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Alternative relay and converter domains tune native muscle myosin isoform function in *Drosophila*

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Abstract

Myosin isoforms help define muscle-specific contractile and structural properties. Alternative splicing of myosin heavy chain gene transcripts in *Drosophila melanogaster* yields muscle-specific isoforms and highlights alternative domains that fine tune myosin function. To gain insight into how native myosin is tuned, we expressed three embryonic myosin isoforms in indirect flight muscles lacking endogenous myosin. These isoforms differ in their relay and/or converter domains. We analyzed isoform-specific ATPase activities, *in vitro* actin motility and myofibril structure/stability. We find that dorsal acute body wall muscle myosin (EMB-9c11d) shows a significant increase in MgATPase $V_{\max}$ and actin sliding velocity, as well as abnormal myofibril assembly compared to cardioblast myosin (EMB-11d). These properties differ as a result of alternative exon-9 encoded relay domains that are hypothesized to communicate signals among the ATP binding pocket, actin-binding site and the converter domain. Further, EMB-11d shows significantly reduced levels of basal Ca- and MgATPase as well as MgATPase $V_{\max}$ compared to EMB (expressed in a multitude of body wall muscles). EMB-11d also induces increased actin sliding velocity and stabilizes myofibril structure compared to EMB. These differences arise from exon 11-encoded alternative converter domains that are proposed to reposition the lever arm during the power and recovery strokes. We conclude that relay and converter domains of native myosin isoforms fine-tune ATPase activity, actin motility and muscle ultrastructure. This verifies and extends previous studies with chimeric molecules and indicates that interactions of the relay and converter during the contractile cycle are key to myosin isoform-specific kinetic and mechanical functions.

Keywords

myosin; *Drosophila*; ATPase; motility

Background

While vertebrates have muscle myosin heavy chain gene families, *Drosophila melanogaster* alternatively splices transcripts from its single muscle *Mhc* gene to produce multiple myosin heavy chain (MHC) isoforms that are expressed in a muscle-specific fashion.¹⁻⁵ The *Drosophila* system is advantageous in that it highlights specific alternatively-encoded

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protein domains that are responsible for myosin functional diversity. *Mhc* contains two alternative versions of exon 3, four of exon 7, three of exon 9, five of exon 11, two of exon 15, and alternative C-terminal coding regions in exons 18 and 19.² All of the alternative myosin domains expressed in the fast indirect flight muscle (IFM) isoform (IFI) differ compared to a slower embryonic body wall muscle isoform (EMB).^{3,5} Previously we showed through *P* element-mediated transformation that transgenic expression of EMB in myosin-null IFM affects flight ability, fiber mechanical properties, myofibril stability, ATPase activity and *in vitro* actin sliding velocity compared to IFI.⁶⁻¹⁰

The ATP-binding pocket, actin-binding domain and alpha-helical lever arm are key elements of the myosin motor that are located in the S-1 head. Conformational changes driven by the state of the ATP-binding pocket affect actin binding and lever arm movement during the power stroke.¹¹ These regions are encoded by constitutive exons in *Drosophila*. However, exons 3, 7, 9 and 11 encode alternative portions of the S-1 head that could modulate constitutive domain functions (Fig. 1A).¹² The two alternative versions of exon 3 encode a domain that may communicate with the reactive sulfhydryls near the ATP binding site and the light chain binding region on the lever arm (Fig. 1A). The four alternative regions of exon 7 encode residues near the ATP binding pocket and on the adjacent surface of the molecule (Fig. 1A). The three alternative versions of exon 9 encode the relay domain (Fig. 1A and B), which is hypothesized to communicate among the ATP-binding pocket, the actin-binding site and the converter domain.¹³⁻¹⁸ The five alternative versions of exon 11 encode the central portion of the converter domain (Fig. 1A and C). The converter is hypothesized to change orientation as a result of interacting with the relay domain.¹¹ This swings the adjacent lever arm, which permits production of the power stroke during the strong actin binding state.¹⁹

We previously expressed myosin molecules composed of EMB with a single IFI domain or IFI with a single EMB domain to gain a better understanding of how alternative isoform domains in the myosin S-1 head influence functional properties of myosin. We showed that alternative versions of exon 3 affect actin-activated ATPase activity and actin filament *in vitro* motility.²⁰ We found that alternative versions of exon 7 differentially influence ATPase activity but not actin sliding velocity.²¹ We showed that the EMB relay domain encoded by exon 9b did not affect myosin kinetic properties in the IFI backbone, but that the opposite chimera (EMB-9a) dramatically reduced actin-activated ATPase activity and disabled *in vitro* motility.²² Finally, we observed that alternative versions of the converter domain encoded by exon 11 influenced myosin ATPase activity and *in vitro* motility.^{6,9} Except for the exon 3 studies, these previous findings were based on expressing chimeric myosin isoforms that are not naturally occurring.

A difficulty with the aforementioned studies is that artificial chimeras may produce inaccurate conclusions about domain function since they contain alternative domains that have not evolved to work coordinately. For example, EMB-9a MHC, which is embryonic MHC containing the IFI relay domain, eliminates *in vitro* motility of actin filaments²², even though the EMB and IFI molecules both support this activity. This may be due to incompatibility of the IFI relay domain with the EMB converter. Hence naturally occurring isoforms offer a superior opportunity to directly test the functions of alternative domains of *Drosophila* MHC.

To assess how alternative domains tune myosin function, we engineered genes encoding naturally occurring *Drosophila* embryonic myosin isoforms, inserted them into the genome by *P* element-mediated transformation and expressed them in an IFM myosin-null background. We compared dorsal acute body wall muscle myosin (EMB-9c11d) to cardioblast myosin (EMB-11d) to an embryonic isoform that is expressed in many body wall

muscles (EMB) (note that acute muscles attach dorsal anterior to ventral posterior positions, whereas oblique muscles go from dorsal posterior to ventral anterior²³). We found that the interacting relay and converter domains that differ among these isoforms fine tune myosin ATPase and actin filament motility. This extends previous conclusions about isoform domains to additional alternative exons and provides the first indication that alternative relay domains help define actin filament velocity. In addition, we observed isoform-specific domain effects on myofibril assembly and stability, suggesting the relay and converter play unique roles in dictating myosin filament assembly and/or myosin interaction with other myofibrillar components. Overall, our comparison of naturally occurring MHC isoforms provides novel insights into the role of the converter and relay domains in modulating myosin function.

Results

P element transformation and generation of transgenic lines

The EMB-11d and EMB-9c11d transgenes contain the 5' and 3' ends of *Mhc* along with a cDNA insert encoding alternative embryonic MHC isoforms. These were constructed as detailed in Materials and Methods. Transgenic lines that express each of the embryonic isoforms were produced by embryo injection. To analyze transgenic myosin in the absence of endogenous MHC, we crossed each first, third or fourth chromosome transgenic line into the *Mhc*¹⁰ background, which is null for MHC in IFM and jump muscle.²⁴ Since the endogenous *Mhc* gene is located on the second chromosome, second chromosome-linked lines were not used. Three transgenic lines were examined in detail for each construct. These are listed in Table 1.

Verification of transgene RNA production and protein expression

Mhc expression was examined for transgenic lines by RT-PCR. RNA was isolated from upper thoraces of two-day-old adults. cDNAs were synthesized for each transgenic line using a forward primer to exon 8 and a reverse primer for exon 12. Each transgenic line produced the expected 1.2-kb cDNA. These were cloned and at least ten independent clones for each transgenic line were sequenced to verify correct alternative exon usage. In the case of EMB-9c11d, each cDNA clone contained alternative exons 9c and 11d. For EMB-11d, each cDNA clone contained alternative exons 9b and 11d. This verifies the expression of the correct clones as well as the lack of expression of endogenous *Mhc* IFI transcripts that contain alternative exons 9a and 11e.

Myosin expression levels were determined for each transgenic line by calculating the ratio of MHC to actin by polyacrylamide gel electrophoresis and densitometric scanning. Three independent transgenic lines were examined for each construct and compared to wild-type levels of myosin expression. The levels of expression for each transgenic line were essentially identical to wild type (Table 1).

Locomotory ability

We determined flight and jump ability for EMB-9c11d and EMB-11d transgenic lines. We showed previously that expression of the EMB isoform results in a flightless phenotype and significantly reduced jump ability when expressed in the *Mhc*¹⁰ null background.¹⁰ Our current results indicate that neither EMB-9c11d nor EMB-11d rescue flight or jump ability, and that there is no significant difference in the flight or jump ability of EMB-9c11d and EMB-11d lines compared to EMB. Data are given in Table 1. We also assessed the crawling ability of third instar larvae expressing each of the transgenes in all of their muscles. None of the lines differed significantly in crawling scores (number of 0.5 cm grid boxes moved

per 5 min), with EMB-9c11d (line A-31) attaining 12.20 ± 0.7 , EMB-11d (line 2) at 12.17 ± 0.8 , and EMB at 12.22 ± 0.6 ($n = 20$ larvae for each isoform).

Basal and actin-activated ATPase activities

Myosin CaATPase, basal MgATPase, and actin-stimulated MgATPase activities of EMB, EMB-11d and EMB-9c11d transgenic myosins are shown in Fig. 2 and Table 2. Differences between EMB and EMB-11d are attributable to alternative converter domains, whereas differences between EMB-11d and EMB-9c11d are attributable to alternative relay domains. CaATPase and basal MgATPase activities of EMB are significantly higher ($p < 0.05$) than EMB-11d (5.43 ± 0.53 vs. 3.58 ± 1.18 and 0.08 ± 0.03 vs. $0.05 \pm 0.02 \text{ sec}^{-1}$, respectively). Actin-stimulated V_{max} of EMB is also significantly higher ($p < 0.001$) than EMB-11d (0.73 ± 0.04 vs. 0.32 ± 0.05). As shown in Table 2, actin affinity relative to ATPase enhancement (K_m) of EMB-11d is not statistically different from EMB. For the myosins with alternative relay domains, only the actin-stimulated ATPase activities (V_{max}) are significantly different ($p < 0.001$), with EMB-9c11d at 0.78 ± 0.05 compared to EMB-11d at $0.32 \pm 0.05 \text{ sec}^{-1}$.

In vitro motility

As shown in Table 2, actin sliding velocity induced by EMB myosin is significantly lower ($p < 0.001$) than both EMB-11d and EMB-9c11d (0.79 ± 0.01 vs. 1.22 ± 0.05 and $1.50 \pm 0.08 \mu\text{m sec}^{-1}$, respectively). Further, actin sliding velocity for EMB-11d is significantly lower ($p < 0.01$) than EMB-9c11d. Distribution curves of actin sliding velocities for EMB, EMB-11d and EMB-9c11d transgenic myosin are shown in Figure 3. The data indicate that both the relay and converter domains significantly impact *in vitro* actin motility.

Ultrastructure

We used transmission electron microscopy to examine the ultrastructure of the IFM for each transgenic line. We determined the muscle structure of late-stage pupae, two-hour-old adults, two-day-old adults and one-week-old adults. One myofibril is shown for each panel and it is representative of the myofibril population at that given stage of development. We had shown previously that EMB myofibrils assemble similarly to wild-type IFI, but that these structures degenerate as flies age.¹⁰

For EMB, the pupae (Fig. 4A and D) assemble circular myofibrils similar to normal IFM and display hexagonal packing of thick and thin filaments with sarcomeres containing M- and Z-lines. In contrast, the EMB-9c11d pupae (Fig. 4B and E) show apparent assembly defects such as abnormally shaped myofibrils with disrupted hexagonal filament packing, particularly at the periphery. Much like EMB, the pupae of EMB-11d (Fig. 4C and F) assemble myofibrils properly and display normal hexagonal packing of thick and thin filaments. Sarcomeres have well-formed M- and Z-lines. Since the only difference between EMB-9c11d and EMB-11d MHC is in their relay domains (encoded by exon 9c or 9b, respectively), we conclude that relay domains differentially influence myofibril assembly.

In EMB two-hour-old adults (Fig. 4G and J) myofibrils show normal thick and thin filament packing, with an occasional subtle disruption. In EMB-9c11d two-hour-old adults (Fig. 4H and K) the myofibrils continue to show disruption to the myofibril morphology. Sarcomeres display abnormal myofilament packing with disrupted M- and Z-lines. EMB-11d two-hour-old adult myofibrils (Fig. 4I and L) show fairly normal thick and thin filament packing with occasional disruption of the hexagonal lattice. The sarcomeric structure is maintained as well.

In EMB two-day-old adults (Fig. 4M and P) myofibrils show more pronounced abnormalities of the thick and thin filament lattice. Sarcomeres display areas lacking

myofilaments with disruption of M- and Z-lines. In EMB-9c11d two-day-old adults (Fig. 4N and Q) the myofibrils are further disrupted compared to those at two hours. Thick and thin filaments are poorly organized. Sarcomeres appear to be hypercontracted with lattice gaps and peripheral defects. In EMB-11d two-day-old adults (Fig. 4O and R) myofibrils show some disruption of the hexagonal packing of thick and thin filaments. Sarcomeres display gaps and have disruptions in M-lines.

In EMB one-week-old adults (Fig. 4S and V) the myofibrils show further disruption. Thick and thin filaments are scattered throughout the cytoplasm. Sarcomeres have abnormal or absent M- and Z-lines. In EMB-9c11d one-week-old adults (Fig. 4T and W) myofibrils are highly disorganized. Thick and thin filaments are scattered throughout and myofibril morphology is similar to EMB one-week-old adults. Sarcomeres are disrupted with gaps in the lattice. In EMB-11d one-week-old adults (Fig. 4U and X) myofibrils show degeneration with disruption of the hexagonal packing of thick and thin filaments. Sarcomeres appear hypercontracted with disrupted M-lines. Overall, however, structure is maintained better than for EMB or EMB-9c11d myofibrils.

Our ultrastructural results indicate that the alternative relay and converter domains affect myofibril assembly and/or stability. The alternative relay domains present in EMB-9c11d and EMB-11d MHC result in highly abnormal (Fig. 4B) or relatively normal (Fig. 4C) myofibril assembly, respectively. Further, abnormal assembly yields more rapid degeneration during aging (Fig. 4T vs. 4U). The converter domain also influences myofibril stability, in that EMB myofibrils are less stable than EMB-11d (Fig. 4S vs. 4U).

Discussion

In this paper we compared the biochemical, biophysical and *in vivo* assembly/stability properties of three naturally occurring *Drosophila* MHC isoforms: EMB-9c11d is normally expressed in the dorsal acute embryonic body wall muscles, EMB-11d is expressed in embryonic cardioblasts (as well as in dorsal acute and ventral oblique body wall muscles), whereas EMB is expressed in eight different types of body wall muscles.⁵ These three isoforms differ only in their alternative relay and/or converter domains, offering the opportunity to determine the properties dictated by isoform-specific versions of these regions. The observed differences in myosin isoform properties suggest that alternative *Mhc* RNA splicing yields diversity in *Drosophila* muscle function.

The *Drosophila Mhc* gene contains three alternative copies of exon 9, which encode the relay domain, and five alternative versions of exon 11, which encode the central portion of the converter domain.^{2,12} X-ray crystallographic studies reveal that the relay domain is located near the converter, allowing for inter-domain interactions during the chemomechanical cycle (Fig. 1A).¹⁹ Modeling by Fisher et al.^{14,25-29} suggests that repositioning of the lever arm feeds back through the adjacent converter to the relay domain. Bloemink *et al.*¹⁸ used molecular modeling to demonstrate that specific residues could interact between the converter and relay. Interactions between alternative residues in the relay and converter domains in *Drosophila* myosin isoforms could be responsible for some differences in muscle physiology.

Our current study allowed us to define specific functions for alternative relay and converter domains. We show that the naturally occurring dorsal acute embryonic body wall isoform (EMB-9c11d) has significantly higher MgATPase $V_{\max}$ and actin sliding velocity than the embryonic cardioblast isoform (EMB-11d). Further, myofibril assembly and stability are compromised in EMB-9c11d compared to EMB-11d. The only structural difference between the EMB-9c11d and EMB-11d isoforms is the presence of alternative relay domains

encoded by exons 9c or 9b. Hence the relay domain dictates the aforementioned variable parameters of myosin function. In contrast, the embryonic cardioblast isoform (EMB-11d) significantly reduces CaATPase, basal MgATPase, and $V_{\max}$ and significantly increases actin sliding velocity compared to EMB. It also yields minor improvement in myofibril stability. Alternative converter domains are responsible for these differences, as this is the only variance between the two isoforms. A key aspect of the current study is that it allowed the analysis of naturally occurring isoforms that contain compatible alternative domains selected through evolution; this work complements and extends previous studies of the *Drosophila* relay and converter domains that relied on chimeric myosin molecules.^{6,7,9,18,22,30,31}

Molecular differences between relay domains

There are five amino acid variations between exons 9c and 9b (Fig. 1A, upper right), and these are responsible for the biochemical, biophysical and ultrastructural differences seen in EMB-9c11d compared to EMB-11d. Two of these differences flank an invariant tryptophan 510 (Fig. 1B). The residue N-terminal to invariant tryptophan 510 is acidic in both cases, with D509 encoded by exon 9b and E509 encoded by exon 9c. In contrast, the alternative amino acids located C-terminal to invariant tryptophan 510 are not conserved. A hydrophobic alanine residue (A511) is encoded by exon 9b, whereas a polar threonine (T511) is encoded in the same position in exon 9c. The variant residues flanking the invariant tryptophan are located in a region of the relay known to undergo conformational changes during the chemomechanical cycle, since fluorescence levels of the invariant tryptophan increase as a result of ATP binding.³²⁻³⁴ Through molecular modeling it was demonstrated that residues 509 and 511 contact the converter domain^{13,18,35} and previous studies have shown that changes in the relay domain can influence interactions with the converter.^{13,18,35-38} Thus, interactions between residues 509 and/or 511 and the converter domain may modulate conformational changes in the relay and/or converter, yielding some of the functional differences between these two embryonic isoforms. Further, residue 511 reaches toward the base of the molecule and could regulate intramolecular interactions that are important for communicating to the active site. The third amino acid difference between the relay domains encoded by exons 9b and 9c is a non-conservative change at residue 504 (Fig. 1B). A basic lysine (K504) is encoded by exon 9b whereas a polar glutamine (Q504) is encoded by exon 9c. This residue makes no obvious intramolecular contacts in the preand post-power stroke molecules (Fig. 1A, upper right), but could be involved in modulating the see-saw motion of the relay helix proposed to occur during the recovery stroke.¹⁴ The two remaining differences between the relay domains are in adjacent residues 520 and 521 (Fig. 1B). A hydrophobic leucine (L520) is encoded by exon 9b whereas a polar glutamine (Q520) is encoded by exon 9c. Alternative hydrophobic residues are present at the adjacent site, with A521 encoded by exon 9b and L521 encoded by exon 9c. Residues 520 and 521 are located at the region of the relay that leads toward the actin binding site (Fig. 1B, upper right).

Previous studies have shown that conformational changes in the relay domain are necessary for establishing a communication pathway among the converter domain, actin and ATP binding sites, and the lever arm.^{13,14,18,35-38} Thus the above-mentioned changes in key residues of the relay are likely to explain the observed difference in $V_{\max}$, which could arise from alterations in communicating actin binding (residues 520, 521) to the active site (residues 504, 509). Further, differences in *in vitro* motility may arise from variation in relay loop-converter communication (residues 509, 511), required for lever arm re-positioning during the power stroke.

Molecular differences between converter domains

There are nineteen amino acid differences between the converter domains encoded by exons 11c and 11d (Fig. 1C), with 12 of these being non-conservative substitutions. The biochemical variation (reduced CaATPase, basal MgATPase, and $V_{\max}$) and biophysical disparity (increased actin sliding velocity) of EMB-11d compared to EMB isoforms are attributable to these amino acid differences. Molecular modeling of the two converter domains based upon scallop pre- and post-power stroke structures does not reveal obvious changes in residue interactions with the adjacent relay domain or lever arm (not shown). However, intra-domain structure and interactions are affected, seen most notably in disparate loop structures linking two helices (Fig. 1A, lower right). This may change the flexibility of the converter. Muscle mechanical studies identified the converter domain as the major compliant element in the myosin head.^{39,40} Changes in converter compliance between the *Drosophila* myosin isoforms should result in different levels of feedback through the relay to the active site (affecting ATPase) or from the converter to the lever arm (affecting power stroke size or kinetics and hence actin sliding velocity). Modeling studies have shown significant feedback between the lever arm/converter region through the relay domain to the active site of the molecule¹⁴, and biochemical, mechanical and mutant studies have established the requirement for constant interactions between the relay and converter domains for normal progression of the chemomechanical cycle.^{13,14,18,22,35-38}

Ultrastructural and locomotory assessments of organisms expressing alternative relay and converter domains

Indirect flight muscle expressing EMB-9c11d shows significant differences in ultrastructure compared to EMB-11d. Expression of the relay domain encoded by exon 9c resulted in myofibril assembly defects and instability compared to the relay encoded by exon 9b. The myofibril instability may arise from the increased ATPase activity and actin sliding velocity associated with the exon 9c-encoded relay domain, as well as the expectation that poorly formed myofibrils are more likely to degenerate. The interesting observation of defective myofibril assembly arising from the 9c relay domain suggests that this domain is important to myofibrillogenesis. While the defects could arise from contraction in pupal muscle, this appears unlikely since some other mutants that have severe contraction-induced myofibril degeneration in adults (*e.g.*, *Mhc*⁵) show normal myofibrils at the pupal stage.⁴¹ Perhaps altered interaction of the 9c relay-containing myosin head with actin during the myofibril assembly process is responsible for the observed defects. If the relay domain encoded by exon 9c is essential for defining the embryonic myofibril morphology that is required in dorsal acute body wall muscles, it may not be compatible with the myofibrillar architecture of the indirect flight muscles. Thick and thin filaments of embryonic body wall muscles are not well organized and myofibril morphology is significantly different than in indirect flight muscles.⁴² In contrast to the situation with alternative relay domains, the converters do not appear to play a role in this process, since myofibril assembly appears essentially normal in both EMB and EMB-11d. The minor increased level of myofibril degeneration in EMB could result from altered ATPase or actin motility compared to EMB-11d.

It is notable that degeneration is accompanied by hypercontraction, for EMB-11d and EMB-9c11d (Fig. 4) as well as EMB.⁹ This phenomenon has been documented previously to result from mutations in several *Drosophila* contractile proteins⁴³⁻⁴⁵, including myosin.^{46,47} Hypercontraction could arise from the decreased cross-bridge detachment rate and increased duty ratio found in embryonic myosin compared to the endogenous indirect flight muscle isoform.^{6,9} The higher-than-normal forces resulting from the increased cross-bridge “on-time” could lead to the hypercontraction and degeneration seen here.

None of the transgenic lines was able to fly and no significant differences in jump distance were observed (Table 1). Our crawling assay did not detect statistically significant differences in locomotory function among larvae expressing EMB, EMB-11d, or EMB-9c11d. However, our assay may not be sensitive enough to detect subtle differences in larval locomotion.

Comparison to analyses of chimeric isoforms

Our previous studies compared the indirect flight muscle (IFI) and embryonic body wall muscle (EMB) isoforms of myosin.^{8,10} As these two molecules differ in all four alternative domains of the myosin head (Fig. 1A) as well as in their S2 hinges and C-terminal regions, we produced chimeric isoforms in an attempt to tease out the functions of each domain. Studies of the alternative relay domains of these isoforms revealed that when the region encoded by exon 9a (the IFI version of exon 9) is substituted for exon 9b of the embryonic isoform there is a significant decrease in all parameters of ATPase function and actin sliding is abolished.²² Further, myofibril assembly and stability are poor compared to EMB. In contrast, when we substituted exon 9b (the embryonic version of 9) for exon 9a in the indirect flight muscle isoform, there were no significant changes in biochemical and biophysical properties or myofibril stability compared to IFI. The current study corroborates a role of the relay domain in ATPase activity and myofibril assembly. It better documents the relay domain's ability to dictate *in vitro* sliding velocity of actin filaments, since abolishment of motility found in the earlier study could have arisen from incompatibility between the domains of the non-native chimeric molecule.²²

We previously substituted the EMB version of exon 11 (encoding the central portion of the converter domain) into the IFM isoform IFI. This chimera showed a significant reduction in actin velocity⁹, increased CaATPase, MgATPase and $V_{\max}$ and a reduction in K_m ⁶, suggesting that the embryonic version of exon 11 converter domain influences biochemical and biophysical properties. When the IFI version of exon 11 was substituted into EMB there was a significant increase in actin velocity and improved *in vivo* myofibril stability, but no difference in myofibril assembly.⁹ Our previous results show that the converter domain, in the context of chimeric isoforms of MHC, influences biochemical, biophysical and myofibril properties. Our current data corroborate these findings, extending them to naturally occurring isoforms.

The functional differences among the naturally occurring isoforms that we measured *in vitro* and in the indirect flight muscles were significant. However, functional differences of embryonic isoforms in their natural locations may be subtle, based upon the absence of severe locomotion defects observed when each isoform is mis-expressed in all larval muscles. This seems an unexpected finding, as one might conjecture, for instance, that an isoform used in the embryonic cardioblast with its rhythmic beating should differ dramatically from one expressed in body wall muscles. Although our analyses detected differences, it is important to note that the cardioblast isoform is expressed in some body wall muscles, which demonstrates its plasticity. This is not unlike the situation in humans where the β -cardiac isoform is also expressed in slow skeletal muscles.

In conclusion, we used naturally occurring myosin isoforms to demonstrate that discrete regions of the molecule are essential to producing isoform-specific functions. Both the relay domain and the interacting converter affect the ability of myosin to propel actin filaments, likely through the converter domain's influence on the lever arm. The converter is able to feed back through the relay to the active site of the molecule. Thus isoform-specific residues in the converter or relay influence ATPase activity. Actin-activation of this property may also be influenced by interaction of alternative relay residues with helices that reach toward the actin binding site. Interestingly, the relay domain has an influence upon myofibril

assembly, suggesting that myosin head interactions with other contractile proteins is important for myofibrillar architecture. The *Drosophila* system is amenable to further refining isoform-specific domain interactions responsible for differences in functional properties of myosins. Single amino acid substitutions, such as the ones we are currently pursuing at the relay-converter interface, should permit identification of key amino acid residues that dictate isoform-specific properties.

Materials and Methods

Protein structure analysis

The scallop muscle myosin II crystal structure in the pre-power stroke state (PDB# 1qvi) or post-power stroke state (PDB# 1kk8) were used as templates for the *Drosophila* embryonic myosin S-1 amino acid sequence. The Swiss-Model homology modeling server (<http://swissmodel.expasy.org/>) was used to produce the homology coordinates and PyMOL (<http://www.pymol.org/>) was employed to view the output produced by the homology server. We also used this approach to view the alternative relay and converter domains encoded by exons 9c and 11d, respectively.

DNA constructs

cDNAs encoding two naturally occurring myosin isoforms, EMB-11d and EMB-9c11d, were produced in the EMB backbone¹⁰ in a plasmid containing *P* element ends and a selectable *w⁺* eye color marker. The resulting plasmids contain the 5' and 3' ends of *Mhc* along with the requisite cDNA coding sequence. The original EMB construct expresses alternative exons 3a, 7a, 9b, 11c and 15b and lacks exon 18, whereas EMB-11d replaces 11c with 11d and EMB-9c11d replaces 9b and 11c with 9c and 11d.

We constructed EMB-9c11d by subcloning the 5' end of EMB¹⁰ into pBluescriptKS vector (Stratagene, La Jolla, CA) using a gel isolated 4.7-kb Xba I and Apa I fragment to produce Emb5'. We subcloned the 3' end of EMB into the pBluescriptKS vector using a 5.1-kb Apa I and Kpn I fragment to produce Emb3'. Emb5' was cut with Bgl II and Apa I, which removed a 970-bp fragment containing part of exon 8 through 12. We then cut an embryonic cDNA (c52)⁵, which includes exons 9c and 11d with Bgl II and Apa I. The 970-bp Bgl II - Apa I fragment isolated from c52 was ligated into Emb5', which resulted in the Emb5'9c11d subclone. We then sequenced Emb5'9c11d to confirm the presence of exons 9c and 11d and the removal of exons 9b and 11c. Emb5'9c11d was digested with Xba I and Apa I and the 4.7-kb fragment was gel isolated. Emb3' was digested with Apa I and Kpn I and the 5.1-kb fragment was gel isolated. The two gel isolated fragments were then ligated into pCaSpeR 4⁴⁸ which had been digested with Xba I and Kpn I, to yield EMB-9c11d.

We constructed EMB-11d by digesting Emb5'9c11d with Bgl II and Nco I to remove a 183-bp fragment containing the last part of exon 8 through the first part of exon 10. Emb5' was digested with Bgl II and Nco I. The 183-bp fragment which includes exon 9b was gel isolated and ligated into Emb5'9c11d. The resulting subclone, Emb5'11d, was then sequenced to confirm the substitution of exon 9c for 9b. A 4.7-kb fragment was gel isolated from Emb5'11d with Xba I and Apa I. A 5.1-kb fragment was gel isolated from Emb3' with Apa I and Kpn I. The two gel isolated fragments were then ligated into pCaSpeR 4 which had been digested with Xba I and Kpn I, to yield EMB-11d.

To ensure no changes had taken place during the cloning process we sequenced all cloning sites used in constructing EMB-9c11d and EMB-11d.

P element transformation of *Mhc* genes

P element-mediated transformation⁴⁹ was employed by BestGene, Inc. (Chino Hills, CA) to produce transgenic lines for EMB-9c11d and EMB-11d. The entire coding region of each DNA preparation was sequenced prior to injection. The sequencing data confirmed that no mutations had been introduced during the cloning process. Each transgene was mapped to its chromosome location using balancer chromosomes and standard genetic crosses. For EMB-11d, 400 injected embryos resulted in 15 independent lines. Eight of the fifteen inserts mapped to the second chromosome, two to the X chromosome, four to the third chromosome and one to the fourth chromosome. For EMB-9c11d, 400 embryo injections yielded 6 independent lines. Three of the inserts mapped to the second chromosome, one to the X chromosome, one to the third chromosome and one to the fourth chromosome.

RT-PCR

To determine if the appropriate exons were being expressed from EMB-9c11d and EMB-11d transgenic lines, we used RT-PCR along with exon-specific primers. RNA was extracted by the LiCl₂ method.⁵⁰ Briefly, 30 upper thoraces were homogenized in 300 µl of 3 M LiCl₂, 6 M urea. The homogenized upper thoraces were placed on ice overnight and then centrifuged at 10,000 × g. The pellet was dissolved in 150 µl of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA followed by two phenol chloroform extractions. The RNA was then ethanol precipitated and resuspended in a final volume of 20 µl dH₂O. An aliquot of total RNA (0.5 µg) and 3 µmol of reverse primer specific to exon 12 (5'-GAGCTTCTTGAAGCCCTTACGG -3') was used for cDNA synthesis for both EMB-9c11d and EMB-11d RNA, employing a New England Biolabs (Ipswich, MA) Protoscript cDNA synthesis kit. PCR was performed with 1 µl of cDNA and 3 µmol of each primer specific pair under the following conditions: 60 s at 94°C, then 30 cycles of: 30 s at 94°C, 30 s at 55°C and 2 min at 68°C. Primer pairs used for EMB-9c11d and EMB-11d transgenic lines were exon 8 forward primer (5'-CGATACCGCCGAGCTGTACAG-3') and exon 12 reverse primer (as above). To confirm the presence of exons 9c and 11d in EMB-9c11d transgenic lines or 11d in EMB-11d, RT-PCR generated fragments were subcloned using Fermentas's GeneJet PCR Cloning Kit and sequenced.

Myosin expression levels

Expression levels of MHC in each transgenic line in the *Mhc*¹⁰ background were determined by one-dimensional SDS polyacrylamide gel electrophoresis as previously described.⁵¹ Six dissected upper thoraces from two-day-old female flies were homogenized on ice in 60 µl SDS gel buffer then boiled for five min. Ten µl of sample were loaded on a 9% polyacrylamide gel. This was repeated at least 5 times, each time with newly prepared samples. We digitally scanned the Coomassie Blue-stained gels with an Epson Expression 636 flatbed scanner. NIH image software (<http://rsb.info.nih.gov/nih-image/>) was used to determine protein accumulation. The myosin to actin ratio for each lane was normalized to the ratio for wild-type flies.

Flight assay

Each transgenic line expressing EMB-9c11d and EMB-11d in an *Mhc*¹⁰ background was subjected to a flight assay. This determines if a fly is capable of flying upward (U), horizontally (H), downward (D) or not at all (N), as previously described.⁵² All flight assays were performed with two-day-old female flies at 22°C with a minimum of 100 flies for each transgenic line. Flight index was calculated as 6U/T + 4H/T + 2D/T + 0N/T, where T is the total number of flies tested.⁵³

Jump assay

Jump ability is defined as the distance a fly jumps horizontally off of a 10-cm platform onto a piece of paper marked off in 0.5 cm grid lines after wing removal. To determine jump ability, 50 two-day-old female flies for each transgenic line were assayed at 22°C as previously described.¹⁰ The average value for each fly was calculated after ten consecutive jump tests.

Crawling Assay

Mhc transgenes were made homozygous in an X-linked yellow (*y*) background with a second chromosome configuration of *Mhc¹/Cy y⁺*. *Mhc¹* is null for MHC in all tissues.⁴² Organisms arising from crossing adults of this genotype produce some larvae with yellow cuticular markers (mouth hooks), indicating that they lack the *Cy y⁺* balancer chromosome and are homozygous for *Mhc¹*. Third instar larvae showing the *y* phenotype were assessed in the crawling assay by determining the number of 0.5 cm grid boxes they can crawl within 5 min at 22°C.⁴³

Myosin isolation

Dorsolongitudinal IFMs from 180-200 EMB, EMB-11d or EMB-9c11d transgenic flies were removed by microscopic dissection. Purification of myosin was carried out at 4°C using methods essentially as described previously.^{8,22} Concentration of purified myosin was determined spectrophotometrically by measuring the absorption at 280 nm as described previously.^{8,22} Purified myosin was used within two hours for ATPase and *in vitro* motility assays.

G and F actin preparation

G-actin was isolated from acetone powder of chicken skeletal muscle after multiple cycles of polymerization-depolymerization as described previously.^{8,54} Purified G-actin was quantified spectrophotometrically using an extinction coefficient of 0.62 cm⁻¹ (*A*_{310 nm} - *A*_{290 nm}) for 1 mg actin ml⁻¹. Polymerization of G-actin to produce F-actin was carried out by adding 1 volume of 10× polymerization buffer (50 mM Tris-Cl pH 8, 0.5 M KCl, 20 mM MgCl₂, 10 mM ATP) to 9 volumes of G-actin. Prepared F-actin was used for actin stimulated Mg²⁺-ATPase and *in vitro* motility assays.

Determination of steady-state ATPase activity

ATPase activities of EMB, EMB-11d and EMB-9c11d myosin were determined using [γ -³²P]-ATP as previously described in detail.^{8,22} Briefly, 2 μ g of freshly prepared myosin was added to CaATPase buffer (10 mM imidazole pH 6.0, 0.1 M KCl, 10 mM CaCl₂). CaATPase activity of myosin was initiated at 25°C by addition of [γ -³²P] ATP. The reaction was quenched following incubation for 15 min by adding of 1.8 M HClO₄. Extraction and scintillation counting was performed essentially as described previously.⁸ Basal MgATPase assays were carried out in a similar fashion, except in MgATPase buffer (10 mM imidazole pH 6.0, 20 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂ and 1 mM [γ -³²P]-ATP). Actin-stimulated MgATPase assays were performed in MgATPase buffer in the presence of increasing concentrations of F-actin (0.1 to 2 μ M). After 30 min the reaction was quenched using 1.8 N HClO₄. Basal MgATPase activities obtained in the absence of actin were subtracted from all data points. Actin-activated *V*_{max} and *K*_m values for actin were obtained by fitting all data points from several preparations of each myosin with the Michaelis-Menten equation using SigmaPlot. Values were averaged to give mean $\pm$ SD. Statistical comparisons of CaATPase, MgATPase, *V*_{max} and *K*_m were carried out using Student's *t* tests.

Determination of *in vitro* motility

In vitro actin sliding velocity assays for EMB, EMB-11d and EMB-9c11d myosins were carried out as previously described.^{8,22} At least three sets of assays were performed for each isoform studied. Analysis of captured video sequences was performed as described.^{8,22} Distribution curves for EMB, EMB-11d and EMB-9c11d actin sliding velocity were generated using SigmaPlot. Statistical comparisons of actin sliding velocity were carried out using Student's *t* tests.

Analysis of indirect flight muscle ultrastructure

Sample preparation and transmission electron microscopy were performed as previously described.⁴² Cross-sections and longitudinal sections of embedded thoraces for each transgenic line were analyzed at four stages of development (pupa, two-hour-old adult, two-day-old adult and one-week-old adult) using a Tecnai 12 transmission electron microscope. Thoraces from at least three different female samples were examined for each transgenic line (Table 1) to assess the effects of transgene expression on myofibril assembly and stability.

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Abbreviations used

IFM	indirect flight muscle
IFI	indirect flight muscle isoform
EMB	embryonic

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Highlights

- Myosin isoforms help define muscle-specific contractile and structural properties
- We expressed three native embryonic myosin isoforms in *Drosophila* muscles
- Native isoforms differ in their relay and/or converter domains
- Isoforms yield unique ATPases, *in vitro* actin motilities and myofibril structures
- Alternative relay and converter domains fine-tune myosin function

Figure 1.

Mapping of alternatively encoded *Drosophila* regions on the myosin molecule and sequence differences between alternative relay domains and converter domains. (A) Left: Location of alternative domains are mapped on the *Drosophila* EMB sequence threaded onto the scallop myosin molecule in the pre-power stroke state; alternative domains are encoded by exon 3 (red), exon 7 (cyan), exon 9 (blue) and exon 11 (green). Upper right: Exon 9 encodes the relay domain (blue). The five amino acid residue differences between EMB-11d and EMB9c11d are shown as orange spheres. Lower right: exon 11 encodes the central portion of the converter domain (green). The predicted conformations of EMB and EMB-11d differ in a loop connecting two helices, where the EMB-11d version of the loop is shown in black. (B) Two of the three alternative forms of the relay domain encoded by exon 9 are shown (exons 9b and 9c). Asterisks (*) mark the five amino acid differences. (C) Two of the five alternative forms of the converter domain encoded by exon 11 are shown (exon 11c and 11d). Asterisks mark the nineteen amino acid differences. The chicken pectoralis muscle myosin numbering system is used.

Figure 2.

Actin-stimulated MgATPase activity of embryonic myosin isoforms. As described in Materials and Methods, myosin was isolated from A) EMB, B) EMB-11d and C) EMB-9c11d lines and MgATPase activity was determined in the absence and presence of F-actin (0 - 2 μM). Basal MgATPase activities were subtracted from all data points, which were then fit with the Michaelis-Menten equation to determine $V_{\max}$ and K_m (actin), as shown in Table 2.

Figure 3.

Actin sliding velocity induced by embryonic myosin isoforms. Actin sliding velocity of EMB, EMB-11d and EMB-9c11d myosins were determined, as detailed in Materials and Methods. (A) Distribution curves of EMB along with EMB-11d. EMB-11d motility is significantly higher than EMB (see Table 2). (B) Distribution curves of EMB-9c11d along with EMB-11d. EMB-9c11d motility is significantly higher than EMB-11d (see Table 2).

Figure 4.

Effects of embryonic myosin isoforms on myofibril assembly and stability in dorsal longitudinal IFM. (A) Transverse section from EMB late stage pupa. EMB myofibrils show normal hexagonal packing of thick and thin filaments. (B) Transverse section from EMB-9c11d late stage pupa. Myofibrils show disruption in assembly of thick and thin filaments with abnormal myofibril structure compared to EMB. (C) Transverse section from EMB-11d late stage pupa. Myofibrils show normal hexagonal packing of thick and thin filaments similar to EMB late stage pupa. (D) Longitudinal section from EMB late stage pupa shows normal sarcomeric structure with M- and Z-lines (M and Z, respectively). Sarcomeres in panels E, F, J, K, L, P, Q and R are in the same register as this panel. (E) Longitudinal section from EMB-9c11d late stage pupa. The sarcomere structure shows gaps with disruption in the M- and Z-lines. (F) Longitudinal section from EMB-11d late stage pupa. The sarcomere structure appears normal, similar to EMB sarcomeres. (G) Transverse section from EMB two-hour-old adult. Myofibrils show subtle disruption of thick and thin filament packing. (H) Transverse section from EMB-9c11d two-hour-old adult. Myofibril structure is disrupted with abnormalities in myofibril morphology. (I) Transverse section from EMB-11d two-hour-old adult is similar to EMB two-hour-old adult with occasional disruption of thick and thin filaments. (J) Longitudinal section from EMB two-hour-old adult. Sarcomeres appear normal. (K) Longitudinal section from EMB-9c11d two-hour-old adult shows disruption of M- and Z-lines, with peripheral abnormalities of the sarcomere. (L) Longitudinal section from EMB-11d two-hour-old adult. Sarcomeres are mostly normal,

as in EMB. (M) Transverse section from EMB two-day-old adult. Myofibrils show more disruption of hexagonal packing of thick and thin filaments compared to EMB two-hour-old adult. (N) Transverse section from EMB-9c11d two-day-old adult. Myofibril morphology is further disrupted, with thick and thin filaments poorly organized in the myofibril. (O) Transverse section from EMB-11d two-day-old adult. EMB-11d myofibrils show disruption in thick and thin filament packing. (P) Longitudinal section from EMB two-day-old adult. Sarcomeres show gaps in the lattice with disruption of M- and Z-lines. (Q) Longitudinal section from EMB-9c11d two-day-old adult shows filament gaps containing glycogen granules and hypercontraction of the sarcomere with disruption of M- and Z-lines compared to EMB two-day-old adult. (R) Longitudinal section from EMB-11d two-day-old adult. Sarcomeres show gaps in the filament pattern with disruption in the M-line, similar to EMB. (S) Transverse section from EMB one-week-old adult shows complete loss of myofibril shape and hexagonal packing of thick and thin filaments. (T) Transverse section from EMB-9c11d one-week-old adult shows loss of myofibril shape with thick and thin filaments scattered throughout the myofibril similar to EMB one-week-old adult. (U) Transverse section from EMB-11d one-week-old adult shows continued loss of thick and thin filament organization with further disruption of myofibril shape. Myofibril morphology is not as disrupted as in EMB-11d or EMB one-week-old adults. (V) Longitudinal section from EMB one-week-old adult shows filament disorganization and disruption of M- and Z-lines. (W) Longitudinal section from EMB-9c11d one-week-old adult is similar to EMB one-week-old sarcomeres, with filament disorganization and disruption of M- and Z-lines. (X) Longitudinal section from EMB-11d one-week-old adult. Sarcomeres are better organized than in EMB or EMB-9c11d at the same age, but they show a hypercontracted phenotype that lacks clear M-lines. Scale bars, 0.5 μm .

Table 1

Transgenic lines, protein expression, flight index and jump distance

Transgenic line	Chromosome location	Protein ratio $\pm$ SEM	Flight Index $\pm$ SEM	Jump distance $\pm$ SEM (cm)
EMB-9c11d-A14	X	0.95 ± 0.05	0	2.1 ± 0.2
EMB-9c11d-A18	3	0.97 ± 0.07	0	2.0 ± 0.3
EMB-9c11d-A31	4	0.98 ± 0.08	0	2.0 ± 0.2
EMB-11d-2	3	0.97 ± 0.08	0	2.1 ± 0.3
EMB-11d-4	X	0.96 ± 0.10	0	2.3 ± 0.3
EMB-11d-5	3	0.94 ± 0.05	0	2.1 ± 0.5
EMB	3	0.99 ± 0.09	0 ^a	2.2 ± 0.1^a
pwMhc2	X	1.00 ± 0.08	3.8 ± 0.2^a	4.9 ± 0.2^a

Myosin heavy chain protein accumulation relative to actin was determined for each transgene in an *Mhc¹⁰* (IFM and jump muscle myosin null) background as detailed in Materials and Methods. Values are compared to pwMhc2 (wild-type transgenic). Protein amounts are means $\pm$ standard error of the mean (SEM). Flight index equals $6U/T + 4H/T + 2D/T + 0N/T$, where we determined whether each fly is capable of flying up (U), horizontal (H), down (D) or not at all (N), and T is the total number of flies tested in the assay.⁵³

^a from Swank et al., 2002.⁹

Table 2

Ca²⁺, Mg²⁺, actin-stimulated MgATPase kinetics and actin-sliding velocity of myosin isoforms EMB, EMB-11d and EMB-9c11d

Myosin isoform	CaATPase ± SD (s ⁻¹)	Basal MgATPase ± SD (s ⁻¹)	Actin-stimulated V _{max} ± SD (s ⁻¹)	K _m (actin) ± SD (μM)	Actin sliding velocity ± SD (μm s ⁻¹)
EMB (n=4)	5.43 ± 0.53 ^a	0.08 ± 0.03 ^d	0.73 ± 0.04 ^b	0.15 ± 0.02	0.79 ± 0.01
EMB-11d (n=4)	3.58 ± 1.18	0.05 ± 0.02	0.32 ± 0.05	0.13 ± 0.02	1.22 ± 0.05 ^c
EMB-9c11d (n=4)	4.35 ± 0.53	0.06 ± 0.01	0.78 ± 0.05 ^b	0.18 ± 0.06	1.50 ± 0.08 ^{c,d}

^a Significantly higher than EMB-11d (p < 0.05).

^b Significantly higher than EMB-11d (p < 0.001).

^c Significantly higher than EMB (p < 0.001).

^d Significantly higher than EMB-11d (p < 0.01).