Drosophila Paramyosin/Miniparamyosin Gene Products Show a Large Diversity in Quantity, Localization, and Isoform Pattern: A Possible Role in Muscle Maturation and Function

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Abstract. The *Drosophila* paramyosin/miniparamyosin gene expresses two products of different molecular weight transcriptionally regulated from two different promoters. Distinct muscle types also have different relative amounts of myosin, paramyosin, and miniparamyosin, reflecting differences in the organization of their thick filaments. Immunofluorescence and EM data indicate that miniparamyosin is mainly located in the M line and at both ends of the thick filaments in *Drosophila* indirect flight muscles, while paramyosin is present all along the thick filaments. In the tergal depressor of the trochanter muscle, both proteins are distributed all along the A band. In contrast, in the waterbug, *Lethocerus*, both paramyosin and miniparamyosin are distributed along the length of the indirect flight and leg muscle thick filaments. Two-dimensional and one-dimensional Western blot analyses have revealed that miniparamyosin has several isoforms, focusing over a very wide pH range, all of which are phosphorylated in vivo. The changes in isoform patterns of miniparamyosin and paramyosin indicate a direct or indirect involvement of these proteins in muscle function and flight. This wide spectrum of potential regulatory characteristics underlines the key importance of paramyosin/miniparamyosin and its complex isoform pattern in the organization of the invertebrate thick filament.

A LTHOUGH *Drosophila* flight muscles are structurally and physiologically analogous to vertebrate skeletal muscles (Peckham et al., 1990; Tregear, 1977) and have been a model for the study of striated muscle, some important differences have been found. Unlike other muscles types, calcium activation results in low tension and low ATPase activity. The calcium is necessary, but not sufficient, to initiate flight because full activation requires the muscles to be stretched as well. This phenomenon, known as stretch-activation, is present in all muscles; however, only in indirect flight muscles (IFM)[1] and mammalian heart muscles is its effect large enough to be functionally significant (Pringle, 1978). The IFM is also classified as asynchronous since the frequency of wing beats is not synchronized with the frequency of nerve impulses. The presence of specific protein components and/or specific isoforms in these muscles could explain, at least in part, the distinct mechanics of these muscle types. For example, paramyosin, miniparamyosin, arthrin, troponin H, and flightin are found only in invertebrate muscles (Vinós et al., 1991; Becker et al., 1992; Epstein et al., 1985, 1988; Bullard et al., 1988; Ball et al., 1987; Vigoreaux et al., 1993). A common property of the above proteins is that they are members of isoform families. In many cases, the isoform type- or stage-specificity in muscle fibers has been maintained throughout evolution, suggesting specific roles for each isoform (Bernstein et al., 1993; Bandman, 1992). However, the functional significance of expressing specific isoforms in different muscles remains a critical unresolved issue. In *Drosophila*, in contrast with other organisms (Smith et al., 1989), isoforms arise mainly by alternative splicing of one or a few genes. Often this diversity is increased by posttranscriptional modifications (Fyrberg and Beall, 1990; Bernstein et al., 1986; Collier et al., 1990). Recently, the importance of phosphorylation for the regulation of flight muscle activity has been demonstrated in two muscle proteins, the myosin regulatory light chain and flightin (Sparrow, 1995; Tohtong et al., 1995; Vigoreaux and Perry, 1994).

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1. *Abbreviations used in this paper*: DLM, dorsal logitudinal muscle; IFM, indirect flight muscle; mPM, miniparamyosin; PM, paramyosin; TDT, tergal depressor of the trochanter.

The process of assembly of the distinct filaments and the molecular mechanisms involved in the contraction of the different types of fibers in an organism remain to be clarified, and organisms such as *Drosophila*, where genetic investigations are possible, can help in these studies (for example see Fyrberg and Beall, 1990; Epstein and Bernstein, 1992; Ferguson et al., 1994). Myosin is the major component of the thick filament, but in invertebrates paramyosin and a few additional minor proteins have also been identified biochemically among the components (Vinós et al., 1991; Becker et al., 1992; Epstein et al., 1988; Deitiker and Epstein, 1993; Maroto et al., 1995). The assembly of the filament is probably more complex than a simple self-assembly of myosin in vertebrates or myosin and paramyosin in invertebrates (Trinick, 1992). The identification of new components (Becker et al., 1992; Vigoreaux et al., 1993; Ferguson et al., 1994; Deitiker and Epstein, 1993; Maroto et al., 1995) in the thick filament, together with the highly variable length and diameter of the filaments and differences in the amount of the structural proteins (Vinós et al., 1991; Beinbrech et al., 1992; Castellani and Vibert, 1992; Levine et al., 1976) in different muscle types, emphasize the question of how the assembly of the thick filament occurs and what is the real function of each protein in the filament.

The study of the different components of the thick filament will provide insights into these questions (Vinós et al., 1991, 1992; Maroto et al., 1992, 1995). Miniparamyosin (mPM), a distinct paramyosin (PM) isoform of lower molecular weight, previously described as exclusively present in certain types of muscles (Becker et al., 1992), is encoded by the same gene as paramyosin and is widely distributed among invertebrates (Maroto et al., 1995). The gene is organized in 10 exons. Paramyosin and miniparamyosin (107 and 60 kD, respectively) share only the two last exons. The interest in studying the expression of paramyosin and miniparamyosin is increased by the existence of two different promoters regulating the expression of this gene and the fact that while paramyosin is expressed at two distinct stages of development as are most other *Drosophila* muscle proteins, miniparamyosin is present only in the adult musculature. In this article, we show that the relative amounts of paramyosin and miniparamyosin are different in different muscle types. Indirect immunofluorescence and EM analysis suggest interesting differences in the location of miniparamyosin in the sarcomere, which depend on the muscle type and organism. At least six miniparamyosin isoforms are present in adult flies, all phosphorylated. The pattern of PM and mPM isoforms changes with the maturation of the adult fly musculature. Our results suggest that mPM has a possible role in the sequential transition of nonfunctional to functional muscle in general, while the PM transition is more specifically related to the onset of function in thoracic flight muscles.

Materials and Methods

Polyacrylamide Gels, Two-dimensional Protein Gel Electrophoresis, and Immunoblot Analysis

Thorax, heads, legs, proboscis, tergal depressor of the trochanter (TDT), and IFM fibers were microdissected from acetone freeze-dried flies (Fujita

et al., 1987). Then they were homogenized in 140 mM NaCl, 0.4% Triton X-100, and 25 mM Tris, pH 7. Soluble and insoluble fractions were obtained by low speed centrifugation. Electrophoresis and immunoblot analyses were done essentially as previously described (Vinós et al., 1991). The separation of solubilized proteins in the first dimension gels was carried out at 1,000 V for 18 h. Ampholytes used in the isoelectrofocusing separation were in the 3–10 pH range (Pharmacia LKB, Piscataway, NJ). The second dimension was carried out in 10% polyacrylamide gels. The PM and mPM antibodies were raised against fusion proteins made with the polypeptides encoded by exon 5 and exon 1B, specific exons of PM and mPM, respectively (Maroto et al., 1995).

In Vivo Labeling of Adult Flies

Adult flies (~1 g) were collected and labeled with 1 mCi of [^{32}P]orthophosphate (Vinós et al., 1991). After 24 h, flies were collected, and thorax and heads were microdissected (Vinós et al., 1991). Samples were directly homogenized in isoelectrofocusing buffer or Laemmli buffer and separated by two-dimensional gel electrophoresis (Vinós et al., 1991).

Immunocytochemistry

Adult flies were selected soon after eclosion. Abdomens were microdissected, fixed, and stained as described (Currie and Bate, 1991). Fixation was limited to 3 min in 4% paraformaldehyde in PBS. In some cases, to allow the visualization of the internal organs, they were only partially removed or not removed at all. Dissected thoraces were embedded in OCT compound (Tissue Teck; Miles Laboratories, Inc., Elkhart, IN); semithin sections (60 μm) were obtained and processed as described (Currie and Bate, 1991).

Immunofluorescence Microscopy

Fibrillar and tubular fibers (nonfibrillar muscles) were prepared from adult flies as described (Vinós et al., 1991; Saide et al., 1989). Myofibrils, fixed to the slides, were stained by indirect immunofluorescence following standard procedures (Patel et al., 1987). Bodipy-phalloidin (Molecular Probes, Eugene, OR) and rhodamine-labeled secondary antibodies (Nordic Immunological Laboratories, Tilberg, The Netherlands) were used at appropiate dilutions. mAb to *Lethocerus* α-actinin was MAC 276 (Lakey et al., 1990).

Electron Microscopy

Strips were dissected from the dorsal longitudinal muscle (DLM) of *Lethocerus indicus*, which had been stored at −80°C in relaxing solution with 75% glycerol for one month (Lakey et al., 1990). Thoracic leg muscles (tergocoxal) of a freshly killed *Lethocerus* were exposed by bisecting the thorax with a vertical cut at the midline; the dorsal longitudinal muscle was removed, and the leg muscle was fixed in situ with 4% paraformaldehyde in rigor solution. The muscle was then removed from the thorax and embedded. Thoraces were dissected from *Drosophila* and cut in half longitudinally. The DLM, an indirect flight muscle, was fixed in situ and then cut from the cuticle before embedding. The TDT muscle was exposed by removing the DLM under fixative. After fixation, the muscle was cut from the cuticle and embedded. Fibers were processed for sectioning and labeling with antibody as before (Lakey et al., 1990), with some modifications. *Lethocerus* leg muscle was infused with 1.7 M sucrose and 15% polyvinylpyrolidone for 2 h, and the sections were picked up with 1.2 M sucrose and 1% methyl cellulose (Tokuyasu, 1989) to prevent disordering of the filaments, which frequently occurs in this muscle. A pig anti–rabbit second antibody was used (Nordic Immunological Laboratories) followed by 10 nm protein A–gold in 1% BSA and 1% fish skin gelatin. After labeling, sections were fixed in 2.5% glutaraldehyde, rinsed in water, and stained. Grids were examined in an electron microscope (400 T; Philips Electronic Instruments, Inc., Mahwah, NJ).

Results

Different Relative Amounts of Miniparamyosin Are Present in Drosophila Adult Muscles

Drosophila develops two distinct sets of muscles during its life cycle, embryonic/larval and adult musculature. Lar-

val muscles, including the body wall musculature, the gut, and the dorsal heart muscles, are supercontractile nonfibrillar muscles. In the adult, the hypodermic musculature and the TDT are tubular and contract synchronously. TDT is involved in jumping, a preparation step to initiate flying response. In the thorax, however, the majority of the musculature is made up of the large fibrillar IFM that are responsible for flight. As in the larva, the adult gut and all the visceral muscles including the dorsal heart vessel are tubular supercontracting muscles (Bernstein et al., 1993).

Earlier evidence using Northern and Western blot analysis with specific probes and antibodies showed that miniparamyosin was mainly present in the adult fly (Becker et al., 1992; Maroto et al., 1995). In 3d instar larvae, a transient peak of expression was detected that strongly decreased during pupation. To better characterize miniparamyosin function, its distribution in different muscle types has been studied. Immunostaining of dissected flies with an antibody specific to the peptide encoded by the miniparamyosin specific exon 1B indicated that all muscles, somatic and visceral, in the adult fly were stained. In Fig. 1, some examples of this immunolocalization are shown. A similar general expression of miniparamyosin in the 3rd instar larval musculature was also detected (data not shown). Quantitative immunocytochemistry, although feasible, is difficult and time consuming and can be performed only after the accessibility of the proteins in the sarcomeres to the antibodies has been guaranteed. Thus, the quantitative relationship between the different components of the *Drosophila* muscle thick filaments has been established by a biochemical approach. Several *Drosophila* extracts from different body parts, as well as from specific muscle types, were analyzed by Western blotting using the specific antibodies raised against paramyosin and miniparamyosin (Fig. 2). Myosin and paramyosin levels can also be estimated from the Coomassie blue–stained gels. Reaction of mPM with antibody was clearly seen in thorax and legs (Fig. 2). Similar results were obtained in abdomen extracts (not shown). In head samples, mPM was also detected, but in relatively low amounts. The smaller proportion of muscle tissue in this body part can only partially explain this result. The lower amount of mPM expressed in head muscles is confirmed with dissected proboscis (not shown). This muscle shows little reaction with anti-mPM (almost undetectable). This is in contrast to the results of Becker et al. (1992), using in situ hybridization with specific RNA probes where high levels of transcripts could be seen in the proboscis muscle. The reason for the discrepancy is unknown. Although IFMs are the main muscle type in *Drosophila* thorax, tubular muscles, such as TDTs (a nonfibrillar synchronous muscle), are also present in this body part. Miniparamyosin transcripts were previously described as particularly abundant in TDT muscles, but they were not detected in IFMs or the temporary abdominal hypodermal muscles (Becker et al., 1992). The miniparamyosin-specific antibody readily detects the presence of the protein in IFM and TDT muscles (Fig. 2), but the quantity in TDTs greatly exceeds that in IFMs. In fact, TDT is the only sample in which miniparamyosin can be readily identified in Coomassie gels.

A different, larger insect, the giant waterbug *Lethocerus*, endowed with huge flight muscles, has been used to study the structure and physiology of the IFMs. The antibodies developed against the *Drosophila* proteins reacted with paramyosin and miniparamyosin in *Lethocerus* muscles (Fig. 3). The *Lethocerus* thoracic leg muscle, which, like *Drosophila* TDT, is nonfibrillar, and the IFMs do not show the differing ratios of miniparamyosin/paramyosin seen in *Drosophila*. The anti-miniparamyosin antibody also reacted with a protein of ~34 kD in the *Lethocerus* leg muscle preparation. This is probably equivalent to a 30-kD protein detected in *Drosophila* thoraces (not shown). In addition, in preparations of *Drosophila* thoraces and IFMs, the miniparamyosin band is a doublet. Additional properties of the second, lower-mobility isoform of miniparamyosin, specific to IFMs, will be presented below.

It has been proposed (Holtzer, 1986) that the coiled-coil α-helix proteins are dimers stabilized by disulfide bridges. To test if miniparamyosin, like paramyosin (Vinós et al., 1991), may be a homodimer joined by a disulfide bond in vivo, thorax extracts prepared in Laemmli buffer in the presence or absence of β-mercaptoethanol were run in denaturing polyacrylamide gels, and the protein was visualized by immunoblotting (data not shown). In the absence of reducing agents, miniparamyosin ran with a lower mobility, which corresponds to that of a protein of twice its apparent molecular weight, suggesting that miniparamyosin, like paramyosin, may be a dimer cross-linked by a disulphide bond in vivo.

Drosophila Miniparamyosin in IFMs Is Localized Mainly in the M Line and at Both Ends of the Thick Filament

Double indirect immunofluorescence of isolated adult muscles was carried out to localize miniparamyosin and paramyosin in the sarcomere. Polyclonal antibodies, made previously against whole purified paramyosin, recognized both PM and mPM in adult fibers. The specific antibodies raised in our laboratory (Maroto et al., 1995) allowed us to distinguish the specific location of both PM and mPM in the sarcomere.

The immunofluorescence of nonfibrillar muscles with anti-mPM (Fig. 4, *e* and *g*) was intense, and a strong diffuse fluorescence was regularly seen. In fibrillar fibers (Fig. 4, *a* and *b*), the fluorescence was consistently weaker than in nonfibrillar fibers and was mainly in the M band region and, at lower intensity, in the region of the Z disc. Stretched fibrillar fibers labeled with anti-mPM showed signal located at both sides of the Z disk, possibly at the end of the thick filament (Fig. 4, *c* and *d*). A similar staining pattern was seen with anti-PM in fibrillar and nonfibrillar fibers. Furthermore, double immunofluorescence with anti–α-actinin antibody (Fig. 4, *b* and *j*) and bodipy phalloidin (Fig. 4, *d*, *f*, and *h*), which stains actin, clearly differs from the immunofluorescence of miniparamyosin and paramyosin antibodies. These results suggest that PM and mPM are not uniformily distributed in the thick filament but are more abundant at the ends of the thick filaments close to the Z discs and at the M band region in the center of the thick filament.

The fluorescent labeling pattern may be due to nonuniform distribution of mPM and PM in the A band or to lack of penetration of the antibody into the sarcomere. Frozen
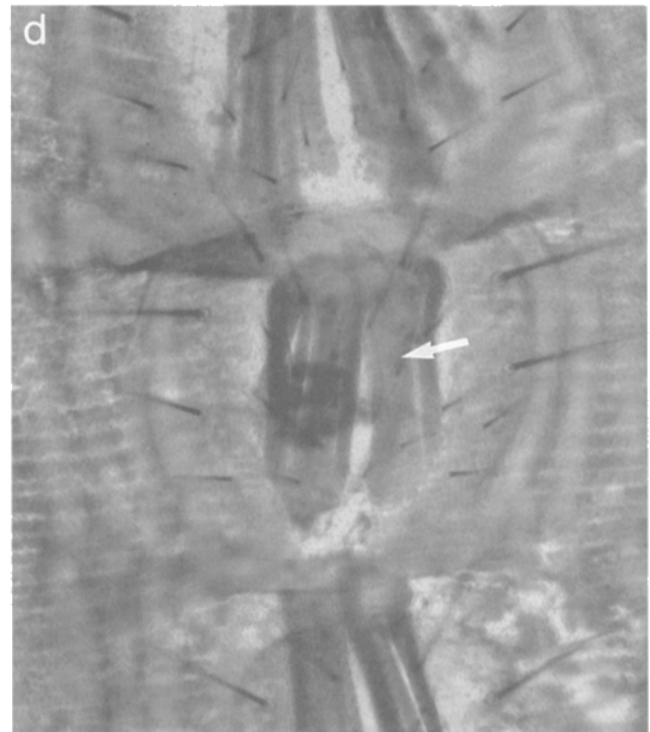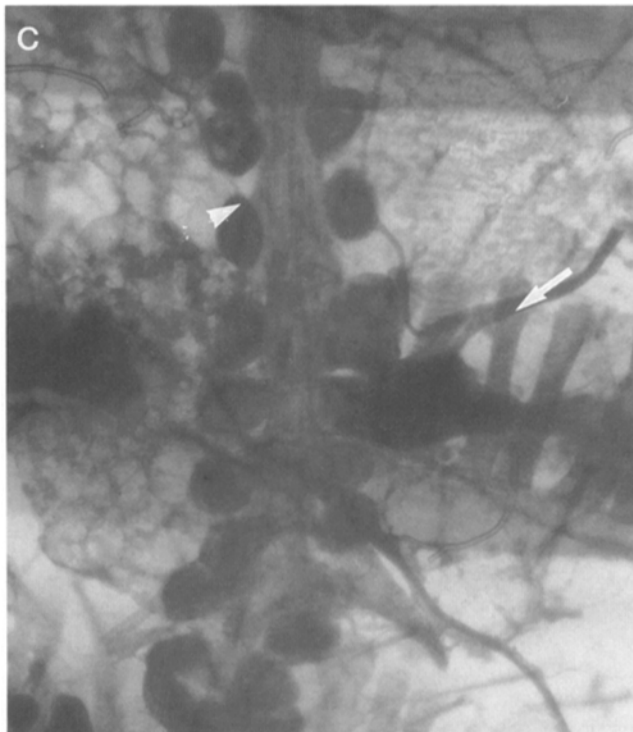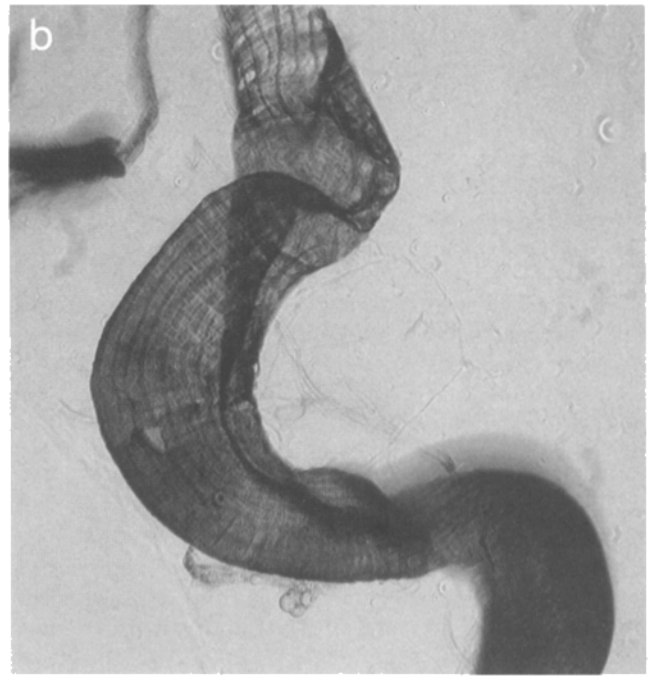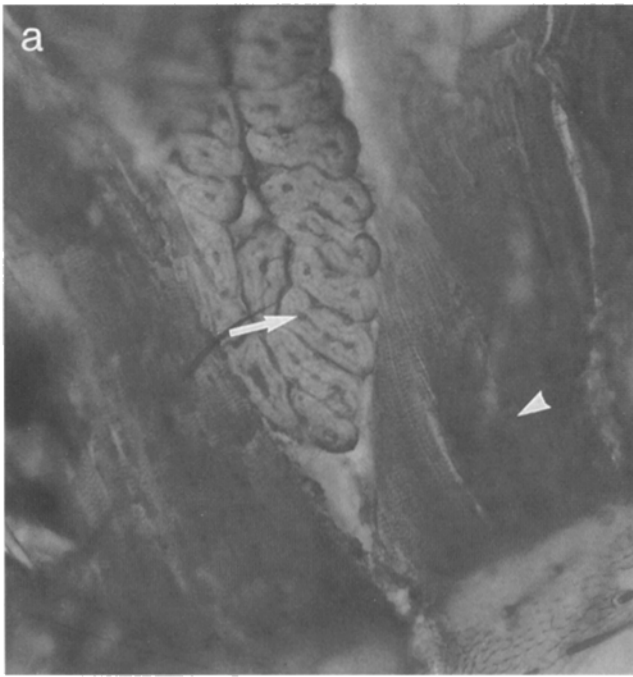
Figure 1. Miniparamyosin is present in all *Drosophila* adult muscles. Sections of flies were labeled with anti-mPM. (*a*) Semithin section of thorax. TDT muscle (*arrow*) and IFM (*arrowhead*). (*b*) A stained visceral muscle where it is possible to visualize the longitudinal and transverse fibers. (*c*) The heart dorsal vessel present in the dorsal part of the abdomen with the pericardial cells on both sides. Pericardial cells (*arrow*) and the dorsal hypodermic muscles of the abdomen (*arrowhead*). (*d*) A view of the stained ventral hypodermic muscles of the abdomen. Controls without first, second, or both antibodies gave no staining.

sections were labeled with antibodies and examined by EM to find the true distribution of miniparamyosin and paramyosin. The sectioning procedure exposes sites in the core of the thick filament that might not be accessible to antibody in intact fibers. Fig. 5 shows micrographs of IFM and TDT of *Drosophila*. In the IFM, miniparamyosin is concentrated in the M line and close to the ends of the thick filaments, whereas paramyosin is uniformly distributed along the thick filaments. In TDT muscles, both proteins are uniformly distributed along the A band. To see if
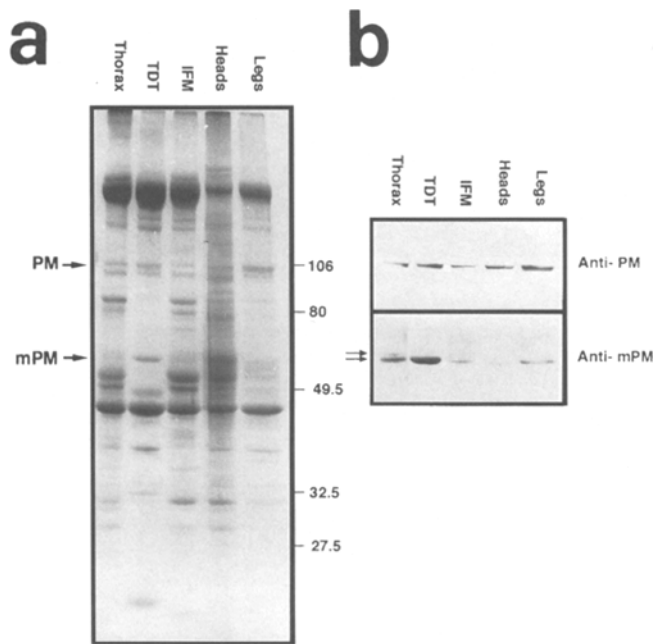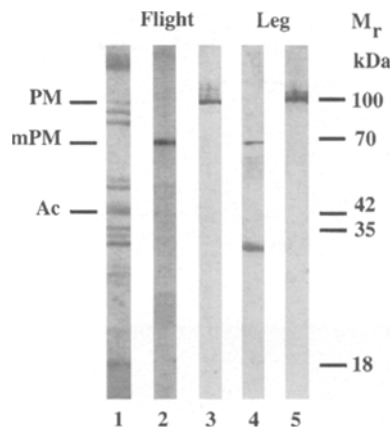
Figure 3. Miniparamyosin and paramyosin in *Lethocerus* muscles. Immunoblots of *Lethocerus* myofibrils were incubated with antibodies against *Drosophila* paramyosin and miniparamyosin. Lanes were loaded with similar amounts of protein. (Lanes *1–3*) Flight myofibrils; (lanes *4* and *5*) thoracic leg myofibrils. (Lane *1*) Ponceau-stained nitrocellulose blot; lanes *2* and *4* were incubated with anti-miniparamyosin, and lanes *3* and *5* with anti-paramyosin. Antibodies to the *Drosophila* proteins reacted with PM and mPM in *Lethocerus*. Anti-miniparamyosin reacted with a protein of ∼34 kD in leg muscle in addition to miniparamyosin. *PM*, paramyosin; *mPM*, miniparamyosin; *Ac*, actin.

Figure 2. The amount of miniparamyosin varies depending on the *Drosophila* muscle type. Several *Drosophila* extracts representing different muscles types as well as body parts of flies were run in SDS 12% polyacrylamide gels (see Materials and Methods). (*a*) Coomassie blue–stained gel of the following dissected samples: thorax, TDT, IFM, heads, and legs. Lanes were loaded with similar amounts of protein (∼20 µg) except in the case of the head sample (∼25 µg). Arrows on the left indicate the position of the paramyosin and miniparamyosin. (*b*) The corresponding Western blots with anti-paramyosin– and anti-miniparamyosin–specific antisera. Arrows indicate the distinct mobility mPM isoforms.

the localization of miniparamyosin in IFMs was the same in *Lethocerus*, sections of the waterbug muscle were labeled with antibodies (Fig. 6). Curiously, in this case, both PM and mPM have the same distribution in the two muscle types, thoracic leg muscle and IFM. Antibody labeling shows that both proteins are all along the thick filaments. This agrees with the distribution of paramyosin in *Lethocerus* IFM seen previously in sectioned fibers (Ferguson et al., 1994; Bullard et al., 1977).

Multiple Phosphorylated mPM Isoforms: The Transition Toward More Basic Isoforms Is Related to Muscle Function

Mogami et al. (1982) numbered 186 spots by two-dimensional gel analysis of proteins in *Drosophila* thorax and myofibrilar IFM. Only ∼25-30 of these spots have been identified (Bernstein et al., 1993). We tried to identify the miniparamyosin in Mogami's two-dimensional gels analysis of thorax and head proteins. In Fig. 7, the Coomassie blue–stained gels and the corresponding Western blots are shown. At least six miniparamyosin isoforms are present in both head and thorax preparations with approximately the same mobility in the second dimension under denaturing conditions (∼60 kD). The unexpectedly wide range of pH (from pH 6 to 8) in which these isoforms focused in the first dimension is interesting and was confirmed several

times. In the immunoblots presented in Fig. 7, the similar intensity of the spots in head and thorax samples was due to differences in the times of antibody staining development. Comparison with Mogami's gels indicates that spots 65 and 67 could correspond to miniparamyosin. Additional miniparamyosin isoforms could correspond to unnumbered spots in Mogami's gels. The predicted pI of the miniparamyosin as deduced from its sequence is 7.83 (Becker et al., 1992). Our data indicate that the most basic isoform focuses around pH 8, but as mentioned above, at least five more acidic isoforms were visualized in Western blots (the most acidic spot was ∼pH 6).

To find out if phosphorylation could generate some of these isoforms, in vivo ^{32}P-labeling experiments were performed. Fig. 8 shows an autoradiograph of the Western blot membrane from dissected thorax preparations, showing that all isoforms are phosphorylated (*arrows*). The phosphorylation seems to be higher in three of the isoforms and did not exactly correlate with the amount of protein. A test with an anti-phosphotyrosine antibody was negative, suggesting that serine or threonine were possibly the labeled amino acids, in agreement with the fact that most of the phosphorylation sites revealed by sequence analysis with MacPattern and Prosite (Fuchs, 1991; Bairoch, 1990) are at serine or threonine.

In an attempt to identify the possible roles of these isoforms, thoraces and heads were microdissected from different stages of development (late pupae, nonflight, and flight imagoes) and analyzed by bidimensional gel electrophoresis. Although some relative quantitative differences are visible, a pattern resembling miniparamyosin isoforms was present in both head and thorax samples, as already described in the adult stage, when the same stage of development was compared (Fig. 9, *upper and lower left panels*). When mPM isoforms were analyzed during thorax or head
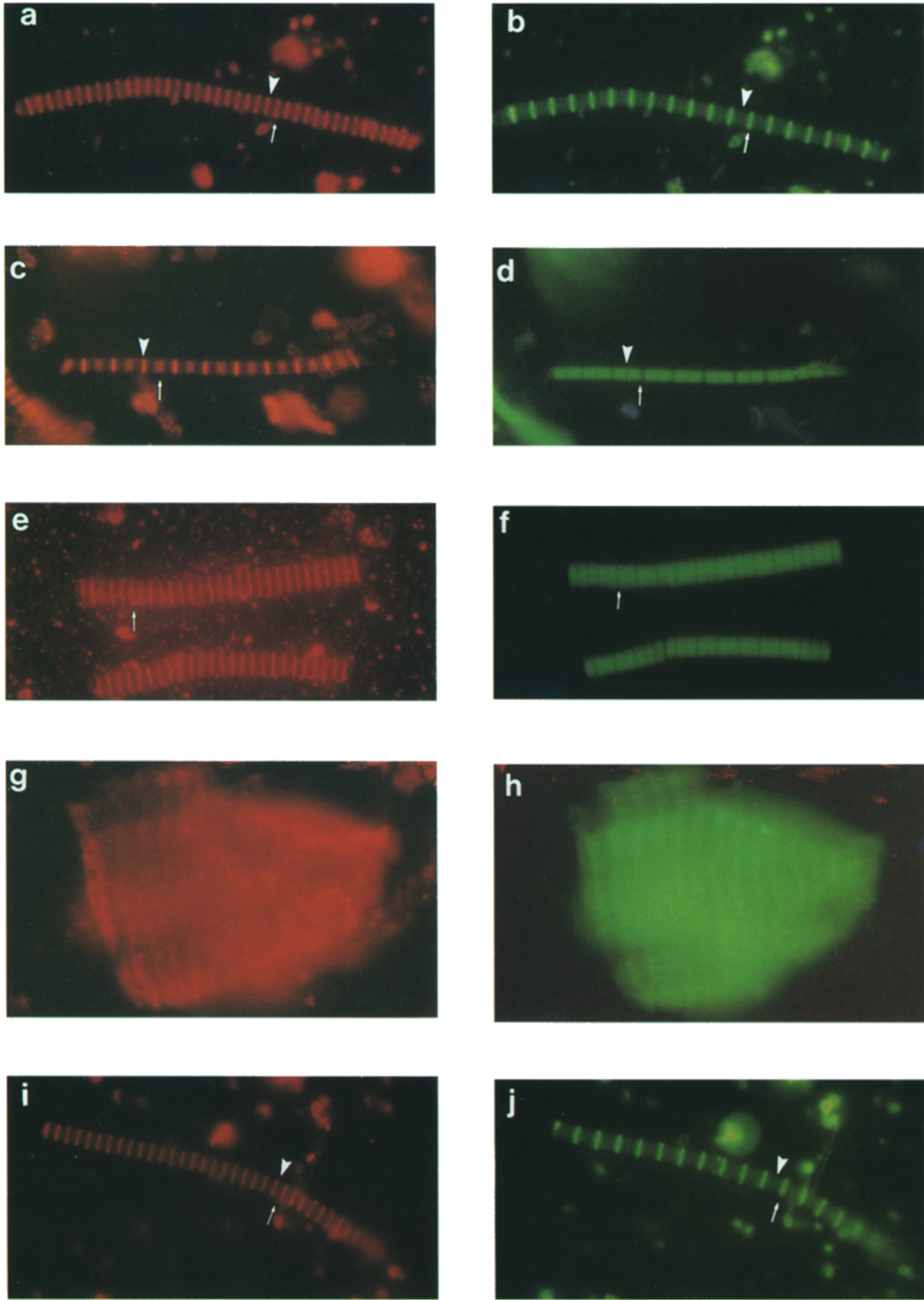
Figure 4. Double immunofluorescence of fibrillar (*a–d, i,* and *j*) and nonfibrillar (*e–h*) *Drosophila* myofibrils. (*a, c, e,* and *g*) Micrographs of myofibrils reacted with the specific anti-miniparamyosin antibody (rhodamine-labeled second antibodies were used). *b* corresponds to the myofibril on the left (*a*) stained with the monoclonal *Lethocerus* anti–a-actinin. *d, f,* and *h* correspond to the same myofibrils seen on the left stained with bodipy phalloidin. Myofibrils in *a* and *b* are more contracted than those in *c* and *d. i* is a micrograph of a myofibril reacted with the specific anti-paramyosin antibody; *j* corresponds to the fiber on the left (*i*) stained with the monoclonal *Lethocerus* anti–α-actinin. (*Arrowheads and arrows*) Position of the M and Z bands, respectively, in the sarcomere. Bar, 25 μm.
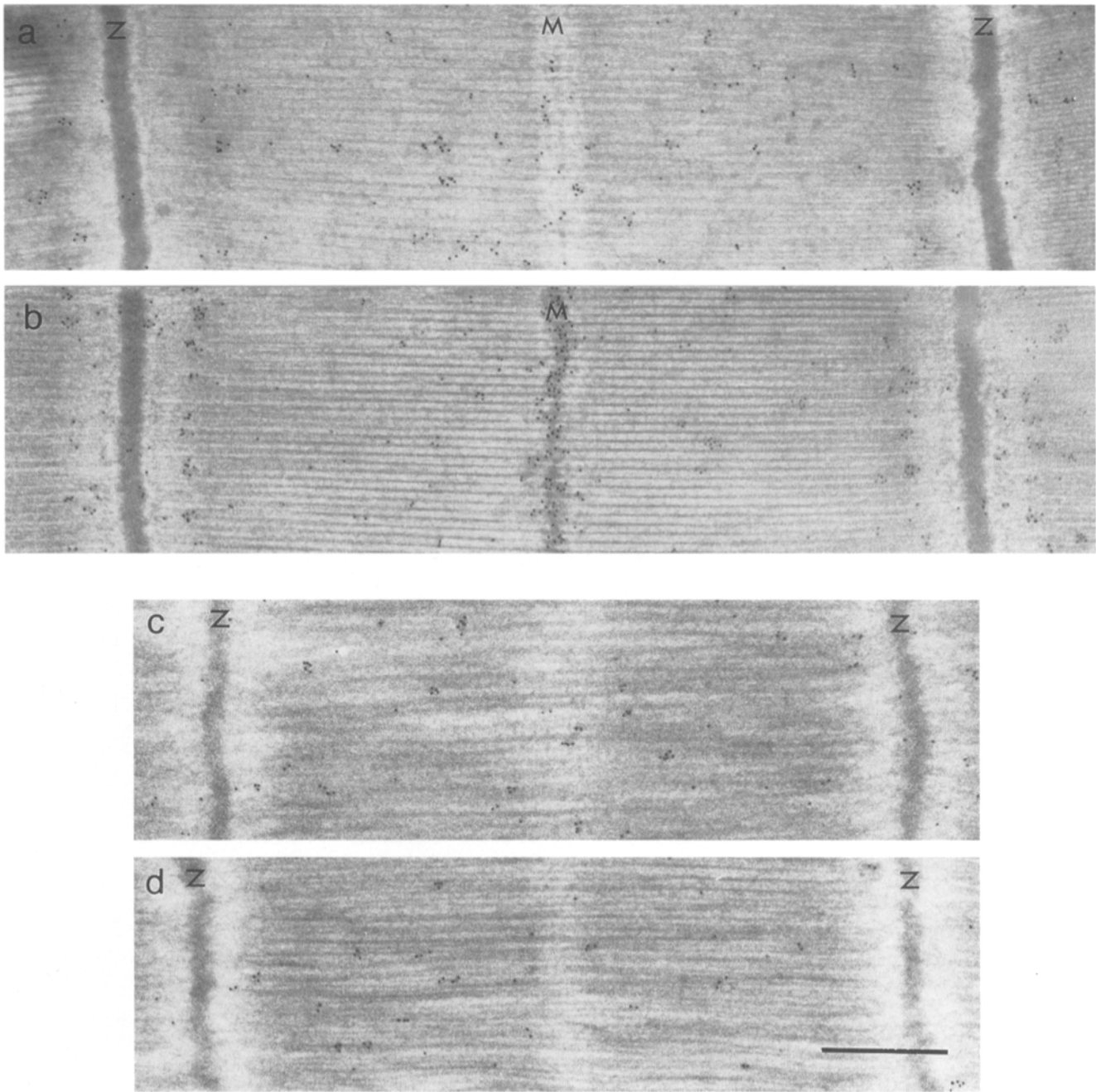
Figure 5. Electron microscope distribution of miniparamyosin and paramyosin in *Drosophila* muscles. Cryosections of fibers were labeled with antibody. (*a*) DLM labeled with antiparamyosin; (*b*) DLM labeled with anti-miniparamyosin. Paramyosin and miniparamyosin are distributed across the A-band, but miniparamyosin is more concentrated at the M-line and toward the end of the A-band. (*c*) TDT labeled with antiparamyosin; (*d*) TDT labeled with anti-miniparamyosin. Both proteins are evenly distributed across the A-band of the TDT muscle. Bar, 500 nm.

development, the more acidic mPM isoforms were detected in pupae (the same pattern is seen in third instar larvae). The more basic isoforms appeared progressively, both in thorax and head samples, from pupae to flight competent imagoes. PM distribution was also studied in the same or parallel gels, and interestingly, head and thorax PM isoforms patterns were different. Several PM isoforms were thorax specific and may be, directly or indirectly, related to flight and jump muscle functionality (* in Fig. 9, *lower left panels*).

A particular type of isoform of lower mobility, $\sim$62 kD, is detected exclusively in thorax and IFM preparations. In one-dimensional gels, the miniparamyosin antibody recognizes two polypeptides with mobilities of 60 and 62 kD. The 62-kD isoform is a minor isoform soluble in homogenization buffer with 0.4% Triton (see Materials and Methods) (Fig. 10 *a*). To verify that the indirect flight muscle–specific low-mobility isoform was phosphorylated, ^{32}P-dissected whole thorax and its soluble and insoluble fraction were run in a 12% polyacrylamide gel. The autoradiogra-
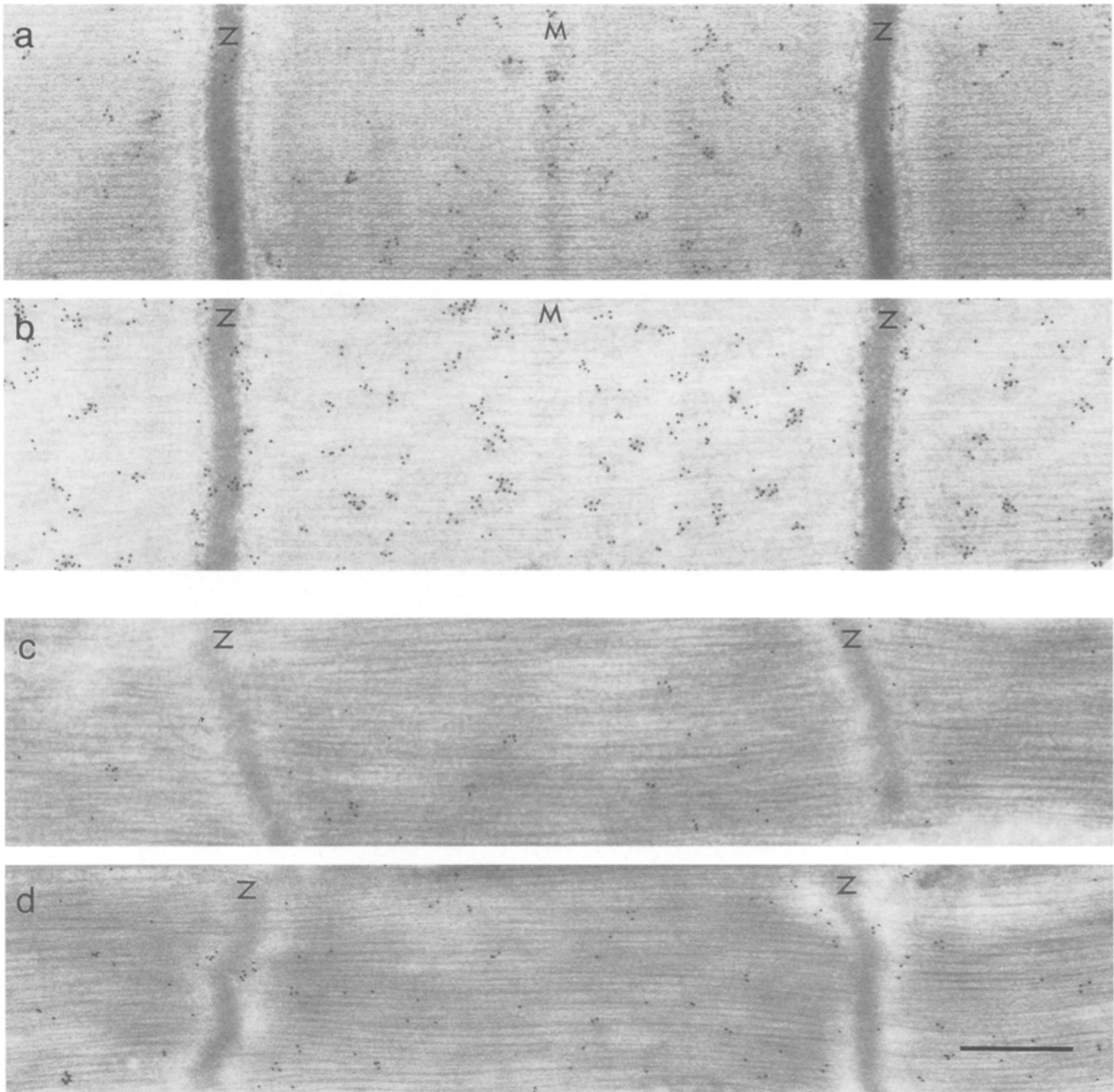
Figure 6. Electron microscope distribution of miniparamyosin and paramyosin in *Lethocerus* muscles. Cryosections of fibers were labeled with antibody. (*a*) DLM labeled with antiparamyosin; (*b*) DLM labeled with anti-miniparamyosin. Both proteins are distributed across the A-band but, unlike *Drosophila* DLM, there is little miniparamyosin in the center of the sarcomere. (*c*) Thoracic leg muscle labeled with antiparamyosin; (*d*) thoracic leg muscle labeled with anti-miniparamyosin. Both proteins are distributed across the A-band. Bar, 500 nm.

phies of the Western blot clearly revealed that a protein with the same mobility as the lower-mobility isoform was also phosphorylated (Fig. 10 *b*). Since in this region several proteins may be present, immunoprecipitation experiments with protein A–Sepharose and mPM antibody confirmed the phosphorylation of the soluble isoform (data not shown). Our attempts to identify this isoform by two-dimensional gels were unsuccessful. The amount of this particular isoform is small, and to detect the signal in the one-dimensional Western blots, we had to increase the developing time. As already shown (Fig. 2), in the dissected

TDT, the only isoform found was the one with higher mobility (60 kD), whereas in the dissected IFM, the two isoforms were present. The higher-mobility isoform (60 kD), insoluble in 0.4% Triton, was also the only one found in leg extracts, other tubular muscle, and head preparations (Fig. 2).

Discussion

Determining the detailed organization and function of the proteins in the invertebrate thick filaments has proven to

Thorax   **Heads**

106 —
80 —

49.5 —

32.5 —

anti-mPM
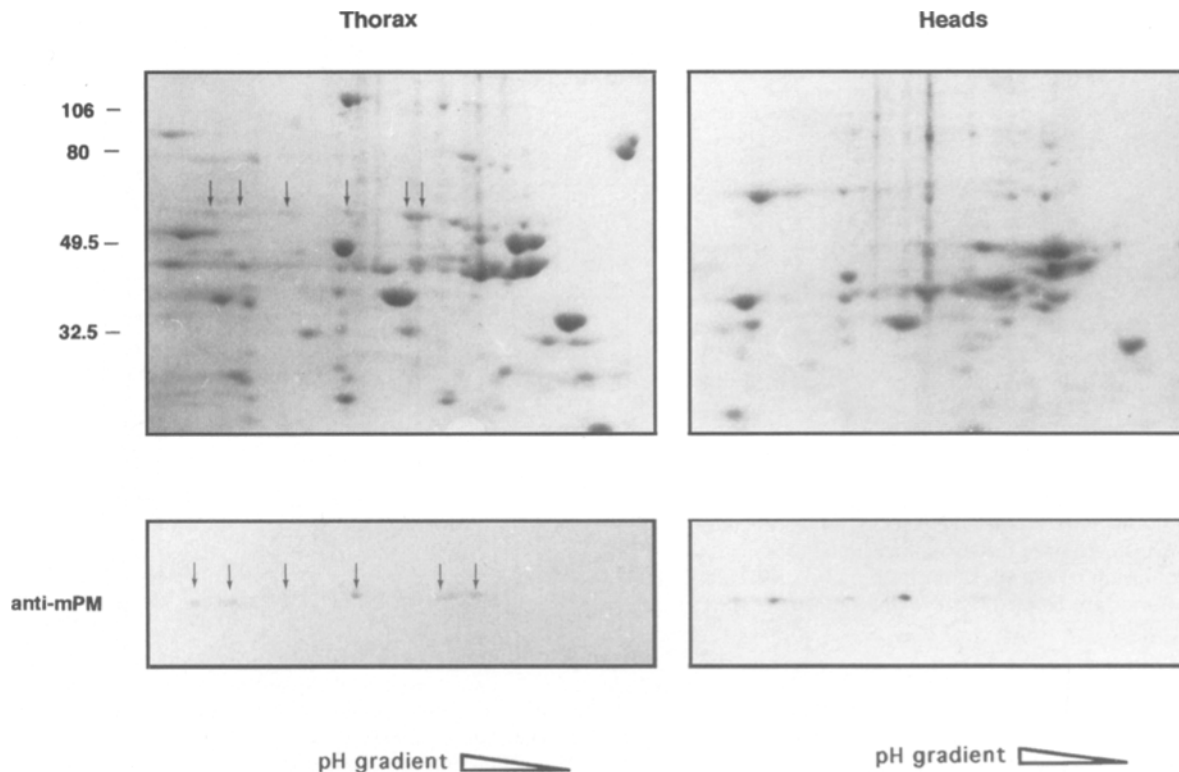
pH gradient ▭◁———   pH gradient ▭◁———

Figure 7. Six isoforms of miniparamyosin can be identified in heads and thorax from *Drosophila* adults. Two-dimensional Coomassie blue–stained gels and the corresponding Western blots are shown. 20 dissected thorax and 50 dissected heads were directly homogenized in isoelectrofocusing buffer and run as described in Materials and Methods. Arrows indicate the localization of the miniparamyosin isoforms in the Coomassie blue–stained gels.

be a complex task. Several models have been proposed (Deitiker and Epstein, 1993; Beinbrech et al., 1992; Castellani and Vibert, 1992; Armitage et al., 1972; Squire, 1992), but they fail to explain fully the complex situation in invertebrate muscles. Even for the same functional type of muscles, as for example, flight muscles, different levels of complexity in the thick filament have been described in different species. The length and diameter show large variations compared with the relative uniformity found in vertebrates (Bernstein et al., 1993; Beinbrech et al., 1992; Levine et al., 1976; Bullard et al., 1973a; Beinbrech et al., 1985). The density of the filament core varies, and filaments may be hollow or solid as revealed in electron microscope images of cross-sections of thick filaments. In some cases, both filament types occur in the same muscle (Bernstein et al., 1993; Beinbrech et al., 1992; Castellani and Vibert, 1992; Levine et al., 1976; Bullard et al., 1973a, b; Beinbrech et al., 1985). The protein composition of the thick filament varies, and not only are different proteins present, but also the relative amount of the main components, myosin and paramyosin, changes (Beinbrech et al., 1992; Levine et al., 1976 ; Beinbrech et al., 1985). The position of proteins may differ depending on the muscle type; for example, projectin is in the I band in IFMs and in the A band in other muscle types (Saide et al., 1989; Lakey et al., 1990), and we show here that mPM is differently distributed in the A band of the same type of muscles (IFM) in *Drosophila* and *Lethocerus*.

In contrast with the conclusions of Becker et al. (1992),

our results indicate that miniparamyosin, like paramyosin, is present in all adult muscle types. In previous work, we have shown that the relative amounts of myosin and paramyosin vary in different types of *Drosophila* muscle (Vinós et al., 1991). When the relative amounts of paramyosin and Miniparamyosin are compared in different *Drosophila* muscles, at least four situations can be identified. (*a*) Miniparamyosin is an abundant protein component, as in TDT, an adult tubular muscle. (*b*) Paramyosin is expressed at higher levels than miniparamyosin, and there is a relatively high ratio of paramyosin to myosin. This is the situation in leg (see Fig. 2) and abdomen muscles. (*c*) There is a low ratio of paramyosin and miniparamyosin to myosin as in asynchronous indirect flight muscles. (*d*) There is no miniparamyosin and relatively large amounts of paramyosin as in embryonic supercontractile muscles. Furthermore, the relatively large number of miniparamyosin isoforms described in this work (nonfibrillar muscles have several 60-kD isoforms and IFMs have an additional 62-kD IFM-specific isoform) completes the picture of thick filament diversity in adult invertebrate muscles. Most, if not all, of these isoforms are phosphorylated, suggesting an additional level of regulation. Phosphorylation has been shown to play several roles in the regulation of *Drosophila* muscle contraction (Vigoreaux et al., 1993; Sparrow, 1995; Tohtong et al., 1995; Takahashi et al., 1990a, b). The analysis of the miniparamyosin sequence, a dimer with a predicted α-coiled-coil structure in the carboxy terminus, shows that there are many potential phosphorylation sites,
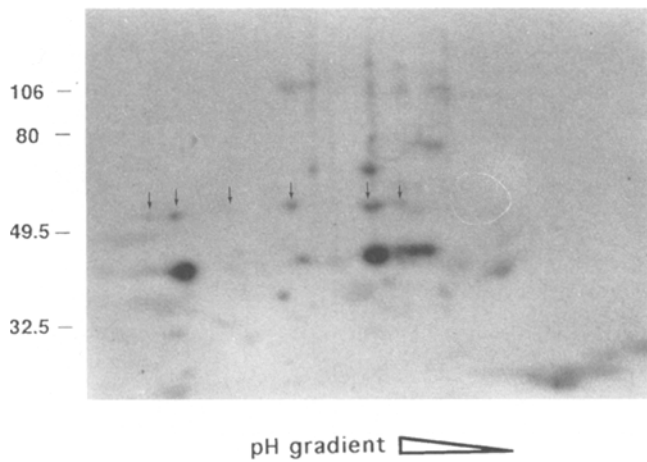
Figure 8. Miniparamyosin isoforms are all phosphorylated. Autoradiography of the in vivo ^{32}P-labeled dissected thorax preparation analyzed by Western blot of the two-dimensional gel reacted with the mPM antiserum. Overexposure of the autoradiographs shows that all isoforms are labeled. Arrows indicate the labeled mPM isoforms.

27 of which are potentially specific for serine/threonine kinases. Several protein kinases have been identified in *Drosophila* (Beinbrech et al., 1985). Probably, phosphorylation would occur at the NH$_2$ terminus, as in the case of the *C. elegans* paramyosin (Schriefer et al., 1989; Dey et al., 1992), even though the molecule has many more potential phosphorylation sites because most of these sites are localized in positions that are probably not accessible to the kinases.

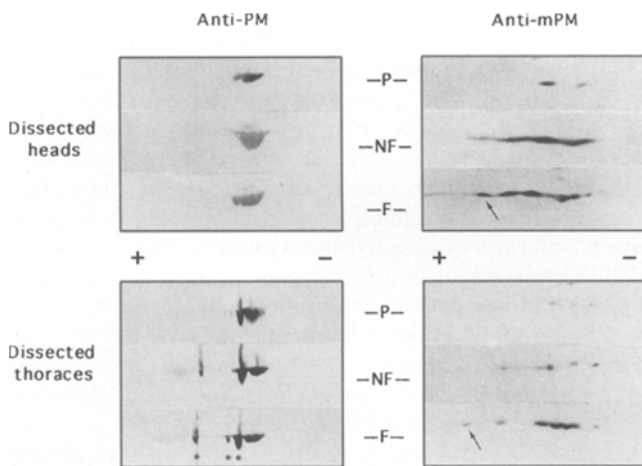The wide range of isoelectric points of miniparamyosin

Figure 9. Variation in the paramyosin and miniparamyosin isoform patterns during maturation of *Drosophila* imagoes. Head and thorax were dissected from late, red-eyes pupae (*P*), from recently hatched nonflying imagoes (*NF*) and young, already flying imagoes of <1 d of age (*F*). The specimens were directly homogenized in isoelectrofocusing buffer and run as described in Materials and Methods. Western blots developed with anti-PM (*left*) and those with anti-mPM (*right*). Arrowheads point to the new basic mPM isoforms, and asterisks (*) mark the new PM isoforms that appear in thorax flight muscles. + and −, the basic and acid Western regions.
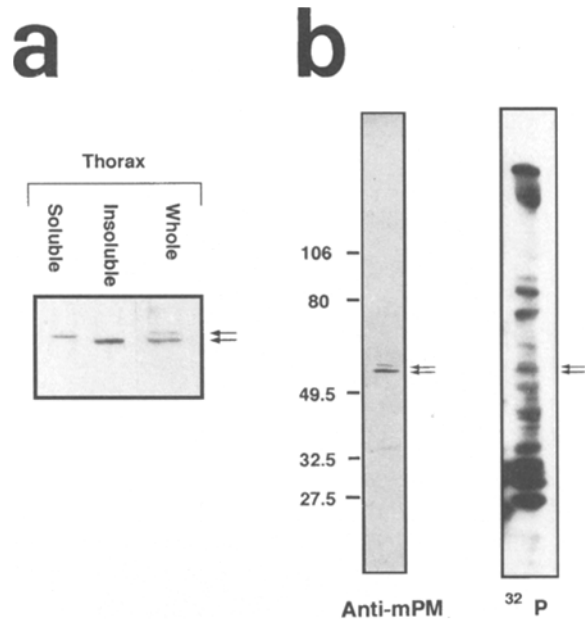

Figure 10. A different mobility isoform is found in the indirect flight muscles, and it is also phosphorylated. (*a*) Western blot with the miniparamyosin-specific antiserum of the following samples: a preparation of *Drosophila* dissected thorax directly homogenized in Laemmli sample buffer (*Whole*) and the fractions (*Soluble* and *Insoluble*) of thorax homogenates (see Materials and Methods). Arrows indicate the position of the two different isoforms of the miniparamyosin. (*b*) Separation in 12% polyacrylamide gels of dissected thorax preparations of in vivo–labeled flies (see Materials and Methods). Western blot reacted with the anti-miniparamyosin antibody (*left*), and autoradiography of the same gel (*right*). Arrows mark the two mPM mobility isoforms.

isoforms is intriguing. Our results suggest the possibility that all phosphovariants originate from a single precursor by sequential phosphorylation. Curiously, we have identified new isoelectric mPM and PM variants that appear sequentially during development of muscle functional capability. Since this process is observed for PM in thorax muscles, but for mPM appears both in head and thorax muscles, we suggest that mPM has a possible role in the sequential transition of nonfunctional to functional muscle in general, while the PM transition is more specifically related to the functional onset of flight-related thoracic musculature. Moreover, since in both cases more basic variants appear, we suggest a possible role of dephosphorylation in the process. The importance of phosphorylation in the acquisition of flight capability has been already demonstrated, but our results suggest that phosphorylation/dephosphorylation is not only an important process for flight but also for muscle function.

The function of the diverse organization of thick filaments is not known, nor is the function of the differing position of mPM in the filaments. Our results analyzing *Drosophila* muscles with the paramyosin- and miniparamyosin-specific antibodies indicate that both proteins are all along the thick filament in TDT muscles in *Drosophila* or tergocoxal (a thoracic leg muscle) in *Lethocerus*. The colocalization of paramyosin and miniparamyosin could indicate a potential interaction between these proteins in the thick fila-

ment. In fact, paramyosin and miniparamyosin share the α-coiled-coil domain containing the same repetitive 28-nucleotide sequences of alternating positive and negative charges that are involved in the interactions between myosin tails and paramyosin in thick filament assembly (Kagawa et al., 1989; McLachlan and Karn, 1983; Cohen and Parry, 1990). Thus, the three thick filament components of invertebrates contain extensive α-coiled-coil domains, which are likely to be involved in thick filament assembly. On the other hand, the head domains are different for each protein. These unshared domains are involved in key functions such as regulation of filament assembly or contraction in the case of myosin (Epstein and Bernstein, 1992; Bernstein et al., 1993). The role of these domains in paramyosin and miniparamyosin remains to be determined. The interaction between myosin and paramyosin in invertebrate muscles could be mediated and/or modulated by miniparamyosin.

The situation in fibrillar flight fibers is very peculiar. In the IFM of *Drosophila*, miniparamyosin antibodies labeled mainly the M band and both ends of the thick filaments, indicating a different localization of this protein in these muscles from that in TDTs. Moreover, when the same analysis was performed on the IFMs of *Lethocerus*, the localization of miniparamyosin was similar to that in TDT muscles. This result can be linked to the model suggested by Crossley (1978), based on earlier work in *Drosophila* by Goode (1972) in which the thick filaments of insect flight fibers are hollow except at the M line and at the end of the filaments. However, this would only apply to *Drosophila* and not to *Lethocerus*. Although IFMs from *Drosophila* and *Lethocerus* are of the same type, the different localization of the miniparamyosin needs an explanation that may be found in functional differences. The IFMs of *Drosophila* and *Lethocerus* have the same filament lattice, but the paramyosin content of the thick filament differs. The myosin/paramyosin ratio in *Drosophila* IFMs is 34, wt/wt (Vinos et al., 1991), and in *Lethocerus* IFMs, it is 8, wt/wt, (Bullard et al., 1973a). Differences in the amount of paramyosin in the thick filament and in the position of miniparamyosin in the two insects may be related to differences in the function of the muscles. *Drosophila* and *Lethocerus* IFMs share many physiological properties such as asynchronous stretch-activation, but the rate constant for the increase in delayed tension is 10 times greater in *Drosophila*, correlating with the fivefold greater wingbeat frequency (Peckham et al., 1990). In *Drosophila*, Vigoreaux et al. (1993) indicated that flightin, which is an IFM-specific protein located in the A band, sometimes appears more concentrated in discrete regions of the A band in each half sarcomere. It is tempting to speculate that flightin in the thick filament of IFM interacts not only with myosin (Kronert et al., 1995) but also with miniparamyosin and paramyosin. In *Lethocerus*, flight muscle zeelin I (35 kD) and zeelin 2 (23 kD) are two additional proteins associated with thick filaments (Ferguson et al., 1994). Zeelin I is close to the core of thick filaments and might interact with paramyosin and miniparamyosin. The diversity of the thick filaments produced by these interactions may require the complex regulation of these proteins at the transcriptional, posttranscriptional, and posttranslational levels described in this work and in previously reported ar-

ticles (Epstein and Bernstein, 1992; Bernstein et al., 1993), and the elucidation of these mechanisms remains one of the main goals of the current work in our laboratories.

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