Two Muscle-specific LIM Proteins in *Drosophila*

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**Abstract.** The LIM domain defines a zinc-binding motif found in a growing number of eukaryotic proteins that regulate cell growth and differentiation during development. Members of the cysteine-rich protein (CRP) family of LIM proteins have been implicated in muscle differentiation in vertebrates. Here we report the identification and characterization of cDNA clones encoding two members of the CRP family in *Drosophila*, referred to as muscle LIM proteins (Mlp).

*Mlp60A* encodes a protein with a single LIM domain linked to a glycine-rich region. *Mlp84B* encodes a protein with five tandem LIM-glycine modules. In the embryo, *Mlp* gene expression is spatially restricted to somatic, visceral, and pharyngeal muscles. Within the somatic musculature, *Mlp84B* transcripts are enriched at the terminal ends of muscle fibers, whereas *Mlp60A* transcripts are found throughout the muscle fibers. The distributions of the *Mlp60A* and *Mlp84B* proteins mirror their respective mRNA localizations, with *Mlp84B* enrichment occurring at sites of muscle attachment.

Northern blot analysis revealed that *Mlp* gene expression is developmentally regulated, showing a biphasic pattern over the course of the *Drosophila* life cycle. Peaks of expression occur late in embryogenesis and during metamorphosis, when the musculature is differentiating. *Drosophila* *Mlp60A* and *Mlp84B*, like vertebrate members of the CRP family, have the ability to associate with the actin cytoskeleton when expressed in rat fibroblast cells. The temporal expression and spatial distribution of muscle LIM proteins in *Drosophila* are consistent with a role for Mlps in myogenesis, late in the differentiation pathway.

*The LIM domain is a modular protein motif present in single or multiple copies in a wide variety of eukaryotic proteins that generally appear to regulate gene expression and cell differentiation during development (for review see Sadler et al., 1992; Sanchez-Garcia and Rabbitts, 1994; Dawid et al., 1995). The LIM motif is defined by a cysteine-rich consensus sequence, CX2CX16-23HX2CX2CX2CXI6-23(C,H,D) (Freyd et al., 1990; Karlsson et al., 1990; Sadler et al., 1992). Together the conserved Cys, His, and Asp residues coordinate two zinc atoms per LIM domain, giving rise to a double zinc finger (Michelsen et al., 1993, 1994; Kosa et al., 1994). The LIM domain has been shown to mediate specific protein-protein interactions and, in this way, may regulate protein activity and localization (Feuerstein et al., 1994; Schmeichel and Beckerle, 1994; Valge-Archer et al., 1994; Wu and Gill, 1994). Interestingly, recent structural studies have revealed that one of the two zinc-binding modules of the LIM domain displays a tertiary fold similar to DNA-binding domains in known transcription factors, raising the possibility that LIM domains might also be capable of interacting with nucleic acids (Perez-Alvarado et al., 1994).

The LIM motif was first identified in three developmentally regulated transcription factors, *Caenorhabditis elegans* *Lin-11*, rat *Isl-1*, and *C. elegans* *Mec-3*, from which the name LIM was derived (Way and Chalfie, 1988; Freyd et al., 1990; Karlsson et al., 1990). LIM domain proteins fall into two general categories: proteins in which LIM domains are associated with functional domains, such as homodomain or kinase domains, and proteins that are comprised more or less exclusively of LIM domains. Notably, even LIM-only proteins, which lack obvious DNA-binding or catalytic sequences, have been implicated in the control of cell differentiation. For example, targeted disruption of the gene encoding rhombotin 2, a protooncogene product with two LIM domains, eliminates erythroid differentiation in mice (Warren et al., 1994). Likewise, experiments using a cell culture model system have revealed that the muscle LIM protein (MLP)

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1. Abbreviations used in this paper: CRP, cysteine-rich protein; GST, glutathione-S-transferase; MEF, myocyte enhancer factor; MLP (mlp), muscle LIM protein; nt, nucleotide; REF, rat embryo fibroblast.
CRPs exhibit tissue-specific distributions and temporally regulated expression during embryogenesis (Wang et al., 1992; Arber et al., 1994; Crawford et al., 1994). For example, in the developing chick, CRP1 is most prominent in tissues rich in smooth muscle, and expression levels increase dramatically during smooth muscle maturation (Crawford et al., 1994). In contrast, a dramatic reduction in the levels of transcripts encoding both CRP1 and CRP2 correlates with the transformation of fibroblast cells by both chemical carcinogens and viral oncoproteins (Weiskirchen and Bister, 1993; Weiskirchen et al., 1995). CRPs are associated with elements of the actin cytoskeleton and can bind directly to another LIM protein called zyxin, which has been postulated to play a role in signal transduction at sites of membrane–substratum attachments enriched in integrin receptors (Sadler et al., 1992; Crawford et al., 1994). Collectively, the biochemical features, expression characteristics, and functional properties of the CRP family members lend credence to the hypothesis that CRPs are involved in promotion or maintenance of cell differentiation, particularly in muscle. However, the specific role(s) of CRPs in these developmental events is still unknown.

A number of discrete steps in muscle development have been defined in Drosophila melanogaster. As in vertebrates, myogenesis involves specification of mesoderm, commitment of cells to differentiate, and then expression of contractile proteins that mark terminal differentiation. Cell movements associated with gastrulation in Drosophila lead to the invagination and specification of cells that form the presumptive mesoderm (for review see Campos-Ortega and Hartenstein, 1985; Bate, 1993). These cells undergo several rounds of mitosis and ultimately become committed to differentiate into one of several major mesodermal derivatives: the somatic or body wall muscles, the visceral mesoderm or gut musculature, the cardiac mesoderm or dorsal vessel, and the fat body (Bate, 1993). In the somatic muscle lineage, fusion of myoblasts occurs midway through embryogenesis to produce sycnctial myotubes (Bate, 1990). These newly formed myotubes migrate toward their proper attachment sites in the epidermis and make formal attachments to extracellular matrix via integrin receptors (Bogaert et al., 1987; Leptin et al., 1989). Integrins also link the visceral musculature to basal lamina surrounding the gut epithelium (Bogaert et al., 1987). Finally, completion of the terminal differentiation program in the striated body wall and gut muscles involves the assembly of functional myofibrils. Although many of the early events involved in the specification and subdivision of the mesoderm are fairly well understood, aside from the expression of structural components of the contractile machinery, relatively few regulatory genes have been described that act late in the differentiation program.

Based on the observation that a CRP family member appears to be required for terminal differentiation in vertebrate muscle development (Arber et al., 1994), we have undertaken a molecular genetic approach to study the role of CRPs using Drosophila melanogaster as a model system. Here we describe the identification and developmental expression of two genes, Mlp60A and Mlp84B, that encode muscle-specific LIM proteins related to vertebrate CRP family members. Our analysis has revealed striking conservation of sequence, timing of gene expression, tissue distribution of gene products, and subcellular localization among LIM proteins of vertebrates and invertebrates. Mlp60A and Mlp84B are both expressed during periods of significant cell differentiation during development of the fly. The restricted temporal and spatial expression of the muscle LIM proteins in Drosophila is consistent with a role in myogenesis, late in the muscle differentiation pathway.

Materials and Methods

Southern Genomic Blots

10 μg of genomic DNA, purchased from Promega Corp. (Madison, WI), was processed according to standard procedures (Sambrook et al., 1989) and transferred to Hybond N+ nylon membrane (Amersham Corp., Arlington Heights, IL) overnight in 20× SSC. Blots were subsequently hybridized and washed according to the manufacturer’s protocol at 60°C (heterologous) or 65°C (homologous) with random-primed (Stratagene, La Jolla, CA) 32P-labeled chicken CSRP1 DNA (Crawford et al., 1994), Mlp60A DNA, or Mlp84B DNA. The CSRP1 probe consisted of a PCR-generated fragment, nucleotides (nt) 72-650 of the cDNA, corresponding to the coding region. Mbp probes were full-length cDNAs.

Library Screening

The same CSRP1 DNA probe was used to screen an adult Drosophila melanogaster cDNA library (KEXLOX) derived from mRNA in bodies (Novagen Inc., Madison, WI). Plaque plating and growth were carried out according to the manufacturer’s protocols. Filter lifts and hybridization were performed essentially as for genomic blots except the final wash was more stringent. After plaque purification, we used the lropCRE recombinase system to isolate plasmid DNA containing the clones of interest. In subsequent library screens, Drosophila clones identified in the first screen were used as probes to isolate more clones representing the gene.

Sequencing

Double-strand DNA was sequenced using the dideoxy chain termination method (Sanger et al., 1977) with Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH) and α-[32P]dATP, or PCR Cycle Sequencing (GIBCO BRL, Gaithersburg, MD) with β-[32P]-ATP according to the manufacturer’s directions. We sequenced a combination of full-length clones, restriction fragments subcloned into pBluescript (Stratagene), and deletion clones generated using ExoIII nuclease (New England Biolabs, Beverly, MA), using primers against vector sequences and specific internal primers. Both strands were sequenced in entirety. Sequence comparisons were generated using the GAP program within the GCG sequence analysis software package (version 7; Genetics Computer Group, Madison, WI) based on the algorithms derived from Needleman and Wunch (1970).

In Situ Hybridization to Polytene Chromosomes

Drosophila larval salivary gland dissection and squashes, as well as pre-treatment of chromosomes on the slides before hybridization, were essentially as described (Pardue, 1986) but without heat or RNAse treatment. Double-strand DNA was random primer labeled using the Genius system (Boehringer Mannheim Biochemicals, Indianapolis, IN). Probes were as follows: #20 clone for Mlp60A, #21 clone for Mlp84B. Hybridization was carried out at 62°C overnight in 5× SSC, 1× Denhardt’s reagent, 5 mM MgCl2, 0.5% Genius blocking reagent. Washes, processing, and detection were performed essentially according to the Genus detection protocol, but with anti-digoxigenin antibody diluted 1:500, and reacted with chromosones 2 h at room temperature. Finally, the chromosomes were stained briefly in acetor-orcein and observed with phase-contrast optics. Images of chromosomes were captured using a microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY) equipped with a video camera (DC-151 GB; Sony Corp., Park Ridge, NJ).

Northern Blots

Staged embryos, larvac, pupae, or adult females were used to isolate poly A+ RNA using either of two procedures: (a) a combination of RNA agents...
total RNA isolation kit (Promega Corp.) followed by poly A+ selection using PolyATtract mRNA Isolation System III (Promega Corp.) according to the supplier; or (b) total RNA was isolated using the hot phenol method (Jowett, 1986), and subsequent poly A+ selection was carried out using oligo dT cellulose (Collaborative Research, Inc., Waltham, MA) according to the manufacturer. 5-6 µg mRNA from each developmental stage was electrophoresed through a denaturing formaldehyde gel in 1x MOPS buffer (Sambrook et al., 1989). After processing, the mRNA was transferred to Hybond N+ membrane (Amersham Corp.) overnight and subsequently hybridized with random primer 32P-labeled probes as we had for the Southern, but at higher stringency. The same blot was hybridized independently with each probe; after data were collected for each probe, the blot was stripped with boiling 0.5% SDS for 10 min and reused. LIM probes consisted of the entire coding regions of the cDNAs. rp49 probe was a gift from A. Letsou (University of Utah, Salt Lake City, UT), containing rp49 coding sequences cloned into pBluescript.

Whole Mount In Situ Hybridization

Canton-S embryos were collected overnight on apple juice plates and dechorionated in 90% bleach. Embryo processing and hybridization were carried essentially as described by Tautz and Pfeifle (1989), with the following modifications for use with RNA probes. Hybridization solution consisted of 50% formamide, 5x SSC, 50 µg/ml heparin, 100 µg/ml yeast tRNA, 0.1% Tween-20, pH 4.5. Hybridization was carried out overnight at 65°C with digoxigenin-labeled riboprobes added to 0.25 ng/ml. Subsequent washes were performed at 65°C. Just before adding anti-digoxigenin antibody, embryos were blocked with 1x PBS, 0.1% Tween-20, and 1% blocking reagent. probed reagent, and rehybridization were performed using the Boehringer Mannheim Genius RNA labeling kit according to the manufacturer. For detecting Mlp60A RNA, we used an antisense riboprobe corresponding to the 3' untranslated region, nt 320-428, generated by digesting the full-length clone with NarI enzyme (New England Biolabs) and transcribing run-off transcripts using an appropriate enzyme, and run-off transcripts were used to generate the T3 promoter. Embryos were mounted in JB-4 resin (Polysciences, Inc., Warrington, PA) and photographed using differential interference contrast optics on a Zeiss Axiopt phot microscope.

Antibody Production, Western Blot Analysis, and Immunofluorescence

Mlp coding sequences were cloned into the pGEX-2T expression vector (Pharmacia, Upsala, Sweden) and expressed in bacteria as fusion proteins with glutathione-S-transferase (GST) sequences. Fusion protein purification was performed according to standard procedures (Ausubel et al., 1994). The C-terminal 205 amino acid sequence of MlpA was cleaved from GST using thrombin (Sigma Chemical Co., St. Louis, MO), whereas for MlpB48, the intact GST fusion was used as an immunogen. Purified protein for immunizing rabbits was obtained by separation on a preparative SDS polyacrylamide gel, followed by electrophoresis of the protein and extensive dialysis against PBS. For characterization of resulting polyclonal antibodies, 16-24 h Drosophila Canton-S embryos were collected, washed, and homogenized in Laemmli sample buffer (Laemmli, 1970). SDS-PAGE was performed according to the method of Laemmli (1970) with modifications described previously (Schmeichel and Beckerle, 1994). Subsequent Western immunoblots were carried out as described (Beckerle, 1986) using 125I-protein A to detect primary antibody binding. For Western blots, anti-Mlp60A and anti-MlpB48 antibodies were used at dilutions of 1:600 and 1:1500, respectively.

Immunostaining of whole mount embryos was carried out essentially as described (Patel, 1994) using antibodies precipitated against fixed, early-stage embryos. Antibodies to Mlp60A were used at 1:100, anti-MlpB48 antibodies were used at 1:200, and HRP-conjugated goat anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were used at 1:500. Embryos were mounted in 70% glycerol and photographed using differential interference contrast optics on a Zeiss Axiopt microscope. A similar muscle pattern was observed in embryos using an independently generated anti-peptide antibody against Mlp60A sequence. Confocal immunofluorescence microscopy was performed using similar procedures, and antibody dilutions except embryos were fixed for 4 min in a fixative composed of 9 ml 37% formaldehyde and 1 ml 0.5 M EGTA, pH 8.0, plus an equal volume of heptane (Kiehart and Feghali, 1986). Anti-muscle myosin antibody was kindly provided by D. Kiehart (Duke University, Durham, NC) and diluted to 1:400. A Texas red-conjugated goat anti–rabbit secondary antibody (Cappel Laboratories, Durham, NC) was used at 1:200. Images were captured using the confocal system (MRC-600; Bio-Rad Laboratories, Cambridge, MA) attached to an epifluorescence microscope (Nikon Inc., Garden City, NY). Low magnification images (×20) represent 6.7-μm sections, and high magnification images (×40) represent 4.2-μm optical sections. Images were assembled and labeled using software (Adobe Photoshop, Adobe Systems, Inc., Mountain View, CA) and subsequently printed on a printer (XLS 6800 PS; Eastman-Kodak Co., Rochester, NY).

Heterologous Expression and Immunofluorescence

Expression vector construction involved amplifying Mlp coding regions from full-length cDNAs using PCR. Primers encoded BamHI (5' end) or NotI (3' end) restriction sites, and Pfu polymerase (Stratagene) was used to minimize the likelihood of errors. Amplified fragments were digested and ligated into a pcDNA1/Neo vector (Invitrogen, San Diego, CA) that was modified (gift from D. Nix, University of Utah, Salt Lake City, UT) by inserting sequences encoding the FLAG epitope downstream from the NotI site. Ligation at that site would generate an in-frame Mlp fusion with FLAG. Triplicate PCR samples were used to generate three independent constructs for microinjection. Plasmid DNA was isolated using a polyethylene glycol precipitation procedure (Sambrook et al., 1989) and finally resuspended in PBS. REF52 cells were grown to 50–70% confluence on coverslips in growth medium and microinjected with plasmid DNA at 250 ng/μl. Cells were fixed 24 h later and processed for fluorescence microscopy with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR), and indirect immunofluorescence (Beckerle, 1986) with anti-FLAG M2Ab primary antibody (IBI/Alpha Diagnostics, New Haven, CT) at 1:600 and FITC-conjugated goat anti–mouse secondary antibody (Jackson Immunoresearch Laboratories) at 1:500.

Results

Identification of CRP-related Sequences in Metazoans

Members of the CRP family are characterized by the presence of two LIM domains, each followed by a glycine-rich repeat with the sequence GPKG(Y/F)G(Y/F)G(M/Q) GAG. The presence of this glycine-rich repeat distinguishes CRP family members from other small LIM-only proteins such as rhombotin. In addition, CRP family members display a potential nuclear targeting signal (KKYGPK) that partially overlaps the glycine-rich repeat.

To determine whether sequences related to those specifying avian CRPs are present in other organisms, we used a cDNA encoding CRP1 (referred to as CSRPI2) to probe genomic Southern blots of DNA from chicken, fly, human, mouse, yeast, and frog. As can be seen in Fig. 1, cross-hybridizing genomic DNA fragments are detected in all metazoan species examined using a CSRPI2 probe. No specific hybridization is observed with yeast genomic DNA, although yeast are known to possess genes encoding LIM domain proteins (Muller et al., 1994). The cross-hybridizing band observed in yeast genomic DNA (Fig. 1, lane 5) corresponds to an intense band of repetitive DNA observable in the ethidium bromide–stained agarose gel (not shown).

2. Proteins of the vertebrate CRP family are designated CRP1, CRP2, and MLPCRP3; the corresponding genes are designated with the symbols CSRPI, CSRPI, and CSRPI3. Stronach et al. Muscle LIM Proteins in Drosophila
Molecular Cloning of cDNAs That Encode Drosophila Proteins Related to CRP

In an effort to identify and to characterize CRP family members in Drosophila, an adult Drosophila cDNA library was screened with an avian CSRP1 probe. From 600,000 recombinants screened, two cross-hybridizing clones were isolated and characterized by sequence analysis (Figs. 2 and 3). The resulting cDNAs were shown to encode distinct, but closely related, proteins referred to initially as DmLIM-2 and DmLIM-3 (Stronach, B.E., T.B. Macalma, and M.C. Beckerle. 1994. 35th Annual Drosophila Research Conference. Chicago. 370a). Both DmLIM-2 and DmLIM-3 display features that are hallmarks of the CRP family, being comprised more or less exclusively of LIM-Gly repeats. The gene encoding DmLIM-2 was independently isolated by Arber and colleagues in a search for Drosophila sequences related to MLP/CRP3 (Arber et al., 1994). These authors have referred to this gene as Mlp1. We suggest renaming this gene, Mlp60A, to include information about the genomic location and to be consistent with standard Drosophila nomenclature (Flybase, 1994). Similarly, DmLIM-3 is hereafter referred to as muscle LIM protein 84B. Mlp84B corresponds to a novel gene sequence named for its relationship to Mlp60A, its tissue-specific expression, and its genomic location. Because the members of the CRP family have been most extensively characterized in birds, we use those sequences here for comparison with the Drosophila CRP family members. It should be noted that the avian CRPs are >90% identical to their counterparts in mouse and human (Weiskirchen et al., 1995).

Characterization of an Mlp60A cDNA

The nucleotide and deduced amino acid sequences of Mlp60A are shown in Fig. 2 B. The cDNA sequence is 428 nucleotides in length. Nine additional clones isolated and characterized in a subsequent screen provide only six additional nucleotides 5' to what is presented in Fig. 2 B (not shown). The ATG (nt 60–63) is postulated to be the initiation codon since flanking sequences conform well to the consensus translation initiation site in Drosophila, cacaca-cAaaATGgc (Cavener and Ray, 1991). A polyadenylation signal that partially overlaps the glycine-rich region (double underline). These sequence data are available from EMBL/GenBank/DDBJ under the accession number X91244.

The Mlp60A cDNA is predicted to encode a protein of 92 amino acids. The derived protein product is comprised of a single LIM domain linked to a glycine-rich repeat that closely resembles the glycine-rich sequence observed in CRP1 (Fig. 4, A and C). Like vertebrate CRPs, the LIM domain of Mlp60A exhibits the sequence CX2CX17HX2

Characterization of Mlp84B cDNAs

The nucleotide and deduced amino acid sequences of

![Figure 1. Metazoan species contain sequences related to avian CSRP1. CSRP1 coding sequences were used to probe a genomic Southern blot of DNA isolated from various species. Each species, except for yeast, shows a unique set of cross-hybridizing fragments.](image-url)
Figure 3. Nucleotide and predicted protein sequences encoded by *Drosophila* Mlp84B cDNAs. (A) Schematic representation of cDNA clones encoding Mlp84B. Clone #21 contains the entire open reading frame (thickened line). Several restriction enzyme sites, used in subcloning, are shown. Additional cDNAs encoding Mlp84B are indicated, with the lines representing their length and position relative to clone #21. (B) Nucleotide and derived amino acid sequence of Mlp84B, a five LIM domain protein. Nucleotide and amino acid positions are indicated by numbers in the left margin preceding each row. Translational start and stop codons (underlined). The conserved cysteine and histidine residues of the five LIM domains (circled). Glycine residues that comprise the glycine-rich regions following each LIM domain (boxed). Putative nuclear targeting signals found adjacent to the first and second LIM domains (underlined twice). These sequence data are available from EMBL/GenBank/DDBJ under the accession number X91245.
Figure 4. Drosophila muscle LIM proteins are closely related to vertebrate CRP family members. (A) Schematic representation of vertebrate CRPs and the *Drosophila* Mlp8s showing LIM domain and glycine-rich (stippled) regions. Alignment of all the proteins at the amino terminus gives the highest sequence conservation. (B) Pairwise sequence comparisons among avian and *Drosophila* CRP family members. Numbers depict amino acid identity derived from analysis using the Genetics Computer Group GAP program with default parameters based on the algorithm of Needleman and Wunsch (1970). (C) Amino acid alignment of all the individual LIM/glycine modules shown in A. The L1–L5 designation after the protein names in the left margin defines which LIM domain of the protein is displayed in that row and is consistent with the nomenclature shown in A. Amino acid positions are numbered to the left of each row. Highlighted residues are identical or conserved in at least 90% of the domains, and the exceptions are not highlighted. The LIM region is marked by a dotted line below the last entry; similarly, the glycine-rich region is marked by a solid line. The cysteine and histidine residues that define the LIM domain are indicated by a shaded square at the bottom of a column. (Open circles) Conserved residues potentially involved in hydrogen bonding in the three-dimensional structure of a LIM domain (Perez-Alvarado et al., 1994). Similarly, crosses indicate conserved residues thought to contribute to a hydrophobic core (Perez-Alvarado et al., 1994). Avian CRP sequences are given in the following references (MLP/CRP3: Arber et al., 1994; CRP1: Crawford et al., 1994; CRP2: Weiskirchen et al., 1994).
In situ Localization of Mlp60A and Mlp84B to Polytene Chromosomes

The genomic locations of Mlp60A and Mlp84B have been mapped using in situ hybridization to the larval salivary gland polytene chromosomes. Mlp60A, encoding the single LIM protein, is detected within subdivisions 60A5-6; 60B11 on the distal tip of the right arm of chromosome 2 (Fig. 5 A). Mlp84B, coding for five LIM domains, is localized to subdivisions 84B3;84C2-6 near the centromere on the right arm of chromosome 3 (Fig. 5 B). Each gene appears to be unique, as the hybridization signal is seen at only a single site in the genome. This is consistent with the results of genomic Southern blotting, which reveal a simple pattern of restriction fragments hybridizing with cDNA probes derived from each gene (Fig. 5 C).

Expression of Mlp60A and Mlp84B during Drosophila Development

We have examined the expression of Mlp60A and Mlp84B by developmental Northern analysis (Fig. 6). Both Mlp60A and Mlp84B display a biphasic pattern of expression, with peaks late in embryogenesis and again during metamorphosis of the fly. The Mlp60A gene encodes a single abundant transcript of ~0.5 kb (Fig. 6 A). Transcripts are first detectable in 8–12-h embryos and peak strongly in 16–24-h embryos. A significant decrease in steady state RNA levels occurs during the larval stages. A second, less robust peak of expression is observed in pupae. Mlp60A transcripts persist in adults. The Mlp84B gene encodes a moderately abundant transcript of ~2.3 kb. Mlp84B RNA expression is strikingly similar to Mlp60A in its biphasic nature. Like Mlp60A, Mlp84B RNA is first detectable in 8–12-h embryos. Peak expression is observed in 16–24-h embryos. Transcript levels decline dramatically in larvae and elevate again during the larval to pupal transition. RNA levels are decreased, but still detectable, in adults. Neither Mlp60A nor Mlp84B mRNA is maternally inherited. The mRNA levels for each gene have been quantified using Phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA), and the data were normalized with respect to the amount of mRNA loaded per lane (Fig. 6 B). The ribosomal protein gene, rp49, was used as a probe to assess the general quality and quantity of RNA loaded. Detailed analysis of the steady state levels of rp49 transcripts revealed that rp49 expression is not constant throughout development (Andres and Cherbas, 1992) but, rather, varies in a manner consistent with what we observed. The fluctuations in rp49 transcript levels may reflect global changes in gene transcription during embryogenesis and metamorphosis. Although we detect the greatest amount of rp49 transcripts at 16–24 h of embryogenesis, the increases in Mlp60A and Mlp84B expression are substantially greater than that observed for rp49.

Muscle-specific Expression of Mlp60A and Mlp84B

We have analyzed the distributions of Mlp60A and Mlp84B transcripts during embryogenesis of the fly by in situ hybridization to whole mount embryos (Figs. 7 and 8). The results obtained using this technique were completely consistent with the timing of expression of Mlp60A and Mlp84B determined by developmental Northern analysis (Fig. 6). The hybridizations were performed on embryos at early and late stages of development, as well as on adult flies.

Figure 5. Cytological locations and genomic Southern blot analysis of Drosophila Mlp60A and Mlp84B. (A) Mlp60A maps to subdivision 60AB on the distal tip of the right arm of chromosome 2. (B) Mlp84B maps to subdivision 84BC on the right arm of chromosome 3. (C) Southern hybridization to Drosophila genomic DNA digested with five different restriction enzymes using either an Mlp60A or Mlp84B probe. Enzymes: B, BamHI; Bg, BglII; RI, EcoRI; RV, EcoRV; H, HindIII. Positions of molecular weight markers in kb (left).
Figure 6. Drosophila Mlp gene expression is developmentally regulated throughout the Drosophila life cycle. (A) Developmental Northern blot analysis of poly A+ RNA from embryonic (numbered as hours of development at 25°C), larval, pupal, or adult stages. RNAs were hybridized with a 32P-labeled Mlp60A, Mlp84B, or ribosomal protein, rp49, probe. Numbers to the right refer to the size of the hybridizing band. (B) Quantitation of Northern blot data. Expression levels are indicated as a percentage of maximum (100%), calculated individually for Mlp60A (circles) and Mlp84B (squares). The data have been normalized to account for the amount of RNA loaded per lane in micrograms.

Although both Mlp60A and Mlp84B are coexpressed in the somatic muscles, their patterns of hybridization are distinct. Mlp60A mRNA appears to be distributed throughout mature myotubes, whereas Mlp84B mRNA hybridization is concentrated at the terminal portions of the myotubes near where they are making attachments to the epidermis. The difference in transcript distributions is easily visualized in the ventral-lateral longitudinal muscles, in which a significant proportion of Mlp60A staining is found in the middle of a muscle fiber between the segment boundaries (Fig. 7, C and E). This contrasts with the polarized distribution of Mlp84B transcripts seen as segmentally repeated double stripes with significant exclusion of signal in the middle of the segment (Fig. 7, compare C with D, and E with F). The difference in the distributions of Mlp60A and Mlp84B transcripts is also particularly striking in the large cephalic muscles located ventrally in a stage 16 embryo (Fig. 7, G and H).

Mlp60A and Mlp84B are also coexpressed in the visceral musculature surrounding the fore-, mid-, and hindgut of stage 14 and older embryos (Fig. 8). By the beginning of stage 14, the visceral muscles have already attached to the developing gut epithelia (Skaer, 1993; Tepass and Hartenstein, 1994). Mlp expression begins to be observed as the muscle cells spread and encircle the gut during stages 14 and 15 (Fig. 8, A–D). In addition to the presence in visceral mesoderm, both Mlp60A and Mlp84B are strongly expressed in pharyngeal muscle (Fig. 8, E and F). In contrast with what we observed in the somatic musculature, at this level of resolution, we do not detect a polarized distribution of Mlp84B transcripts in visceral or pharyngeal muscle.

Protein Distribution of Mlps in the Developing Musculature

To analyze the distributions of Mlp gene products during...
Figure 7. *Drosophila* Mlp genes are expressed in embryonic somatic muscles. (A and B) Stage 14 embryos hybridized with antisense Mlp60A (A) or Mlp84B (B) probes to reveal gene expression in developing syncytial myotubes positioned dorsally (d), laterally (l), and ventrally (v) within posterior segments. Dorsal groups are just out of focus in (B). (C and D) Stage 16 embryos showing staining of Mlp60A (C) or Mlp84B (D) in the completed pattern of larval musculature. (E and F) Higher magnification of embryos shown in C and D to reveal localization of Mlp60A (E) or Mlp84B (F) transcripts in muscles of two abdominal segments. (Asterisk) Ventral-lateral longitudinal muscles in one segment; (arrowheads) segment boundaries of one segment. Note that the majority of Mlp60A transcripts are found in the middle of the muscle fibers within each segment (E), whereas Mlp84B transcripts are largely excluded from the middle of the segment, being localized more prominently at the ends of muscle fibers near the segment boundaries (F). (G and H) High magnification of large cephalic muscles (c) positioned ventrally in stage 16 embryos, probed with either Mlp60A (G) or Mlp84B (H). Note the distinct patterns of mRNA localization relative to the asterisk; Mlp60A transcripts are found throughout the muscles, and Mlp84B transcripts localize near muscle attachment sites. In all frames, embryos are oriented with anterior to the right. (A–F) Lateral views of embryos with dorsal side up. (G and H) Ventral views of embryos. Bars: (A–D) 50 μm; (E–H) 25 μm.

*Drosophila* embryogenesis, we raised antibodies to Mlp60A and Mlp84B sequences that were expressed as fusion proteins in bacteria. Western blot analysis using rabbit anti-Mlp60A or rabbit anti-Mlp84B probes shows the specificity of the individual antibodies (Fig. 9). Anti-Mlp60A antibodies detect a single protein of ~9 kD in 16–24-h *Drosophila* embryonic lysates (Fig. 9 B). Anti-Mlp84B antibodies detect a single protein of ~53 kD in a duplicate lysate (Fig. 9 C). The preimmune sera harvested from both rabbits fail to show any reactivity with proteins in the 16–24-h embryo lysate (Fig. 9, B and C).

Immunocytochemical staining of embryos reveals that the distribution of muscle LIM proteins mimics the distribution of transcripts in various mesodermally derived tissues, including all somatic, visceral, and pharyngeal muscles. In these tissues, Mlp60A and Mlp84B are first observed in late stage 14 embryos. In the visceral musculature, although Mlp60A is seen reproducibly, the intensity of stain-
Figure 8. Drosophila Mlp60A and Mlp84B are expressed in embryonic visceral mesoderm. (A and B) Early stage 14 embryos showing Mlp60A (A) or Mlp84B (B) expression in the visceral mesodermal cells (v) surrounding the gut epithelium. (C and D) Later in stage 15 embryos, when the middle gut constriction has formed, Mlp60A (C) and Mlp84B (D) genes continue to be expressed in visceral mesoderm (v) attached to the developing fore-, mid-, and hindgut as well as in the somatic muscles (s) located laterally close to the body wall. (E and F) In late stage 16 embryos, Mlp60A (E) and Mlp84B (F) transcripts are seen in the visceral musculature (v), the pharynx (p), and the somatic muscles (s). In all frames, embryos are viewed ventrally and oriented with anterior to the right. Bar, 50 µm.

Expression is never as robust as that seen for Mlp84B in the gut muscles and may reflect differences in the levels of protein expression in this tissue (not shown). When the mature pattern of somatic muscles is evident in stage 16 and older embryos, intense immunoreactivity is detected with both anti-Mlp60A and anti-Mlp84B antibodies. Both proteins are found throughout the myotubes (Fig. 10, A and B). Upon closer examination of the immunostained embryos, we discerned more intense staining for Mlp84B at the ends of muscle fibers at the point of attachment to the epidermis (Fig. 10, see arrows in C and D).

To further characterize the subcellular distributions of the Drosophila muscle LIM proteins, we used confocal microscopy to visualize embryos that were fluorescently labeled with anti-Mlp antibodies in parallel with an anti-muscle myosin antibody (Kiehart and Feghali, 1986) (Fig. 11). Examination of embryos by confocal optical sectioning allowed us to discern several prominent differences in protein distribution between the Mlps and myosin. First, the Mlps, although not enriched in muscle cell nuclei, do not show a significant nuclear exclusion as does myosin (Fig. 11, compare A with B and C). Second, Mlp84B, uniquely, becomes associated with the developing myotendinous junction, visualized as bright staining at the ends of myotubes (Fig. 11, see arrowheads in B and C). This enrichment at muscle attachment sites is largely absent before stage 16 (Fig. 12, A and B), when the midgut has constricted but is not yet convoluted. The redistribution of Mlp84B to the ends of muscle fibers after 14 h of development (Fig. 12, C and D) correlates with early signs of the development of functional myotendinous junctions, including somatic muscle attachment and visible muscle contractions. It appears then that the association of Mlp84B with the muscle attachment sites could serve as an early marker for the assembly of this junction. Finally, immunofluorescent detection of Mlps using confocal microscopy also revealed that both muscle LIM proteins appear to associate with linear cytoplasmic elements within the muscle cell syncytium, suggestive of the sarcomeric actin filament network (Figs. 11 and 12).
Muscle LIM Proteins in Drosophila

Drosophila Mlp60A and Mlp84B Localize to the Cytoskeleton in Vertebrate Cells

Previous work has shown that CRP family members colocalize with the actin cytoskeleton in various cell types including muscle (Sadler et al., 1992; Arber et al., 1994; Crawford et al., 1994). Based on the extensive sequence conservation of the Drosophila LIM proteins with respect to their vertebrate counterparts and our observations regarding their subcellular distributions in Drosophila muscles, we were interested in evaluating the ability of the fly proteins to associate with the actin cytoskeleton. Therefore, we expressed FLAG epitope-tagged versions of the full-length Drosophila cDNAs under the control of a mammalian viral promoter in rat embryo fibroblast (REF) cells (REF52). When either Drosophila Mlp60A or Mlp84B is expressed in the REF52 cells, each shows significant colocalization with rhodamine-phalloidin–labeled actin bundles (Fig. 13), illustrating that the LIM-glycine repeats found in the fly proteins share with their vertebrate relatives the ability to associate with the actin cytoskeleton. The cytoskeletal staining observed with the anti-FLAG antibody can be attributed to the recognition of the expressed Drosophila sequences since no staining appears in untransfected cells (Fig. 13). Moreover, the localization of Mlp60A and Mlp84B to the actin cytoskeleton is specific since the majority of LIM-containing proteins do not associate with the cytoskeleton. Although we occasionally observe Mlp60A in cell nuclei, the physiological significance of this distribution is not clear. We never observed Mlp84B in the nuclei of REF52 cells.

CRP Proteins Are Conserved in Drosophila

We have identified two new members of the CRP family in Drosophila melanogaster. Sequence analysis revealed a high degree of conservation within the LIM domains of both Mlp60A and Mlp84B in comparison with vertebrate CRPs. In addition to the identity and spacing of zinc-binding residues characteristic of the LIM motif consensus, many of the nonmetal coordinating residues are also conserved. In particular, residues that have been shown by nuclear magnetic resonance spectroscopic analysis of avian CRP1 (Perez-Alvarado et al., 1994) to be involved in hydrogen bonding and establishment of a hydrophobic protein core are highly conserved in the Drosophila muscle LIM proteins (Fig. 4 C); these residues are postulated to promote the proper overall fold of the LIM domain. The availability of the sequences of the Drosophila CRP family members has also pointed out a lack of conservation at some sites that were believed to be critical for establishing or maintaining the tertiary fold of the LIM domain of CRP1. These positions appear to accommodate more variability than previously thought, based on sequence comparisons of vertebrate proteins only. The overall sequence conservation, however, suggests that the global structural fold of the Drosophila Mlps is likely to be similar to their vertebrate counterparts and supports the notion that the proteins are functionally related.

A glycine-rich region follows each LIM domain in all the CRP family members and serves to distinguish CRPs from other LIM-only proteins. Interestingly, the glycine-rich region is the most highly conserved feature of the Drosophila muscle LIM proteins in comparison with vertebrate CRPs. The consensus sequence, GPKG(F/Y)GF(F/Y)GAG, overlaps with a putative nuclear targeting sequence, KKYGPK, and displays a sequence that resembles an RNA-binding motif, (K/R)G(F/Y)(G/A)FVX(F/Y), found in many ribonucleoproteins (Burd and Dreyfuss, 1994). Although we do not yet understand the role of the glycine-rich repeats, the high degree of conservation among all the family members shows that this region has been restricted from changing over time and is therefore likely to be functionally significant.

The regions between the LIM-glycine modules of vertebrate CRPs and Drosophila Mlp84B exhibit substantial heterogeneity in both length and sequence. It is not clear whether this heterogeneity is an indication that the linker regions represent functionally inert spacers or that the sequence divergence reflects key functional differences among the family members.

Discussion

The CRP family of LIM domain proteins consists of at least three highly related isoforms: CRP1, CRP2, and MLP/CRP3 (Liebhaber et al., 1990; Arber et al., 1994; Crawford et al., 1994; Weiskirchen et al., 1995). To investigate the possible role of CRP proteins in differentiation during development, we have initiated a reverse genetic approach in Drosophila melanogaster. Here we have reported the identification and initial characterization of two LIM genes in the fly, Mlp60A and Mlp84B. These genes encode proteins that share many features with vertebrate members of the CRP family.
Immunostaining reveals muscle LIM protein localization in the differentiating somatic musculature. Mlp60A is detected in all somatic muscles of a stage 16 embryo (A and C). Mlp84B protein is also expressed in the mature pattern of somatic muscles (B and D). A higher magnification view of five segments reveals that Mlp84B (D) immunoreactivity is enhanced at the muscle attachment sites, while Mlp60A (C) is not. (Arrows) Ventral-lateral longitudinal muscle attachment sites that are coincident with the segment borders (arrowheads). In all panels, embryos are oriented with anterior to the right and dorsal up. Bars: (A and B) 50 μm; (C and D) 25 μm.

The general structures of the *Drosophila* MIps are unique among the CRP family members because of the number of LIM-glycine modules. All vertebrate CRPs identified to date exhibit two LIM-glycine motifs, whereas *Drosophila* Mlp60A exhibits only one, and Mlp84B displays five complete LIM-glycine repeats. It is clear that a single LIM domain can act independently as a functional protein-binding unit (Schmeichel and Beckerle, 1994). If a single LIM domain is capable of mediating protein–protein interactions, then perhaps a protein like Mlp84B, with five repeats, could serve to dock five copies of the same protein or multiple proteins simultaneously. Recent work has highlighted the versatility and importance of modular protein-binding domains for protein function. Like LIM domains, the Src-homology domains (SH2 and SH3) may be found alone or in tandem with other functional domains within proteins. Even proteins comprised exclusively of SH2 and SH3 domains can function as adaptors that mediate the localized assembly of multimeric signaling complexes (for review see Pawson, 1994; Schlessinger, 1994). Thus, it seems plausible that the *Drosophila* Mlp84B protein, which displays five tandemly arrayed LIM domains, may act as a molecular scaffold that serves to juxtapose key signaling or structural components in a complex. Given the striking sequence similarity between the first LIM domain of Mlp84B and the only LIM domain of Mlp60A, it is possible that Mlp60A serves as a competitive inhibitor of Mlp84B function in muscle cells.

In fibroblasts and muscle cells, CRPs associate with the cellular actin cytoskeleton (Sadler et al., 1992; Arber et al., 1994; Crawford et al., 1994). We have shown that the *Drosophila* muscle LIM proteins retain the ability to associate with actin bundles when expressed in mammalian fibroblast cells. Since the regions of highest sequence conservation in the fly proteins correspond to the LIM-glycine repeats, it is likely that colocalization with actin is a conserved function that can be attributed to these regions. CRPs also interact with zyxin, a protein with LIM domains found at sites of cell adhesion where transmembrane signals are generated via integrin extracellular matrix recep-
tors. We have identified a Drosophila gene that encodes a protein related to zyxin (Macalma, T.B., B.E. Stronach, and M.C. Beckerle, unpublished results), suggesting that the function of CRP-zyxin complexes in vertebrate cells may also be conserved in the fly.

Mlp Expression and Muscle Development

Like the vertebrate CRP family members, we have observed that the expression patterns of Mlp60A and Mlp84B are both spatially restricted in the fly embryo and developmentally regulated throughout the life cycle of Drosophila. Mlp60A and Mlp84B display tissue-specific gene expression in a subset of muscular tissues in the developing embryo. In particular, we observed Mlp gene products in somatic, visceral, and pharyngeal muscles late in embryogenesis.

Although Mlp60A and Mlp84B are coexpressed within the somatic musculature, both the transcript and protein distributions are unique. Whereas Mlp60A mRNA is distributed throughout the muscle fibers, Mlp84B mRNA exhibits a polarized subcellular distribution, being localized at the ends of muscle fibers where they make attachments to the epidermis through the action of the PS integrins (Bogaert et al., 1987; Leptin et al., 1989). It is known that distribution of a specific mRNA can parallel the distribution of the cognate protein. For example, both Drosophila crumbs mRNA and protein are localized to the apical ends of polarized epithelial cells where Crumbs function is thought to be required (Tepass et al., 1990). Indeed, further analysis of the subcellular distribution of Mlp84B revealed an enrichment of protein at the muscle attachment sites. Thus, the polarized distribution of Mlp84B transcripts may serve as a source of localized protein, some of which remains associated with the attachment sites, while the rest is free to diffuse throughout the cytoplasm. The distinct subcellular distributions of Mlps in somatic muscle cells raise the intriguing possibility that Mlp84B functions within muscle cells at the attachment sites, or myotendinous junctions. This observation is consistent with the observation that vertebrate CRP family members interact with a constituent of integrin-rich junctional complexes (Sadler et al., 1992). In addition, all of the muscle tissues that express Mlp genes exhibit integrin-dependent attachment to extracellular matrix and highly ordered actin filament arrays (Crossley, 1978; Bogaert et al., 1987; Tepass and Hartenstein, 1994).

Of particular interest is the regulated entry of Mlp84B

Figure 11. Confocal immunofluorescence microscopy shows the subcellular localization of Mlp60A and Mlp84B with respect to muscle myosin. (A) Drosophila muscle myosin protein is detected in the somatic muscles of a stage 16 embryo. Note the cytoplasmic expression and exclusion of myosin from muscle cell nuclei; a few nuclei are marked with an asterisk. In contrast, Mlp60A (B) and Mlp84B (C) are detected in both the cytoplasm and the nuclei of somatic muscle cells in stage 16 embryos. (Arrowheads) Enrichment of Mlp84B (C) at the myotendinous junction, as opposed to Mlp60A that is not enriched there (arrowheads in B). (D) Representation of the ventral and lateral muscles of one abdominal segment that can be observed in A–C. This panel has been adapted from Bate (1990). (Arrowheads) Ventral-lateral longitudinal muscle that corresponds to those similarly labeled in B and C. Preimmune sera from rabbits immunized with either Mlp60A (E) or Mlp84B (F) fail to stain embryos. In all panels, embryos are oriented with anterior to the right and dorsal up. Bars: (A–C) 20 μm; (E and F) 40 μm.
Figure 12. Mlp84B is an early marker of functional myotendinous junctions. (Left panels) Immunofluorescent detection of Mlp84B in dorsal body wall muscles. (Right panels) Deeper confocal sections of the same embryos to assess the midgut morphology and, therefore, the stage of development. (A) Mlp84B is not enriched at the muscle fiber termini (arrowheads) in an early stage 16 embryo, when the midgut (B) is constricted but not convoluted. This section also reveals that Mlp84B protein is expressed in the visceral and pharyngeal muscles. Note that Mlp84B protein begins to accumulate at the developing myotendinous junctions (arrowheads in C) of somatic muscles at ~14 h after egg lay, midstage 16, when the midgut becomes convoluted (D). In all panels, embryos are viewed dorso-laterally and oriented with anterior to the right. Bar, 40 \( \mu \text{m} \).

The muscle-specific expression patterns of Mlp60A and Mlp84B in Drosophila, coupled with the extensive sequence conservation with vertebrate MLP/CRP3, a protein clearly involved in muscle cell differentiation (Arber et al., 1994), suggest that Mlp genes function in myogenesis. Examination of the temporal expression of Mlp genes during embryogenesis has provided a context for considering their roles in myogenesis relative to other genes expressed in muscle. The expression of the Mlp genes is most coincident with those processes that occur late in the muscle differentiation program, after specification, proliferation, and subdivision of the mesoderm, but just before markers associated with overt differentiation, like the contractile proteins (Fig. 14). The onset of Mlp expression in both somatic and visceral mesoderm occurs between 10 and 11 h of development (stage 14). Both Mlp60A and Mlp84B levels continue to increase, peaking between 16 and 24 h of embryogenesis (stage 17). Events that specify and subdivide the mesoderm are completed by 7 h of development (Bate, 1993), long before the onset of Mlp expression. Similarly, determination of the final fates of mesodermal cells, influenced by positional cues in the embryo (Frasch, 1995; Maggert et al., 1995) and requiring the restricted expression of transcription factors (Bate, 1993; Bernstein et al.,
cells with rhodamine-phalloidin (D) confirms colocalization with the actin cytoskeleton. Note that expression of Drosophila Mlp sequences does not appear to adversely affect actin filament arrays. Bar, 30 μm.

1993), temporally precedes expression of Mlp60A and Mlp84B by a few hours, effectively precluding their involvement in commitment or patterning. Instead, the timing of Mlp60A and Mlp84B expression is concomitant with late events in myogenesis, such as cell migration, attachment, and cytoskeletal rearrangements. Although some cell fusion is still occurring in the somatic lineage during the time the Mlps are expressed, we believe it is unlikely that Mlp60A or Mlp84B functions in this process because fusion begins at least 2 h before their expression, and, also, both genes are expressed in visceral and pharyngeal muscles that are mononucleate.

Other late myogenic events involve transcriptional up-regulation of genes required for terminal differentiation, such as those that encode proteins of the contractile apparatus. Recent work has highlighted the importance of Drosophila myocyte enhancer factor (MEF) 2, a MADS box-containing transcription factor homologous to the vertebrate MEF proteins, in this process (Bour et al., 1995; Lilly et al., 1995). Although Drosophila MEF2 is expressed in the developing mesoderm from very early on, mutations in the gene exert their effects late in the myogenic program, after myoblasts have been specified. The phenotype manifests as a disrupted muscle pattern and loss of terminal differentiation markers, such as myosin heavy chain, with the implication that MEF2 may regulate expression of late structural genes. The Mlp genes are expressed just before the expression of myosin heavy chain but concomitant with the lethal phase of MEF2 mutants (Michelson et al., 1990). MEF2 may therefore be a reasonable candidate for participation in regulating or in interacting with the muscle LIM proteins, and we cannot rule out a role for the Drosophila muscle LIM proteins in transcriptional control. Compatible with this notion is the observation that Mlp can be found in muscle cell nuclei, although the proteins do not appear to be concentrated there. Nonetheless, the hypothesis that Drosophila Mlps function late in myogenesis is consistent with studies showing that rat MLP/CRP3 is required for muscle differentiation subsequent to determination by the action of the MyoD family members (Arber et al., 1994). Likewise, expression of avian CRP1 protein coincides with the maturation of smooth muscle cells (Crawford et al., 1994).

The Mlp genes show a biphasic expression pattern, with a second peak of expression during metamorphosis, when additional myogenic events occur. The transition from a
larva to an adult fly requires massive changes in body and tissue morphology. Adult muscles are not simply remodeled from the existing larval musculature; rather, a second round of myogenesis occurs in preexisting groups of cells set aside during embryogenesis (Bate, 1993). We observed an increase in Mlp transcript levels during the transition from larval to pupal stages, at which time additional myogenic events take place, suggesting the Mlps are again required for proper differentiation. Interestingly, other myogenic genes, such as MEF2 and nautilus, show biphasic expression patterns that presumably also reflect a requirement for their function in both rounds of myogenesis (Michelson et al., 1990; Nguyen et al., 1994).

In summary, Drosophila muscle LIM proteins display muscle-specific distributions and developmentally regulated gene expression with peak expression corresponding to times when the musculature is differentiating. Mlp84B transcripts and protein are enriched at muscle attachment sites in the embryo, and both Mlp60A and Mlp84B have the ability to associate with the actin cytoskeleton when expressed in vertebrate cells. Based on our observations, together with evidence about the physiological function of vertebrate CRP proteins, we postulate that Mlp60A and Mlp84B play a role in cell differentiation late in myogenesis. Examination of the phenotypic consequences of eliminating Mlp60A and Mlp84B function will be required to define the functional significance of these proteins in vivo.

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References

Abmayr, S.M., M.S. Erickson, and B.A. Bour. 1995. Embryonic development of the larval body wall musculature of Drosophila melanogaster. Trends Genet. 11:153-159.

Andres, A.J., and P. Cherbas. 1992. Tissue-specific ecdysone responses: regulation of the Drosophila genes Eip28/29 and Eip40 during larval development. Development (Camb.). 116:865-876.

Arber, S., G. Halder, and P. Caroni. 1994. Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. Cell. 79:221-233.

Ausubel, F.M., R.B. Brent, R.E. Kingston, D.D. Moore, J.G. Siedman, J.A. Smith, and K. Struhl. 1994. Current Protocols in Molecular Biology. John Wiley & Sons, Boston, MA.

Bate, M. 1990. The embryonic development of larval muscles in Drosophila. Development. (Camb.). 117:791-804.

Bate, M. 1993. The mesoderm and its derivatives. In The Development of Drosophila melanogaster. M. Bate and A. Martinez Arias, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1013-1060.

Beckerle, M.C. 1986. Identification of a new protein localized at sites of cell-substrate adhesion. J Cell Biol. 103:1679-1687.

Berget, S.M. 1984. Are U4 small nuclear ribonucleoproteins involved in polyadenylation? Nature (Lond.). 309:179-182.

Bernstein, S.L., P.T. O’Donnell, and R.M. Craig. 1993. Molecular genetic analysis of muscle development, structure, and function in Drosophila. Int. Rev. Cytol. 143:63-152.

Bogatin, T., N. Brown, and M. Wilcox. 1987. The Drosophila Pk2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. Cell. 51:929-940.

Bour, B.A., M.A. O’Brien, W.L. Lockwood, S.G. Goldstein, R. Bodmer, P.H. Taghert, S.M. Abmayr, and H.T. Nguyen. 1995. Drosophila MEF2, a transcription factor that is essential for myogenesis. Genes & Dev. 9:730-741.

Boursnell, G., and G. Drewrys. 1987. Conserved structures and diversity of functions of RNA-binding proteins. Science (Wash. DC). 265:615-621.

Campos-Ortega, J.A., and V. Hartenstein. 1985. The Embryonic Development of Drosophila melanogaster. Springer-Verlag, Berlin, Germany. 227 pp.

Cavener, D.R., and S.C. Bay, editors. Academic Press, London. 499-560.

Dawid, I.B., R. Toyama, and M. Taira. 1995. LIM domain proteins. C.R. Acad. Sci. Ser. III. Sci. Vie. 318:295-306.

Feuerstein, R., X. Wang, D. Song, N.E. Cooke, and S.A. Liebhaber. 1994. The LIM/double zinc-finger motif functions as a protein dimerization determinant. Proc. Natl. Acad. Sci. USA. 91:10655-10659.

Flybase. 1994. The Drosophila genetic database. Nucleic Acids Res. 22:4356-4358.

Franch, M. 1995. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early Drosophila embryo. Nature (Lond.). 374:484-487.

Freyd, G., S.K. Kim, and H.K. Horvitz. 1990. Novel cytosine-rich motif and homoeodomain in the product of the Caenorhabditis elegans cell lineage gene lin-14. Nature (Lond.). 344:876-879.

Jowett, T. 1986. Preparation of nucleic acids. In Current Protocols in Molecular Biology. T.R.F. Wright, editors. Academic Press, London. 49%560.

Kiehart, D.P., and R. Feghali. 1986. Cytoplasmic myosin from Drosophila melanogaster. J. Cell. Biol. 103:1517-1525.

Kruse, L.J., J.W. Michelsen, H.A. Louis, J.I. Olsen, D.R. Davis, M.C. Beckerle, and D.R. Winge. 1994. Common metal ion coordination in LIM domain proteins. Biochemistry. 33:468-477.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (Lond.). 227:680-685.

Leptin, M., T. Bogaert, R. Lehmann, and M. Wilcox. 1989. The function of PS integrins during Drosophila embryogenesis. Cell. 56:401-408.

Liebhaber, S.A., J.G. Emery, M. Urbanke, X. Wang, and N.E. Cooke. 1990. Characterization of a human cDNA encoding a widely expressed and highly conserved cytosine-rich protein with an unusual zinc-finger motif. Nucleic Acids Res. 18:3871-3879.

Lilly, B., B. Zhao, G. Ranganarayakula, B.M. Paterson, R.A. Schultz, and E.N. Olsen. 1995. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila. Science (Wash. DC). 267:688-692.

Maggert, K., M. Levine, and M. Franch. 1995. The somatic-vascular subdivision of the embryonic mesoderm is initiated by dorsal gradient thresholds in Drosophila. Development (Camb.). 121:207-216.

Michelson, J.W., K.L. Schmeichel, M.C. Beckerle, and D.R. Winge. 1993. The LIM motif defines a specific zinc-binding protein domain. Proc. Natl. Acad. Sci. USA. 90:4404-4408.

Michelson, J.W., A.K. Sewell, H.A. Louis, J.I. Olsen, D.R. Davis, D.R. Winge, and M.C. Beckerle. 1994. Mutation analysis of the metal sites in an LIM domain. J. Biol. Chem. 269:11108-11113.

Michelson, A.M., S.M. Abmayr, M. Briz, A. Martinez Arias, and T. Maniatis. 1992. Expression of a Myc family member prefigures muscle pattern in Drosophila embryos. Genes & Dev. 4:2086-2097.

Muller, L., G. Xu, R. Wells, C.P. Hollenberg, and W. Piepersburg. 1994. LRG1 is expressed during sporulation in Saccharomyces cerevisiae and contains motifs similar to LIM and rhoactGAP domains. Nucleic Acids Res. 22:3151-3154.

Neddleman, S.B., and C.D. Wunch. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. 48:443-453.

Nguyen, H.T., R. Bodmer, S.M. Abmayr, J.C. McDermott, and N.A. Spoerel. 1994. D-mef2: a Drosophila mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis. Proc. Natl. Acad. Sci. USA. 91:7520-7524.

Pawson, T. 1994. SH2 and SH3 domains in signal transduction.

Sadler, I., A.W. Crawford, J.W. Michelsen, and M.C. Beckerle. 1992. Zyxin and PS2 antigen are two interactive LIM domain proteins associated with the cytoskeleton. J. Cell Biol. 119:1573-1587.
Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 545 pp.

Sanchez-Garcia, I., and T.H. Rabbitts. 1994. The LIM domain: a new structural motif found in zinc-finger-like proteins. Trends Genet. 10:315–320.

Sanger, F., S. Nicklen, and A.R. Coulsen. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463–5467.

Schlessinger, J. 1994. SH2/SH3 signaling proteins. Curr. Opin. Genet. Dev. 4: 25–30.

Schmeichel, K.L., and M.C. Beckerle. 1994. The LIM domain is a modular protein-binding interface. Cell. 79:211–219.

Skaer, H. 1993. The alimentary canal. In The Development of Drosophila melanogaster. M. Bate and A. Martinez Arias, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 941–1012.

Tautz, D. and C. Pfeifle. 1989. A nonradioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals a translational control of segmentation gene hunchback. Chromosoma (Berl.) 98: 81–85.

Tepass, U., and V. Hartenstein. 1994. The development of cellular junctions in the Drosophila embryo. Dev. Biol. 161:563–596.

Tepass, U., C. Therès, and E. Knust. 1990. crumbs encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. Cell. 61:787–799.

Valge-Archer, V.E., H. Osada, A.J. Warren, A. Forster, J. Li, R. Baer, and T.H. Rabbitts. 1994. The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells. Proc. Natl. Acad. Sci. USA. 91:8617–8621.

Wang, X., G. Lee, S.A. Liebhaber, and N.E. Cooke. 1992. Human cysteine-rich protein. A member of the LIM/double-finger family displaying coordinate serum induction with c-myc. J. Biol. Chem. 267:9176–9184.

Warren, A.J., W.H. Colledge, M.B. Carlton, M.J. Evans, A.J. Smith, and T.H. Rabbitts. 1994. The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. Cell. 78:45–57.

Way, J.C., and M. Chalfie. 1988. mec-3, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in C. elegans. Cell. 54:5–16.

Weiskirchen, R., and K. Bister. 1993. Suppression in transformed avian fibroblasts of a gene (crp) encoding a cysteine-rich protein containing LIM domains. Oncogene. 8:2317–2324.

Weiskirchen, R., J.D. Pino, T. Macalma, K. Bister, and M.C. Beckerle. 1995. The cysteine-rich protein family of highly related LIM domain proteins. J. Biol. Chem. 270:28946–28954.

Wu, R.Y., and G.N. Gill. 1994. LIM domain recognition of a tyrosine-containing tight turn. J. Biol. Chem. 269:25085–25090.