Flightin, a Novel Myofibrillar Protein of Drosophila Stretch-activated Muscles

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Abstract. The indirect flight muscles of Drosophila are adapted for rapid oscillatory movements which depend on properties of the contractile apparatus itself. Flight muscles are stretch activated and the frequency of contraction in these muscles is independent of the rate of nerve impulses. Little is known about the molecular basis of these adaptations. We now report a novel protein that is found only in flight muscles and has, therefore, been named flightin. Although we detect only one gene (in polytene region 76D) for flightin, this protein has several isoforms (relative gel mobilities, 27-30 kD; pIs, 4.6-6.0). These isoforms appear to be created by posttranslational modifications. A subset of these isoforms is absent in newly emerged adults but appears when the adult develops the ability to fly. In intact muscles flightin is associated with the A band of the sarcomere, where evidence suggests it interacts with the myosin filaments. Computer database searches do not reveal extensive similarity to any known protein. However, the NH₂-terminal 12 residues show similarity to the NH₂-terminal sequence of actin, a region that interacts with myosin. These features suggest a role for flightin in the regulation of contraction, possibly by modulating actin–myosin interaction.

The indirect flight muscles (IFM) of Drosophila melanogaster are a group of thoracic fibers whose oscillatory contractions power wing beats at high frequencies, far greater than the firing rate of motor nerves. With steady neural input intracellular calcium levels remain high in the IFM, and the muscle responds to slight changes in length with delayed changes in tension (35, 51). This property, called stretch activation, allows the IFM to drive the mechanically resonant wing/thorax system at its very high natural frequency. Since skinned fibers also do oscillatory work in the presence of calcium and ATP, it is assumed that components of the myofibril itself are responsible for this adaptation (18).

The mechanism underlying stretch activation is not known. Wray (64) has suggested that a slight longitudinal displacement of thick and thin filaments increases the number of cross-bridges recruited by optimally aligning the matching actin and myosin filament periodicities. Recently, however, Squire (45) has challenged this hypothesis on the basis of detailed analysis of filament organization. Alternative proposals are that longitudinal displacement of filaments might influence tension development by changing the angle of attachment of cross-bridges bound to actin (45, 51, 52), or might alter cross-bridge dynamics by placing stress on the thick filaments through their attachments, via connecting filaments, to the Z band (35, 51, 52). Such models will undoubtedly be refined as the function of components of the contractile apparatus are further clarified.

The mechanical properties of the IFM distinguish it from other Drosophila muscle types and reflect not only differences in ultrastructure but also in the isoforms of most myofibrillar proteins. The IFM isoforms are produced either by differentially spliced transcripts from genes that produce the isoforms for other muscles (e.g., myosin heavy and light chains) (7), or by specific genes expressed only in flight muscle (e.g., actin) (7). For most proteins, the IFM isoforms appear to perform essentially the same functions that the related isoforms perform in other muscles. In some cases the IFM contains forms of myofibrillar proteins so significantly modified that they may have new functions. One of these proteins is arthrin, a stable ubiquinated actin (8). Another is troponin H, a modified tropomyosin with an extended hydrophobic carboxy terminus (22). In the case of the myofibrillar protein projectin, the IFM isoform seems to have a very different function from the products of the same gene in other muscles. In the IFM projectin is located in the I band while in other muscles this protein is found in the A band (60).

We have now characterized a protein that is found only in the IFM and does not appear to be related to any protein found in other muscle types. Because of its specific association with flight muscle, we have named the protein flightin. It is tempting to think that such a protein might be involved...
in the mechanism of stretch activation in the IFM. The possibility of such involvement is strengthened by the localization of flightin to the A band and by the production of a subset of additional flightin isoforms at the time the fly becomes competent to fly.

Materials and Methods

Antibodies

mAbs 5b7, 7c10, and 7f8 are specific for flightin. mAb α:2 is directed against α-actinin. mAB C is an IgGl that does not recognize any fibrillar proteins. All of these mAbs were derived from mice immunized with preparations of Drosophila 2 disks which were contaminated with small amounts of other fibrillar proteins (38). Antibodies against arginine kinase and glycerdehyde-3-phosphate dehydrogenase (gpd) were kindly provided by Dr. David Maughan (University of Vermont, Burlington, VT).

Gel Electrophoresis and Immunoblot Analysis

Dissection of tissues and sample preparations were performed as described by Vigoreaux et al. (60). SDS-PAGE was performed with the discontinuous buffer system of Laemmli (24) with the modifications reported by Vigoreaux et al. (60). Two-dimensional gel electrophoresis was done according to the technique of O'Farrell (31) with the modifications described by Saide et al. (60). Immunoblot analyses were performed as described previously (38).

Indirect Immunofluorescence Microscopy

Frozen Sections. Fixing and immunostaining of frozen sections of Drosophila tissue were done exactly as described in our previous study (60).

Myofibrils. Fresh flight muscle myofibrils were prepared from whole Drosophila thoraces by homogenization in 0.1 M KCl, 1 mM MgCl2, 2.5 mM EGTA, pH 7.9 (Hodge's-EGTA), containing protease inhibitors (38). Dispersed fibrils were centrifuged and resuspended in Hodge's-EGTA but not further purified. Some preparations were suspended in 0.1 M KCl, 5 mM Mg2+-ATP, 10 mM Tris, pH 6.8, containing either 5 mM EGTA or 1 mM CaCl2 (contracting solution) or 1 mM Mg2+ (relaxing solution). Fibrils were labeled with a mixture of anti-flightin mAbs (5b7, 7c10, 7f8), each at a dilution of 1:2,000 and transferred to nitrocellulose and probed with a mixture of mAbs 5b7, 7c10, 7f8, each at a dilution of 1:200 in suspension buffer, using procedures described by Saide et al. (39).

Biochemical Fractionation of Fiber Proteins

This method is used in the laboratory of Dr. David Maughan (University of Vermont, Burlington, VT) and is described in detail by Warnke et al. (62). IFM fibers are dissected from adult fly thoraces under cooled water-saturated mineral oil and then transferred to a droplet of relaxing solution (18 mM MgATP, 1 mM free Mg2+, 5 mM EGTA, 20 mM N,N′-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid, pH 7.0 (BES), 150 mM K methanesulfonate. After 20–30 min, the drop is collected and diluted in an equal volume of modified 2× SDS Laemmli sample buffer. This first drop corresponds to the cytosolic fraction. The remaining fiber is transferred to a drop of relaxing solution containing 0.5% wt/vol Triton X-100. The drop is then collected and diluted in modified 2× Laemmli sample buffer. This second drop corresponds to the membrane/organelle fraction. The residual fiber (myofibrillar/cytomatrix fraction) is sonicated in 50 mM Tris-HCl, pH 8.0, 150 mM KCl, 2 mM MgCl2, 1 mM EGTA, and 1 mM ZnCl2. Bacterial alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was used at 1 U per reaction. After incubation, the fibers were precipitated with cold 15% TCA, rinsed with acetone and resuspended in SDS Laemmli sample buffer containing protease inhibitors (60).

Phosphatase Treatment of Fibers

Adult flight muscle fibers were dissected as described above and immediately transferred to a drop of relaxing solution containing 0.5% wt/vol Triton X-100, 1 mM PMSF and 100 μM leupeptin. After incubating on ice for the indicated time, the fibers were transferred to a solution containing 20 mM Tris-HCl, pH 8.0, 150 mM KCl, 2 mM MgCl2, 1 mM EGTA, and 1 mM ZnCl2. Bacterial alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was used at 1 U per reaction. After incubation, the fibers were precipitated with cold 15% TCA, rinsed with acetone and resuspended in SDS Laemmli sample buffer containing protease inhibitors (60).

Isolation of cDNA Clones and Subcloning

A Agt11 cDNA expression library provided by P. Salvaterra (30) was screened with mAb 7c10 following the method of Young and Davis (65) as modified by Goldstein et al. (16). A single positive plaque was obtained from a screen of ~50,000. We attribute the low recovery of clones to the fact the library was made from fly head RNA. Since we cannot detect flightin RNA in heads, the clone must have come from muscle tissue containing the head preparation. The phage selected by the antibody was grown by the plate lysis method and the DNA purified following standard protocols (40). The phage DNA was digested with EcoRI. The Drosophila DNA insert was isolated by agarose gel electrophoresis and subcloned into the EcoRI site of pGem 4Z (Promega Corp., Madison, WI) to produce clone p5b, containing a 960-bp insert with a potential open reading frame of 181 amino acids and a 3' noncoding region of 411 nucleotides. The p5b clone lacks sequences 5' to the open reading frame.

Additional flightin cDNA clones were isolated from a Agt10 cDNA library from Drosophila pupal RNA given to us by C. Emerson (14). The library was screened with a probe corresponding to the 5' region of p5b, a 360-bp Smal-EcoRI fragment. The gel-isolated fragment was labeled with 32P using random primers (New England Biolabs, Beverly, MA) and then separated from unincorporated nucleotides by Sephadex G50 spin column chromatography (40). The library was plated and the filters processed following standard methods (40). Filters were prehybridized for 2 h at 68°C in 4× SET (1× SET = 0.15 M NaCl, 30 mM Tris-HCl, pH 7.0, 2 mM EDTA), 1× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA and 0.5% SDS followed by an overnight hybridization at 68°C in the same solution including 2× 106 cpm of probe. Filters were then washed once for 30 min at 68°C in 2× SSC/0.1% SDS followed by three to four washes at 68°C in 0.2× SSC/0.1% SDS for 2–3 h. 15 positive clones were identified from ~50,000 plaques by matching autoradiographs of duplicate filters. DNA was purified from each phage grown singly by the plate lysis method (40). After digestion with EcoRI followed by electrophoresis on low melt agarose gels, the insert DNA was recovered and cloned into the EcoRI site of pBluescript SKII+ (Stratagene Corp., LaJolla, CA). We selected one of the clones with the longest 5' end for the most extensive analysis. The sequence of this clone, named p5b, is very similar to p5b, but pl3 extending 16 nucleotides further in the 5' direction and encodes a full length protein.

In vitro Transcription and Translation

Bluescript phagemid II SK+ (Stratagene Corp.) containing the insert from clone p5b was linearized with BamHI, extracted with phenol-chloroform-isomyl alcohol, and precipitated with ethanol. The DNA was suspended in water and transcribed in vitro using T7 RNA polymerase according to instructions provided by the manufacturer (Promega Corp.). After digestion with RNase-free DNase, the preparation was extracted and precipitated as above. RNA transcripts were translated by the rabbit reticulocyte lysate system from Promega Corp. using the manufacturer's protocol.

Lysate aliquots were electrophoresed on 11% polyacrylamide SDS gels, transferred to nitrocellulose and probed with a mixture of mAbs 5b7, 7c10, and 7f8, each at a dilution of 1:2,000.

DNA Sequence Analysis, Genomic Southern Blots, and Chromosomal Localization

DNA Sequence. The DNA sequence of pl was determined from a series of overlapping deletion fragments that were generated using an Exonuclease III/mung bean nuclease deletion kit (Stratagene). Single stranded DNA templates were obtained by infecting XLI-blue bacterial cells containing pl with the helper phage VCSM13 according to the instruction provided by the manufacturer (Stratagene). All templates were sequenced by the dyeoxy method using a KS primer (Stratagene).

Genomic Southern. Hybridization to restriction fragments of Drosophila DNA was performed as described by Mischke and Purdie (27).

In Situ Hybridization to Polytenic Chromosomes. The chromosomal localization of the flightin clones was determined by probing polytene chromosome preparations with isolated fragments of the clones as described by Mischke and Purdie (27).

RNA Analysis

Northern Blots. RNA from different Drosophila developmental stages was analyzed by Northern blotting as described by Toffenetti et al. (53).

In Situ Tissue Hybridization. RNA probes labeled with 3H-UTP were generated by in vitro transcription from a pGem 4Z clone using SP6 RNA polymerase (for the sense probe) or T7 RNA polymerase (for the antisense probe). For the sense probe, p5b was linearized with HindIII and for
the anti-sense probe, the same clone was linearized with NcoI. The probes were labeled as described in (34) except that the final purification steps were done by repeated ethanol precipitation using 2 M ammonium acetate.

Frozen sections of adult tissue were fixed, hybridized and processed as described by Ayme-Southgate et al. (4).

**Staging of Developing Organisms and Flight Testing**

Pupae were staged following the criteria devised by Bainbridge and Bownes (5). Young adults were collected immediately after eclosion and aged at 25°C in individual food vials for the indicated times. For flight testing, each fly was placed in an empty milk bottle and observed for wing-beat and flight. Flies were scored on a scale that never beat their wings and fell straight to the bottom when the bottle was tapped were assigned a score of zero. Newly eclosed adults whose wings had not yet unfolded were also given a score of zero. Flies that landed on the side walls near the bottom when tapped, but that otherwise did not fly were assigned a score of one. Flies that folded their wings on their own and flew sporadically were assigned a score of two. Moderate fliers were assigned a score of three. Normal fliers, with very active wing beat, were assigned a score of four. After the flight test, flies were examined under a stereomicroscope to determine if any wing and/or thorax abnormalities were present. All flies used in this study were found to have normal morphology.

**Results**

**Drosophila Muscle Has Multiple Isoforms of Flightin**

Flightin was initially identified by three mAbs that were raised against *Drosophila* myofibrillar proteins (38). This set of mAbs recognizes a group of isoforms that, on two-dimensional gel electrophoresis, resolves into several spots with relative gel mobilities of ~27–29 kD and pIs between 4.6 and 6.0 (Fig. 1). Two of the three mAbs, 7c10 and 7f8, recognize all isoforms while a third mAb, 5b7, recognizes only a subset of these isoforms (compare Fig. 1, A and B).

By one-dimensional SDS-PAGE the multiple isoforms resolve into two distinct bands (Fig. 1 C). Both bands are detected by mAbs 7c10 and 7f8, but only the top band is recognized by mAb 5b7.

Three of the flightin spots colocalize with spots 158, 159, and 160 on the two-dimensional gels of *Drosophila* flight muscle proteins published by Mogami et al. (29). The ability of the antibodies to detect each of these protein spots indicates that spots 158–160 make up a protein family (Fig. 1 A). The mAbs also detect additional spots with a slightly lower apparent molecular weight than spots 158, 159, and 160 (Fig. 1 B). These additional spots represent low abundance components not easily detected on Coomassie blue-stained gels (29; our own unpublished observation) and correspond to the faster migrating band seen on SDS gels (Fig. 1 C, right lane). The elongated streak seen in Fig. 1 B (i.e., slightly more acidic than spot 160) can sometimes be resolved into eight distinct spots. A spot with a more basic pI than spot 158 is also detected (Fig. 1 B, arrowhead). Thus, it is possible that there may be as many as 12 antigenically related variants of this protein.

In preliminary accounts of this work, we had referred to these protein isoforms as mp27 for muscle protein of ~27 kD (Vigoreaux, J. O., J. D. Saide, A. Ayme-Southgate, K. Valgeirsdottir, and M. L. Pardue. 1990. *J. Cell Biol.* 14A:19; Vigoreaux, J. O., J. D. Saide, K. Valgeirsdottir; and M. L. Pardue. 1991. *J. Cell Biol.* 115:379a). Because of the characteristics described below, we have now named the protein flightin.

**Flightin Is Found Only in Asynchronous Flight Muscles**

Both immunostaining of sectioned *Drosophila* and gel analysis of proteins extracted from dissected tissues demonstrate that flightin is found exclusively in the IFM. Frozen sections of *Drosophila* adults stained with each of the three anti-flightin mAbs were visualized by indirect immunofluorescence microscopy. A representative section through an adult thorax is shown in Fig. 2, A and B. Immunostaining is detected over the fibrillar flight muscles but not over the jump muscle, a tubular muscle. Other muscles of the adult fly, for example, the tubular muscles in the leg and head and the super-contractile muscles in the abdomen, also fail to stain with any of the three anti-flightin mAbs. In addition, we have been unable to detect staining of any tissue in sections of developing embryos or larva (data not shown; see also Fig. 4).

**Figure 1.** Identification of flightin variants by immunoblot analysis of total thoracic proteins. (A and B). Proteins were separated by two-dimensional gel electrophoresis and electroblotted. The filter was probed with mAb 5b7 (A) which recognizes three protein spots of ~29 kD and pI between 5.3 and 6.0. (B) The filter from (A) was reprobed with mAb 7c10. This antibody recognizes the same three spots labeled by mAb 5b7 as well as an additional elongated streak that sometimes resolves into eight distinct components. The majority of the spots have a pI between 4.6–5.2. A single spot with a more basic pI than spot 158 is also detected (arrowhead). Identical results were obtained when a similar filter was reprobed with mAb 7f8. (C) Proteins were separated by 12.5% SDS-PAGE and electroblotted. Lane 1, mAb 5b7; lane 2, mAb 7c10. mAb 5b7 recognizes a single band of ~29 kD while mAb 7c10 recognizes an additional, faster migrating band of ~27 kD. A third antibody, mAb 7f8, gives results identical to those of mAb 7c10. The faster migrating band corresponds to the elongated streak seen in two-dimensional gels.
Figure 2. Flightin is a protein specific to the flight muscles. (A and B) Localization of flightin in sections of *Drosophila* thorax by indirect immunofluorescence microscopy. Phase contrast (A) and fluorescence (B) photomicrographs of a frozen section through an adult *Drosophila* thorax stained with mAb 7c10 and visualized with a second antibody–FITC conjugate. Note that the antibody labels the IFM (mf), a fibrillar muscle, but not the tergal depressor of the trochanter (tr), a tubular muscle used primarily for jumping. No other tissue in the adult fly stained with any of the three anti-flightin mAbs. (C) Immunoblot analysis to determine the tissue distribution of flightin. The blot was cut in half, the top portion probed with an anti-α-actinin antibody (α:2, which detects a band of ~90 kD) and the bottom half with an anti-flightin antibody (mAb 5b7) which detects a band of ~27 kD. The muscles of the larval body wall and adult abdomen are examples of super-contractile muscles; the leg and trochanter have tubular muscles and the head has a mixture of tubular and super-contractile muscles. Note the flightin is found only in IFM myofibrils (fibrillar-type muscles), while α-actinin is detected in all tissues, regardless of muscle type. Similar blots probed with mAb 7c10 or mAb 7f8 gave identical results. (D) Autoradiograph showing flight muscle specific expression of flightin RNA. Frozen section of a late pupa was probed with a tritium-labeled antisense RNA derived from a flightin cDNA. Note the high concentration of grains over the fibrillar flight muscle (IFM) and their absence over the tubular jump muscle (TR). We were not able to detect hybridization over any pupal or adult tissue other than the IFM. Bars: (A and B) 6 μm; (D) 10 μm.

When proteins extracted from the three major muscle types (super-contractile, tubular, and fibrillar) are examined by SDS-PAGE and immunoblot analysis, flightin is detected only in the adult fibrillar flight muscles, in contrast to the significant levels of α-actinin which are detected in all muscle types examined (Fig. 2 C).

The IFM specificity of flightin is seen not only in the distribution of the protein but also in the distribution of the mRNA. Fig. 2 D shows the results obtained when a single stranded, anti-sense RNA probe derived from a flightin cDNA (see below) is hybridized to an adult tissue section. This probe recognizes an RNA that is expressed abundantly in flight muscles but is absent from the jump muscle. Examination of other sections has failed to detect RNA accumulation in any adult or pupal tissue other than the flight muscles. The cytological localization studies are supported by studies of RNA extracted from different *Drosophila* stages. Flightin RNA is not detected until late pupal stages when IFM synthesis is occurring (see Fig. 4). This pattern of expression is consistent with that of other flight muscle-specific transcripts (7). No hybridization is detected with a probe derived from the opposite (sense) strand of the cDNA (not shown).
Subcellular Distribution of Flightin

Subcellular fractionation experiments indicate that flightin is closely associated with structural components of the myofibril. Using the technique of Warmke et al. (62) we prepared three fractions from freshly dissected flight muscle myofibrils. These fractions contained: (a) diffusible proteins; (b) detergent-soluble membrane proteins; or (c) insoluble myofibrillar and cytomatrix components. Protein fractions were compared by gel electrophoresis and immunoblot analysis. Flightin was detected only in the insoluble myofibrillar/cytomatrix fraction (Fig. 3 B and C, lanes 1). Note that both size variants of flightin were found in the same fraction. The soluble proteins, arginine kinase (Fig. 3 B, lane 2) and glyceraldehyde-3-phosphate-dehydrogenase (Fig. 3 C, lane 2) were both found predominantly in the diffusible fraction. No evidence of flightin was found in this fraction, however, or in the detergent soluble fraction.

Indirect immunofluorescence microscopy studies show that flightin is a myofibrillar rather than a cytomatrix component and show flightin to be localized in the region of thick filaments. The immunostaining observed in unfixed myofibrils (Fig. 3 A) is specifically within the A band. The Z band appears unlabeled and the M line is either unstained or only weakly labeled. In fixed isolated fibers a slightly higher level of immunofluorescence is seen just flanking the M line. This A band staining pattern is commonly observed in fixed frozen sections as well (see Fig. 2 B). Alternate patterns of staining have also been observed, however. In both fixed and unfixed preparations we have occasionally observed weak A band fluorescence with more intense fluorescence near the M and Z bands. As yet we have been unable to ascribe this variable labeling pattern to specific preparative procedures or state of contraction of the fibrils. These results might be explained if flightin were a soluble protein that redistributed itself. Our subcellular fraction studies demonstrate that flightin is not readily extractable. Nevertheless, it is possible that one or more of our experimental procedures may have affected the association of flightin with other myofibrillar components.

Flightin Variants Are Encoded by a Single Gene

Although Drosophila muscle contains multiple isoforms of flightin, all isoforms are encoded by a single gene. Neither in situ hybridization to polytene chromosomes nor hybridization to restriction enzyme-digested Drosophila DNA detects additional flightin-related sequences in the Drosophila genome. To determine the number and location of the flightin genes we used a flightin cDNA clone (see Materials and Methods) for in situ hybridization to polytene chromosomes (34). The probe hybridized to a single site, at region 76D/E on the left arm of the third chromosome (data not shown).

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Figure 3. Flightin is a myofibrillar component. (A) Localization of flightin in Drosophila flight muscle myofibrils. Phase (upper panels) and fluorescence (lower panels) photomicrographs of unfixed flight muscle myofibrils labeled with anti-flightin mAbs (left) or a nonspecific mAb (mAb C, right). Note the intense staining of the A band in fibrils treated with anti-flightin mAbs and the absence of M and Z line staining. (B and C) Single fibers were dissected from the flight muscles and the proteins fractionated following a diffusion method (see Materials and Methods). The proteins were separated by 15% SDS-PAGE and blotted onto nitrocellulose. Lane 1, cytomatrix fraction containing the myofibrillar proteins; lane 2, cytosolic fraction containing readily diffusible proteins; lane 3, membrane/organelle fraction containing Triton X-100 extractable proteins. (B) The filter was probed first with an anti-glyceraldehyde-3-phosphate dehydrogenase (gpd) antibody and visualized with a second antibody-HRP conjugate. gpd is a soluble glycolytic enzyme which in Drosophila is present as two isoforms of 35.5 and 37 kD. Both isoforms are detected mostly in the diffusible fraction (lane 2, large arrowhead). This filter was then reacted with an anti-flightin mAb and visualized with a second antibody-alkaline phosphatase conjugate. Flightin is detected only in the cytomatrix/myofibrillar fraction (lane 1). (C) A filter similar to the one shown in B was reacted first with an anti-arginine kinase antibody and visualized with a second antibody–HRP conjugate. This antibody detects a single band of ~40 kD primarily in the cytosolic fraction (lane 2, large arrowhead). When the filter is reacted with an anti-flightin mAb followed by a second antibody-alkaline phosphatase conjugate, the typical flightin doublet is detected in the cytomatrix/myofibrillar fraction (lane 1) but not in the cytosolic or organelle fractions (lanes 2 and 3, respectively). f, flightin. Bar, 3 μm.
None of the known *Drosophila* muscle protein genes map to this chromosomal region (7).

The in situ hybridization experiments show that no flightin-related sequences are found outside the 76D/E region. However, they do not eliminate the possibility that this region contains multiple copies of the gene. To determine the number of copies of the gene in the 76D/E region we have used Southern blot analysis of total *Drosophila* DNA. A blot containing samples of *Drosophila* genomic DNA digested separately with four different restriction enzymes was hybridized to a probe representing the entire flightin cDNA. Each sample of digested DNA showed only the hybridization pattern expected of a single copy of the gene (data not shown). Thus, it is unlikely that the multiple isoforms are encoded by tandemly repeated genes. From these results, we conclude that there is only one copy of the flightin gene per haploid genome. All of the hybridization experiments were done under moderately stringent conditions which allow the detection of genes encoding closely related proteins. Therefore, we further conclude that there are no genes encoding flightin-related proteins.

The conclusion that flightin is encoded by a single gene is supported by results from Northern blot analysis (Fig. 4). A flightin cDNA probe detects a single transcript size class of ~1 kb. This RNA accumulates only during late pupal and adult stages, a pattern consistent with the flight muscle-specific expression of flightin protein (see also Fig. 7).

**Characterization of a cDNA Encoding Flightin**

The sequence of flightin was deduced from a cDNA clone isolated from a pupa cDNA library (14; see Materials and Methods). The longest open reading frame of the cDNA extends from nucleotides 36 (the first ATG) to 581 (Fig. 5). If translated, the putative protein would have a molecular weight of ~20 kD and a pl of 5.2. The calculated pl is very close to the pl determined from isoelectric focusing gels (see Fig. 1 B), but the molecular weight is ~7-10 kD smaller than the molecular weight extrapolated from SDS gels for the in vivo protein. This apparent discrepancy in molecular weight appears to be due to anomalous migration of flightin in gels, rather than the absence of a complete coding region on the cloned DNA.

To determine whether the cloned sequence encodes the entire flightin protein, we have analyzed the in vitro translation product of the cDNA. The cDNA-encoded protein is recognized by anti-flightin mAbs and migrates with the same mobility as the upper component of the in vivo protein doublet (Fig. 5 C). These results demonstrate that this cDNA encodes a full length flightin protein and suggest that the faster migrating isofrom may be produced by a modification that does not occur in the reticulocyte lysate.

The in vitro translation experiments also provide suggestive evidence that the difference between the calculated size of flightin (20 kD) and the size measured by gel migration (30 kD for the slower band) is due to effects of the primary protein structure on the rate of migration, rather than to post-translational modifications. It should be noted that the reticulocyte lysate is capable of some forms of posttranslational modification (54). Modifications are not limited to homologous RNAs; *Drosophila* myosin light chain 2 is acetylated by the rabbit reticulocyte lysate (53). However, it seems unlikely that the lysate would correctly perform modifications yielding the large discrepancy between the calculated and the electrophoretically determined molecular weights of flightin.

The ATG starting at position 36 of the cDNA is the first ATG of the open reading frame and is, therefore, likely to encode the amino-terminal methionine. This methionine codon is found within the sequence TAATATG which contains the two highly conserved A residues at positions 2 and 3 of the consensus translational initiation site for *Drosophila* (C^F/A, AA C/A, ATG) (10). Results from primer extension analysis indicate that the 5' end of this cDNA can serve as a transcription initiation site in vivo (our unpublished results). The 5' end sequence of this cDNA does not match the consensus *Drosophila* cap site sequence ATCA^<T>^<T>/c (17). We note, however, that a sequence (ATCAGT) found seven nucleotides downstream from the 5' end of the cDNA closely matches the *Drosophila* cap site sequence. This sequence is 21 nucleotides upstream from the first ATG.

**The cDNA Encodes a Novel Protein**

A computer search of DNA and protein databases did not reveal any sequence with significant similarity to the flightin cDNA or its putative translated product. The DNA sequence search of four databases (nonredundant PIR + SwissProt + GenPep + GPUpdate) using the experimental NCBI program BLAST (l) did not reveal any entries with significant similarities to the flightin DNA sequence. A search for amino acid sequence homology of the GenEMBL database using the UWGGC sequence analysis program TFASTA (12) also failed to reveal any similarities.

There is a short but possibly significant region of similarity between the NH_2-terminal sequence of flightin and the NH_2-terminal sequence of actin. Fig. 6 shows an alignment of the flightin NH_2-terminal sequence with the sequences of vertebrate and *Drosophila* muscle and cytoplasmic actins. Note that both *Drosophila* actins and flightin have acidic amino acids at positions 3, 4, 5, and 12, equivalent to the acidic residues at positions 1, 2, 3, and 10 or 11 of vertebrate actins. Separating the acidic residues between positions 5
Figure 5. The flightin cDNA encodes a novel protein. (A) DNA sequence of cDNA p1A and deduced amino acid sequence. The amino acid sequence was deduced from the nucleotide sequence using the GCG program TRANSLATE. Nucleotide +1 marks the beginning of the open reading frame. Nucleotides upstream of the protein coding region are given negative numbers, beginning with −1 for the first nucleotide preceding the ATG translation initiation codon. Note that the sequence from −4 to +3 matches the Drosophila consensus translational initiation site (10). The sequence from −22 to −28 (broken lines) closely matches the Drosophila consensus cap site sequence (17). The open reading frame extends to the stop codon beginning at nucleotide 547 resulting in a protein of ≈20 kDa with a pI of 5.2. Four consensus polyadenylation signals (AATAAA, thick underlines) (36) are found in the 3′ noncoding region. Five putative phosphorylation sites are indicated: P1, consensus phosphorylation site for cAMP, cGMP-dependent protein kinase; P2 (a, b, and c), casein kinase II, P3, protein kinase C. (B) Kyte-Doolittle hydrophilicity plot of the deduced protein sequence. The five putative phosphorylation sites are indicated by arrowheads. (C) Immunoblot of products from in vitro translation of RNA derived from flightin cDNA clone pla. The blot was probed with a mixture of anti-flightin mAbs (557, 5010, and 788). The RNA derived from pla directs the synthesis of an immunoreactive protein that has the same mobility as the more slowly migrating variant of in vivo flightin, identified in the adult thorax. (left lane). The DNA sequence data are available from EMBL/GenBank/DDBJ under accession number EMBL Z18858.

and 12 (3 and 10 or 11 in vertebrate actins) is a stretch of mainly hydrophobic amino acids.

Analysis of the flightin sequence with the GCG program MOTIF revealed five putative phosphorylation sites (Fig. 5A). Three of these sites correspond to consensus phosphorylation sites for casein kinase II, while the fourth and fifth are potential phosphorylation sites for protein kinase C and cAMP/cGMP-dependent protein kinase, respectively. Four putative phosphorylation sites are indicated in Fig. 5B. Members of all three protein kinase families have been identified in Drosophila, but in most cases their tissue distribution has not been examined (42).

A Subset of Flightin Variants Is Expressed Only after Eclosion

The developmental expression of flightin follows the development of the adult flight muscle (Fig. 7A). Flightin is first detected at pupal stage P8, ≈60 h after pupariation, and continues to accumulate for the remainder of pupal development. These results are consistent with those of the developmental Northern blot analysis (Fig. 4) and demonstrate that expression of flightin is tightly linked to accumulation of its RNA. Interestingly, only the slower migrating subset of variants is detected throughout pupal development. The faster migrating variants are not detected in pupal flight muscles but are very abundant in adult flight muscles. In fact, this subset of isoforms is not present initially in newly eclosed adults; they are first detected a few hours after eclosion (Fig. 7A, lower panel).

Newly eclosed adults are not capable of flight. One reason that they cannot fly is that the wings are partially folded. The time it takes for the wings to unfold and expand varies from a few minutes to several hours (19). In addition, there is evidence that the contractile apparatus continues to develop during the first few hours after eclosion (49). These con-
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The results presented here demonstrate that flightin is a novel...
myofibrillar protein which is expressed only in the asynchronous flight muscles. Because these muscles are stretch activated, a characteristic that distinguishes them from all other muscles of the fly, it is possible that flightin plays a crucial role in stretch activation. This possibility is supported by the A band localization of flightin, by the appearance of new isoforms when flies become competent to fly, and by some features of the protein sequence.

Flightin Appears To Be Associated with Thick Filaments

Results from several experiments indicate that flightin is associated with the myofibril, specifically with structural elements within the A band. We find that flightin copurifies with IFM myofibrils and that anti-flightin mAbs label the A band in the intact muscle. In addition there is genetic evidence that flightin is found only when thick filaments are present. Both Mogami and Hotta (28) and Chun and Falkenthal (11) have noted that spots 158 and 159 (which we identify as flightin) are absent in the IFM of the myosin heavy chain null mutant, Ifm(2)2. We have not been able to detect any flightin isoforms in the IFM of this mutant (our own unpublished observation). Indirect flight muscles in homozygous Ifm(2)2 flies lack thick filaments, though thin filaments appear to assemble normally. One explanation for the lack of accumulation of flightin isoforms in Ifm(2)2 is that in the absence of myosin heavy chain, since thick filaments fail to form, proteins normally associated with thick filaments cannot be properly integrated in the sarcome and are degraded. It should be noted that proteins 158 and 159 are present in the IFM of the actin mutant Ifm(3)7, which lacks thin filaments, but has apparently normal thick filaments (21, 28). Taken together, these studies suggest that flightin isoforms are thick filament-associated components.

Thick filaments contain other protein components in addition to myosin. In vertebrate skeletal muscle, a number of these proteins have been identified (26, 32, 33, 46). In contrast to vertebrate muscle, the only myosin-associated proteins that have been characterized in Drosophila are projectin (38), paramyosin (6, 61), and flightin (this study). However, since at least 10 different proteins fail to accumulate in the thoracic muscles of Ifm(2)2 homozygous it is likely that additional, nonmyosin proteins are found in the thick filaments of Drosophila (11, 28).

A number of questions remain about the association of flightin with the thick filaments. Although unfixed myofibrils and most preparations of fixed fibers show A band staining with all three anti-flightin mAbs, in some preparations we also see some fibers with immunostaining predominantly at or near the Z band and M line. We do not know whether these results have any meaning with respect to the physiology of the muscle or whether they represent artifacts of our experimental procedures.

Flightin Is a Novel Protein

Flightin is the first flight muscle-specific myofibrillar protein that shows no extensive regions of homology to proteins from other muscle types. However, the sequence of the protein has several interesting features that may shed some light on the function of flightin. One of the most striking features of the flightin sequence is the nonrandom distribution of charged amino acids. Overall, flightin has a high frequency of charged residues. The acidic (D + E) and basic (K + R) amino acids account for 28% of the residues. Most acidic amino acids are found towards the NH2-terminal region of flightin, while most basic residues are found towards the C-terminal region of the protein. Nine of the first 17 residues are acidic and 12 of the 13 glutamic acids are found within the first 71 residues of the protein. On the other hand, none of the 11 arginine residues are found in this region.

The estimated pI for the 65 NH2-terminal amino acids is 3.78 while the estimated pI for the 112 COOH-terminal amino acids is 10.55. Separating these two regions is a stretch of five prolines that are found embedded in a region abundant with charged amino acids. This proline-rich region could function as a hinge that separates the oppositely charged amino-terminal and carboxyl-terminal domains. The concentration of charged residues in two separate regions of the molecule may be significant in determining protein-protein interaction. Highly charged domains confer proteins with binding properties through electrostatic interactions. For example, alternating zones of positive and negative charges are found in the myosin rod where they play a role in the staggered packing of myosin molecules in the thick filament (3). Thus, in considering the functional role of flightin in muscle, it is important to understand the structural relationship between the NH2- and COOH-terminal parts of the protein.

One particularly interesting feature of the flightin sequence is in the amino terminus which shows marked similarity to the NH2-terminal region of actin (Fig. 6). This similarity may have implications for flightin function because the NH2-terminal region of actin is part of the contact site with myosin (20). This region of the actin molecule is the least conserved region phylogenetically. However, the presence of 2–4 acidic residues within this region (particularly at positions 1 through 3 and 10 or 11) is extremely conserved (59). Cross-linking experiments with vertebrate proteins have shown that the acidic amino acids in actin's segment 1–11 are in close contact to myosin S1 during rigor (47). Results from site-directed mutagenesis experiments in which these acidic residues have been replaced have shown that they are essential for myosin S1 binding (2) and actin-activated myosin-ATPase activity (48). It should be noted that Solomon et al. (43) also carried out site-directed mutagenesis of actin's acidic residues 3 and 11 and found that substitutions at these sites did not affect binding of actin to myosin S1. However, this latter study did not examine the effect of the substitutions on myosin-ATPase activity.

Flightin: Multiple Isoforms within a Single Muscle Type

A notable feature of flightin is the large number of isoforms of the protein that are present within a single muscle type. Our results indicate that there is a single flightin gene which produces a single transcript and that the flightin isoforms are likely to be differentiated by posttranslational modifications.

The large number of isoforms within an apparently homogeneous muscle raise the questions about how the different forms of flightin reflect its function. Our experiments provide suggestive evidence that one subset of isoforms may have a regulatory role. That subset makes up the faster
migrating band seen in gel electrophoresis. Our studies show that this subset is invariably present in flies that have developed the capability of flight, although it is not produced as a result of this flight. Moreover, our results strongly suggest that phosphorylation of flightin is part of the activation mechanism of flight muscle contraction.

The serine at position 141 is a particularly good candidate for phosphorylation by casein kinase II. This residue is found within a cluster of acidic amino acids and, more importantly, the critical acidic residue three amino acids from the Ser is found in this sequence (9). Casein kinase II has been shown to phosphorylate smooth muscle myosin heavy chain in vivo (23). This enzyme has been purified from Drosophila (15) but it remains to be determined if this protein is expressed in the flight muscles.

Phosphorylation has been shown to have several roles in regulation of muscle contraction (25, 41, 55). In Drosophila, phosphorylation of myosin light chain 2 (MLC2) has been shown to regulate myosin ATPase activity (49, 50). Phosphorylated MLC2 are not detected in pupa but accumulate rapidly shortly after eclosion (50). In addition, mfd mutant flies that cannot phosphorylate MLC2 are flightless (50). Our results demonstrating the appearance of distinct flightin variants shortly after eclosion may be an indication that, like MLC2, modification of flightin is directly involved in activation of flight muscle function.

Our studies have not explained all of the differences that distinguish the flightin isoforms. Another possible modification is acetylation, which is common among muscle proteins. In Drosophila, all actins undergo posttranslational modification by acetylation-dependent removal of the first two NH2-terminal amino acids followed by acetylation of the aspartic acid at position 3, the new NH2-terminal amino acid (37). Acetylation has been shown to account for the shift in pI seen among the different Drosophila actin isoforms (66). The shift in pI seen among the three most basic flightin isoforms (spots 158, 159, and 160) is very similar to the shift in pI seen among the three actin isoforms. Like actin, flightin has an aspartic acid residue at the third position. Thus it is possible that flightin is modified by a mechanism similar to the one that has been demonstrated for actin, although this would only account for a subset of the flightin isoforms.

Is Flightin Involved in the Activation or Regulation of Contraction?

Flightin is the first protein that is found only in stretch-activated muscle and shows no relation to any protein in other types of muscle. Thus, it is tempting to speculate that flightin is involved in the mechanism of stretch activation. One possible scenario is that flightin blocks actin-myosin interaction, either sterically or allosterically. This possibility is suggested by the observed similarity between the amino-terminal region of actin and flightin, particularly in the position of acidic residues. The similarity in the NH2-terminal sequence between flightin and actin suggests that flightin, like actin, may bind to S1 and regulate its interaction with actin. According to this model, the flightin-S1 interaction would be strong in the unstretched muscle providing the mechanical basis for the low active tension that is observed in Ca2+-activated muscles at rest length. This interaction would be relieved by stretch and would allow the binding of myosin to actin and the development of force. One attractive feature of this hypothesis is that it can be incorporated in models proposed to explain the mechanical dynamics of insect flight muscle, namely, the effect of stretch upon rate constants of the cross-bridge cycle (see Thorson and White [52] for a full discussion of this topic).

A second possibility is that flightin may help to maintain the proper lateral alignment and/or orientation between myofilaments. One peculiarity of insect IFM that makes this proposal attractive is that actin filaments and myosin filaments have matching helix periodicities. A high degree of geometrical regularity is likely to be of primary importance for oscillatory contractions.

While the geometrical arrangement of myofilaments is determined to a great extent by complex structures such as Z bands, M lines, and connecting filaments, interactions along the length of the actin and myosin filaments are likely to serve a structural role as well. Flightin could interact with a component of the thin filaments, thus forming a link between thick and thin filaments that may serve to oppose longitudinal displacements and to contribute to the high resting stiffness that is characteristic of these muscles. While White (63) has presented evidence that connecting filaments are responsible for resting stiffness, recent studies of a Drosophila mutant with a defective flight muscle actin have shown that IFM fibers from this strain exhibit considerable resting stiffness despite the absence of Z bands, the presumed anchoring site of connecting filaments (44). One interpretation of these results is that other components of the myofibril contribute to resting stiffness (44).

A thin filament component that could be a candidate to interact with flightin is troponin H. Bullard et al. (8) have suggested that the hydrophobic COOH-terminal of troponin H may extend towards the thick filament and recent studies have shown that troponin H lies very close to myosin heads bound to actin (see Discussion in Bernstein et al. [7]).

In conclusion, we have demonstrated that flightin does not correspond to any previously identified muscle protein. The muscle specificity and the sarcomeric localization of flightin argue that flightin has a role in the activity of the IFM. The sequence of this protein suggests possible models for the role of flightin. More direct proof could come from a mutational analysis of the flightin gene. Isolation of mutants with defects in the flightin gene could help determine whether this novel flight muscle protein is essential for flight or whether it has a more subtle role in modulating activity.

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