclonal antibodies α :3 or Z(210):2. While the density of gold particles was low after staining with P:4, the distribution of the particles was quite distinct and entirely consistent with the localization of projectin in honeybee fibrillar muscle. The P:4 antibody was primarily associated with the region between the ends of the thick filaments and the Z-band. Gold particles within the Z-band were rarely observed (Fig. 8). Among 50 sarcomeres examined in random fields, 78% of 107 gold particles were located between the A-band and the lateral border of the Z-band, 1% were within the Z-band, and the remainder were within the A-band. We note that these results apply only to the fibrillar muscle.

Studies with antibody against the 400/600-kD polypeptides were most intriguing in spite of the fact that the labeling of Lowicryl sections with Z(400):2 was weak and variable with regard to background staining. Culture supernatants provided the most specific labeling, and in our most successful experiments we observed gold particles restricted to the lateral border of the Z-band along the I-band/Z-band junction. This selective binding was evident in oblique sections through the Z-band, as well as in more longitudinal sections (Fig. 8). When more concentrated antibody preparations were used in an attempt to increase labeling, background staining was raised to undesirable levels. Analysis of 75 sarcomeres showed 56% of 154 grains at the I-band/Z-band junction, a region accounting for no more than 2% of the myofibril; 3% of the grains were within the Z-band, 19% within the I-band, and the remainder within the A-band. Taken together with results of light microscope studies in which staining of Z(400):2 was highly specific and restricted to the Z-band region, our findings suggest that the 400/600-kD proteins are, in fact, at the edge of the Z-band distributed along the border defining the I-band/Z-band junction.

Discussion

The monoclonal antibodies described in this paper have allowed us to identify a significant fraction of the high molecular mass proteins (>45 kD) that make up the isolated Z-disks

in the fibrillar flight muscle of *Drosophila*. Most of the major bands on Coomassie blue-stained gels of proteins from isolated Z-disks (Fig. 2) can be accounted for in the antibody collection. The number of different monoclonal antibodies that have been recovered for projectin, α -actinin, and the 210-kD protein suggest that these are the most antigenic components in our system. We have found no Z-band-specific antibodies that detect proteins smaller than α -actinin. It may be that all of the structural components remaining in our isolated Z-disks are high molecular mass proteins. Preparations of Z-disks isolated from giant water bug flight muscle, however, contain a low molecular mass protein, zeelin 1 (35 kD) (36) that has been demonstrated by immunofluorescence microscopy to be a Z-band component (3). It is possible that the smaller structural polypeptides of *Drosophila* Z-disks are less antigenic than the larger ones and thus less likely to be detected when antibodies are raised in response to a mixture of Z-disk proteins. It should be noted, however, that our screen has identified four monoclonal antibodies to low molecular mass muscle proteins (26–80 kD). These, however, lie outside the Z-band and are assumed to be contaminants of our Z-disk preparations (our unpublished observations).

Morphologically, the Z-bands of *Drosophila* fibrillar flight muscle resemble those of the honeybee (16). The studies of the structural components of the Z-band reported in this manuscript reveal marked similarities as well as certain differences between these two insects. As seen in Fig. 5, three of the major proteins, α -actinin, projectin, and the 400/600-kD proteins, have similar sizes in the two insects. These proteins also share at least some antigenic determinants; one of the four anti-*Drosophila* projectin antibodies and three of the four anti-*Drosophila* α -actinin antibodies recognize the corresponding honeybee proteins. None of the monoclonal antibodies in our collection recognizes vertebrate skeletal muscle proteins. However, *Drosophila* α -actinin is sufficiently conserved to be recognized by a monoclonal antibody directed against the vertebrate protein. It is interesting that, although our antibodies do not detect *Drosophila* nonmuscle α -actinin, the vertebrate antibody that we have used was directed against α -actinin from bovine mammary gland epithelial cells.

The 210-kD *Drosophila* protein is a Z-band structural component that has not been previously described. It is recognized by four of the antibodies and appears to be an abundant component of Z-disks, as judged by Coomassie blue-stained gels. No related honeybee antigen has been detected by any of the monoclonal antibodies produced against honeybee isolated Z-disk proteins (35). Furthermore, none of the Z(210) antibodies have been found to cross react with honeybee proteins in either immunofluorescence or immunoblot assays. However, 200,000-D proteins have been found in isolated Z-disk preparations of both honeybee (34) and giant waterbug (2), although in neither case has the protein been confirmed as a Z-band constituent. By immunogold

Figure 8. Immunogold-treated Lowicryl sections of *Drosophila* flight muscle showing localization of the proteins identified in this study. Each of the sections shown has an electron-dense Z-band running through the center flanked by the less dense narrow I-band and a broad A-band. Sections were incubated with culture supernatants from hybridoma clones indicated in the upper right corner of each electron micrograph. Note the reticular appearance and hexagonal symmetry of the obliquely sectioned Z-band (*middle left*). Control section was treated with culture supernatant from a clone (2C3) secreting a nonspecific IgG1. The few gold particles detected in sections treated with this monoclonal antibody appeared randomly distributed. Bar, 0.25 μ m.

staining, the *Drosophila* 210-kD protein is detected within the Z-band with a distribution very similar to that of α -actinin. More work will be necessary, however, to assign these proteins to specific sites within the Z-band lattice and to determine their particular interactions with the proteins that are associated with the Z-band.

The 400/600-kD proteins also represent Z-band components that have not been previously identified in insects. Our results indicate that these polypeptides are not distributed throughout the Z-band as are α -actinin and the 210-kD proteins; instead the 400/600-kD proteins appear to be located along the lateral borders of this structure, perhaps serving to anchor actin or projectin filaments. Since Z(400):2 does not cross react with vertebrate proteins, we are unable to identify the insect antigen with a related vertebrate component. It should be noted, however, that the vertebrate Z-band protein, zeugmatin, migrates on SDS gels as a doublet with an estimated mass >500 kD and is reported to be localized along the sides of the Z-band (20).

One of the proteins associated with *Drosophila* Z-disks has been identified as projectin on the basis of several similarities with projectin from honeybee, the organism in which this protein was first described (33). The *Drosophila* and honeybee proteins have similar mobilities on SDS gels and each is recognized by antibodies raised against projectin of the other species.

Studies of honeybee asynchronous flight muscles have identified projectin with the filaments connecting the thick filaments to the Z-band (33). These "connecting filaments" have been proposed to play a role in stretch activation of oscillatory flight muscle by transmitting tension to the thick filaments when sarcomeres are lengthened (32). It was not expected that projectin would be present in nonfibrillar synchronous muscles in which stretch activation presumably does not occur. We were, therefore, surprised to find that all of our anti-projectin antibodies labeled synchronous larval muscle. Analyses of larval gut muscle showed that the staining was not within the I-band, as in flight muscle, but throughout the A-band. The localization of projectin in *Drosophila* gut muscle is consistent with observations of honeybee leg muscle, another synchronous muscle in which anti-projectin antibodies prominently stain the A-band (35). The apparent differences in location of the antigen in the physiologically distinct muscles raises the possibility that the protein may play different roles in synchronous and asynchronous muscle. It will be interesting to learn whether the antibodies are, in fact, detecting the same protein in both muscles or whether they are recognizing two different but related proteins.

The isolation of this set of monoclonal antibodies has permitted an initial characterization of the proteins associated with the Z-bands of *Drosophila* fibrillar flight muscle. It has also provided us with the tools to begin analysis of the defects in the muscles of flightless mutants, a number of which have Z-band abnormalities (8, 14, 16, 23). Such analyses may give insight into the pathway by which the remarkable structure of the contractile apparatus is assembled.

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