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A new member of the spectrin superfamily may participate in the formation of embryonic muscle attachments in *Drosophila*

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Summary

Myotube migration and the formation of muscle attachments are crucial events for the proper development of muscle patterning in the *Drosophila* embryo. This paper describes the identification of a new embryonic muscle-specific protein, MSP-300, in *Drosophila*. This protein is initially expressed by muscle precursors at muscle-ectoderm and muscle-muscle attachment sites. As development continues, MSP-300 becomes associated with muscle myofibrillar network. Studies of the subcellular localization of this muscle-specific protein in primary embryonic cultured myotubes show that MSP-300 decorates actin filaments, and that it is specifically enriched in sites where actin microfilaments are linked to the plasma membrane. Migrating myotubes exhibit high levels of this protein at their leading edge while, in myotubes that have already developed sarcomeric architecture, the protein is localized mainly at the Z-discs. Sequence of a partial 3.9 kb cDNA clone and molecular analysis of the predicted protein sequence of this protein indicates that it encodes a high relative molecular mass protein (~300×10^3), which exhibits at least five spectrin-like repeats. Several properties are shared by MSP-300 and members of the spectrin superfamily: it is associated with actin microfilaments, its sequence exhibits spectrin-like repeats and it is localized at sites where actin is linked to the plasma membrane. This protein could have a developmental role in the formation of muscle-ectoderm attachments and may be involved in myotube migration on the ectoderm.

Key words: *Drosophila*, muscle, muscle attachments, α-actinin, dystrophin, integrin.

Introduction

Muscle differentiation and the development of muscle patterning in the *Drosophila* embryo occur as a multistage process. Following germ band retraction, fusing myotubes extend their leading lamella over the epidermis and attach to it at specific locations – the muscle attachment sites (Crossley, 1978; Bate, 1990). The molecular mechanisms that allow directed myotube migration towards their attachment sites and the events that lead to their formation are unknown. As attachments between ectoderm and muscle cells or between muscle cells and their counterparts are established, cytoskeletal proteins such as actin, actin-associated proteins and myosin assemble to form muscle-specific sarcomeric architecture (Kiehart, 1990; Fyrberg and Goldstein, 1990). This cytoskeletal rearrangement is stabilized by connections to the extracellular matrix via the integrin receptors (Volk et al., 1990).

The ectoderm was shown to be an important factor in the determination of muscle patterning and the formation of muscle attachments at their proper locations (Williams and Caveney, 1980a,b). Integrins, heterodimeric proteins that mediate cell interactions with the extracellular matrix, may be involved in the establishment of somatic muscle attachments (Wright, 1960; Bogaert et al., 1987; Mackrell et al., 1988; Leptin et al., 1989). The integrin $\alpha_2\beta$ complex, is muscle specific whereas the integrin $\alpha_1\beta$ complex is expressed by the ectoderm. Both heterodimers apparently interact with each other, directly or via an extracellular matrix molecule at the attachment sites (Leptin et al., 1987, 1989; Bogaert et al., 1987). It has not yet been established whether integrin-mediated interactions initiate the formation of muscle attachments, or only stabilize such attachments by connection to the cytoskeleton. Localized integrin ($\alpha_1\beta$ complex) expression at the segmental grooves precedes the formation of muscle attachments (Leptin et al., 1989). However, in l(1)*myospheroid*, an integrin beta chain null mutant, in which neither heterodimer is functional, part of the muscle patterning is initially retained (T. Volk, unpublished observations). It is only later in development, when these muscles start to contract, that the myospheroid phenotype (rounded muscles) is notable (Wright, 1960; Newman and Wright, 1981). These observations suggest that additional factors are involved in the formation of muscle-ectoderm attachments.

Several actin-binding proteins were shown to mediate cellular migration in vertebrates (Bray and White, 1988; Forscher and Smith, 1988; Cunningham et al., 1991, 1992; Rodriguez et al., 1992). Spectrin and α-actinin were identified in the *Drosophila* embryo or larvae (Dubreuil et al., 1989; Byers et al., 1989; Kiehart et al., 1989; Fyrberg et al., 1990). α-actinin, an actin filament cross-linking protein, was identified in *Drosophila* by Fyrberg et al. (1990). A high degree of conservation between the *Drosophila* and

chick α-actinin genes was found. Phenotypic analysis of a null mutation for this α-actinin gene shows that the larval myotubes do not contain Z-discs; this results in muscle dysfunction and larval paralysis, leading to larval death. Interestingly, the embryonic muscle development and the unique muscle patterning is unaffected in these embryos (Fyrberg et al., 1990), suggesting the activity of other related genes. A spectrin β_H chain, which bears some similarity in size to vertebrate dystrophin, has been described in *Drosophila* (Dubreuil et al., 1987, 1990). This β_H spectrin forms heterodimers with α-spectrin and is expressed in adult head tissues, as well as in embryonic muscle and non-muscle tissues.

In this report, a novel *Drosophila* embryonic muscle-specific protein, MSP-300, is described. Evidence is provided to show that, during the early stages of embryonic muscle development, this protein is localized at the submembranous regions of muscle-ectoderm attachment sites, where microfilaments are linked to the membrane. Analysis of the distribution of MSP-300 in primary cultures of wild-type myotubes as well as molecular and biochemical studies indicate that it is a novel member of the spectrin superfamily. We suggest that this MSP-300 is primarily involved in the migration of muscle precursors on the ectoderm and the formation of muscle attachments.

Materials and methods

Fly stocks

Fly stocks used in this study were *y w*, which served as a wild-type strain, and the myospheroid mutant strain l(1)*mys*^{XG43} (Wieschaus et al., 1984).

Immunochemical reagents

Anti-MSP polyclonal antibodies were raised in guinea pigs against a fusion protein that included glutathione transferase sequences and the entire 3.9 kb MSP partial cDNA clone expressed in pGEX-1 vector (AMRAD, Australia) as described (Smith and Johnson, 1988). The serum was usually diluted 1:500 for staining and 1:2000 for western blot analysis.

Anti-beta integrin antibodies (mAb CFGC11), used as hybridoma supernatant for staining, were kindly provided by Dr M. Wilcox (MRC, Cambridge).

Actin was visualized with fluorescein-phalloidin (Sigma, Israel) or with monoclonal anti-actin antibodies (Sigma, Israel).

Secondary antibodies used included goat anti-guinea pig and rhodamine- or fluorescein-conjugated goat anti-mouse IgG or anti-guinea pig IgG (Jackson).

Embryonic cultures

Primary embryonic cultures were obtained essentially as described (Volk et al., 1990). In brief, embryos (6-9 hours old) were dechorionated, washed extensively and dissociated into a single cell suspension using a 7 ml Pyrex homogenizer (Corning no. 21). Following several washes with Schneider medium, the cells were seeded on laminin-coated coverslips. (*Drosophila* laminin was kindly provided by Drs L. and J. Fessler, UCLA). Cultures were maintained for one or two days. The cells were then washed with PBS, fixed and permeablized using a mixture of 3% paraformaldehyde and 0.5% Triton X-100 for 20 minutes, and then immunofluorescently labeled.

Whole-mount embryonic staining

Staining was performed essentially as described previously (Ashburner, 1989). In brief, embryos were collected and incubated as indicated, dechorionated and fixed with a mixture of 3% paraformaldehyde and heptan, and then devitellinized with a methanol-heptan mixture. Permeabilization was performed by incubation in 0.1% Triton X-100 for 2-3 hours, and incubation with primary antibodies was usually performed for 16 hours at 4°C.

Sections

Stained embryos were dehydrated and cleared in methyl salicylate, washed in ethanol and infiltrated with JB-4 embedding media (Polysciences, Inc., USA) according to the manufacturer's instruction. Embryos were oriented in molds and allowed to harden in a desiccator. Sections (2-3 μm width) were obtained in a Sorvall MT2B microtome, mounted on slides and visualized under a Nikon microphot-FX.

Western blot analysis

Membranes were isolated from dissociated embryos, after centrifugation for 30 minutes at low speed (1500 ***g***) to remove cell debris. The supernatant was centrifuged at high speed (Eppendorf centrifuge) for an additional 30 minutes. The resulting pellet was boiled in sample buffer and subjected to SDS-electrophoresis on 6% SDS-polyacrylamide gels according to Laemmli (1970). Protein was transferred to nitrocellulose according to Towbin et al. (1979). The nitrocellulose was blocked with PBS containing 10% milk and 1% Tween-20, reacted with first antibody, washed and reacted with second antibody (HRP-conjugated anti-guinea pig). The ECL Western blotting system (Amersham) was used for detection.

Actin sedimentation assays

Actin sedimentation assays were carried out as previously described (Dubreuil et al., 1987; Pavalko and Burridge, 1991). 1-5 mg of rabbit skeletal muscle G actin were mixed with the RIPA (50 mM Tris pH 7.3, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1% NP-40) soluble fraction protein mixture of embryonic membranes. The RIPA soluble fraction was separated from pelleted insoluble proteins (airfuge at 100,000 ***g***) prior to addition of the G actin. Actin polymerization was induced by adding 1 mM MgCl, 0.1 mM ATP, 0.1 mM 2-β mercapto-ethanol to the actin mixture. After 30 minutes at room temperature, the mixtures were sedimented in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) at 100,000 ***g*** for 30 minutes. Parallel samples were processed without actin. Pellets were analyzed on western blots with anti-MSP and anti-actin antibodies.

Cloning of MSP-300

MSP cDNA was isolated following the screening of a λgt11 expression library from 9-12 hour old embryos (prepared by Zin et al., 1988) with anti-laminin-binding protein monoclonal antibody. The 4 kb insert was excised from the lambda vector with *Eco*RI and subcloned into Bluescript.

Northern blot analysis

RNA was extracted and run over an oligo(dT) column according to Lev and Segev (1986). Poly(A)$^+$RNA (5 μg/lane) was separated on an agarose-formaldehyde gel, blotted to Hybond-N (Amersham) and hybridized with 50% formamide at 58°C. Detection was performed using a radiolabeled 3.5 kb *Eco*RI fragment of the 4 kb cDNA clone.

DNA sequencing

DNA sequencing was carried out using Sequenase kit (US Bio-

chemical), and synthetic 17-mer oligonucleotides were synthesized as primers at ~200 bp intervals. Sequence data were assembled and analyzed using the GCG program (Devereux et al., 1984). Dotplot comparisons were performed using the program Compare at a stringency of 35 identical matches/window of 100 residues, and were displayed using the Dotplot program. Genbank was searched using the program Tfasta.

Results

Identification and isolation of a muscle-specific cDNA clone

In a search for genes that are specifically expressed by myotubes during their migration and the formation of muscle attachments, a muscle-specific cDNA clone was isolated. A cDNA expression library from 9- to 12-hour-old embryos (prepared by Zin et al., 1988) was screened with a monoclonal antibody to a laminin-binding protein (Volk T., unpublished results). Out of several clones identified, one 3.9 kb fragment (MSP-300) was found to be unrelated to the laminin-binding activity. In situ hybridization with this cDNA clone on whole embryos indicated that it is expressed by myotubes by the end of germ band retraction (not shown). This expression pattern prompted us to characterize the clone further.

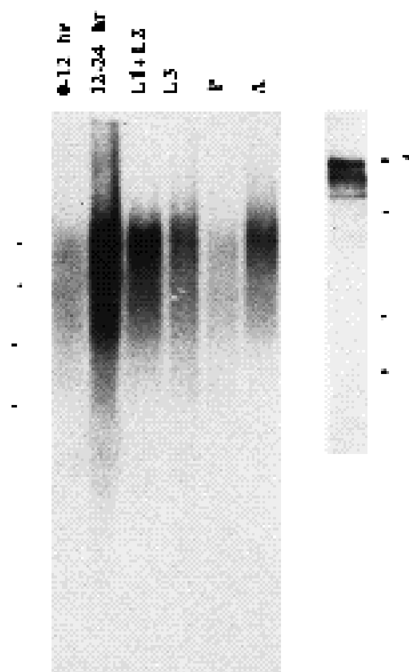

Fig. 1. (A) Northern blot showing developmental expression of MSP-300. Poly(A)$^+$ RNA (5 μg/lane) was extracted from different developmental stages. The blot was probed with a 3.9 kb cDNA antisense RNA probe. Lanes represent 0- to 12-hour embryos; 12- to 24-hour embryos; first- and second-instar larvae (L1+L2); third-instar larvae (L3); pupae (P); adult flies (A). (B) Visualization of MSP-300 by western blot analysis. Proteins from membranes taken from dissociated embryos were separated on a 6% SDS-polyacrylamide gel, transferred to nitrocellulose and reacted with anti-MSP-300 antibodies raised against a fusion protein that included glutathione transferase and the entire 3.9 kb cDNA. Molecular masses are shown at right.

Northern analysis with the muscle-specific cDNA clone indicated that its mRNA size extends to ~13 kb (Fig. 1A). Developmental northern analysis (Fig. 1A) shows that it is highly expressed during the second half of embryonic development. A lower level of expression is maintained throughout development, including all larval and adult stages with the exception of the pupal stage.

Polyclonal antibodies were raised against a MSP-300/glutathione transferase fusion protein in a pGEX vector expression system. Western blot analysis of membrane extracts of embryos (3-16 hours old) with these polyclonal antibodies reveals that the apparent relative molecular mass of this protein is approximately 300×10^3 (Fig. 1B), which is in accord with the size of its mRNA.

To map the gene, a biotinylated probe was prepared from the cDNA clone and hybridized to polytene chromosomes. Hybridization was detected on the left arm of the second chromosome at position 25C, in close proximity to the 25C/D boundary (not shown).

Sequence analysis of the MSP

Sequence analysis of the 3.9 kb cDNA clone was carried out and is shown in Fig. 2. The *Eco*RI site at the 5′ region was in frame with an open reading frame of 3.5 kb, followed by 400 bp of non-coding sequence.

A search for homologous sequences in the database has not revealed high homology to any known protein sequences. A low level of homology was found between the deduced amino-acid sequence of this protein and chick α-actinin (47% similarity, 20% identity) and human dystrophin. Comparison by dotplot of the predicted protein sequence of MSP-300 to itself indicates that it can be subdivided into repetitive and non-repetitive domains (Fig. 3A). Repetitive sequences are found between residues 68 and 460, and between residues 587 and 812 (see also Fig. 4). The predicted protein sequence of MSP-300 was also compared to the sequences of repetitive domains of human dystrophin (Fig. 3B), chick α-spectrin (Fig. 3C), and chick α-actinin (Fig. 3D). The repeat-containing domain of MSP-300 show a low level of homology to that of dystrophin, α-actinin and spectrin. The repetitive regions in MSP-300 were further identified using the Bestfit program. A periodicity of ~120-130 residues was found (see Fig. 4). While proteins from the spectrin superfamily have been shown to exhibit an ‘EF hands’ Ca^{2+} binding motif at their C terminus, no such motif was found in the MSP sequence. Since the NH_2 terminus of the protein has not yet been sequenced, it is not known whether it contains the characteristic globular, actin-binding domain as appears in α-actinin, dystrophin and spectrin.

MSP-300 is an actin-associated protein

The sequence homology found between MSP-300 and the spectrin superfamily raises the possibility that MSP-300 belongs to this superfamily. Proteins from the spectrin superfamily comprise the membrane skeleton, and were shown to be actin-binding proteins (Hammond, 1987; Mimura and Asano, 1987; Baron et al., 1987). The association of MSP-300 with actin filaments was tested in two

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1     GAATTCCCCGAAGGTGAGCTACGCACCACCAGTCTCCCCGTTTTGGAGGAGCAGCTGGCC   60
1     E  F  P  E  G  E  L  R  T  T  S  L  P  V  L  E  E  Q  L  A     20
61    CATTACAAGAAGCTCCTCAGTGATGCCGAGAATAAGGGCGGCCTCATCAACGATGTTTCG   120
21    H  Y  K  K  L  L  S  D  A  E  N  K  G  G  L  I  N  D  V  S     40
121   GAGCAAGGAAAGAGCATCCTGCCCACACTTAGCAATGCCGATAAATTGAAACTCAACGAT   180
41    E  Q  G  K  S  I  L  P  T  L  S  N  A  D  K  L  K  L  N  D     60
181   GATATCAAGAACATGAAGGATCGTTATGGCAGAATCAAGAATACCATCGATGATCGCGTG   240
61    D  I  K  N  M  K  D  R  Y  G  R  I  K  N  T  I  D  D  R  V     80
241   AACGCCTTGGGTGATCACATCAAGAAGTACAAGGATGCCAAGAGCAGGTTGGCCGAGTGC   300
81    N  A  L  G  D  H  I  K  K  Y  K  D  A  K  S  R  L  A  E  C     100
301   AGTCAGTTCTTGGGTAATATTCAACAGAAACTCAGGGAACTCAATCGCCCGATTGGATCC   360
101   S  Q  F  L  G  N  I  Q  Q  K  L  R  E  L  N  R  P  I  G  S     120
361   AGGATCGAAGATGTCCAGGACTTGTTGGGTGCCTATGAGGGAATTCTCAAGGAACTCAAG   420
121   R  I  E  D  V  Q  D  L  L  G  A  Y  E  G  I  L  K  E  L  K     140
421   GACAGCAAGAGCAAAATGGGCGATATGCAGATGGATGATTTGCCCGAGTTGCAGAGCATT   480
141   D  S  K  S  K  M  G  D  M  Q  M  D  D  L  P  E  L  Q  S  I     160
481   TTGGCCCAGCAGGATGATATGATTAAACTGATTGAAGATCAGTTGGCCCATCTTCGCCAG   540
161   L  A  Q  Q  D  D  M  I  K  L  I  E  D  Q  L  A  H  L  R  Q     180
541   CTTCTGCTGCTCCGCGAGCAGTTTATTGCTTTGATTAATGAGATTATTGCTTTTATCATG   600
181   L  L  L  L  R  E  Q  F  I  A  L  I  N  E  I  I  A  F  I  M     200
601   AAGTACACCGATGTTATCATAGACATTGAAAACTCACCTGATAGTCTGGAGGATAAGATC   660
201   K  Y  T  D  V  I  I  D  I  E  N  S  P  D  S  L  E  D  K  I     220
661   AACAAGTACGATGATGTGATTGTGAAGATTCAGGAGTGCGAAGGTGTCTTGGCCTCGGCC   720
221   N  K  Y  D  D  V  I  V  K  I  Q  E  C  E  G  V  L  A  S  A     240
721   AATGATAAGGGCCAGAAGATTGCCTCCGAGGGTAATGCTGCCGATAAGAACAGCATTACA   780
241   N  D  K  G  Q  K  I  A  S  E  G  N  A  A  D  K  N  S  I  T     260
781   GAGCAGTTGCAGTCCCTAAAGAATCAGCTGCAGAATCTCCGAAAGGCGGTAGAATCGCAG   840
261   E  Q  L  Q  S  L  K  N  Q  L  Q  N  L  R  K  A  V  E  S  Q     280
841   CGCCAGAAGCATCAACTCCAACTGGAATCCCACAAGAAGATGGCCGCCGAACTGAGTGAG   900
281   R  Q  K  H  Q  L  Q  L  E  S  H  K  K  M  A  A  E  L  S  E     300
901   ATCCTCGATTGGTTGCACAGCCACGAGGGAGCGGCTAAGTCGCGTCCTCTACTGGATCGG   960
301   I  L  D  W  L  H  S  H  E  G  A  A  K  S  R  P  L  L  D  R     320
961   GATCCCGAGTCCGTGGAGCGCGACGTGCAGAAGCACCAGTCTCTCAGCCAGGACATCGAA   1020
321   D  P  E  S  V  E  R  D  V  Q  K  H  Q  S  L  S  Q  D  I  E     340
1021  TCCTATCTAAATAAATTCAACAAAATCAATGATGGTGTCAAAACGGAAATTGGAATGCCA   1080
341   S  Y  L  N  K  F  N  K  I  N  D  G  V  K  T  E  I  G  M  P     360
1081  AGTTCCCTGTTGGAAATGCTCTCCGAGGGCAGATCCCTGGTGGCTTCACTGCCCCATGAA   1140
361   S  S  L  L  E  M  L  S  E  G  R  S  L  V  A  S  L  P  H  E     380
1141  TTGGAGGAGCGTGAAAAGTATCTGAAGAACAACCGCGACTCTCGTTTGGAGTACATGCAA   1200
381   L  E  E  R  E  K  Y  L  K  N  N  R  D  S  R  L  E  Y  M  Q     400
1201  CTGGTGGCGAAGTTCAATGACTGGGTCCATGAGGCCGAGTTGCGCCTGCAGAACAGCCAG   1260
401   L  V  A  K  F  N  D  W  V  H  E  A  E  L  R  L  Q  N  S  Q     420
1261  CATGGCATCGACTACGAGCACTTGGTCCAGGATCTCGACGAACACAAGATCTTCTTTGGC   1320
421   H  G  I  D  Y  E  H  L  V  Q  D  L  D  E  H  K  I  F  F  G     440
1321  AATGAGGCACCCATCCGTAACCTGGTGCACAAACAGATCCAGGAGGCCGCCGATAAGATC   1380
441   N  E  A  P  I  R  N  L  V  H  K  Q  I  Q  E  A  A  D  K  I     460
1381  TGGTCCTCGCTGAACAACTACGAACAGTCGGAACTCTCGGCGGAGTTGGCTCAGTTCCAA   1440
461   W  S  S  L  N  N  Y  E  Q  S  E  L  S  A  E  L  A  Q  F  Q     480
1441  ACCAAGTTGACCAACACACTGGCCAATGCCAAGACCCAACAGAGTGAACTGGAGAAGGAG   1500
481   T  K  L  T  N  T  L  A  N  A  K  T  Q  Q  S  E  L  E  K  E     500
1501  GCGGAACGTGGCGGGAATACCAACAGTCAATCGATCGCGTCAAGGCCACCATCGAACGTA   1560
501   A  E  R  G  G  N  T  N  S  Q  S  I  A  S  R  P  P  S  N  V     520
1561  CCAAGTTCGTTGATGAGCCCGTCCAAAATTTGGCTGGACTCCACTTCAATATCCAGAAGC   1620
521   P  S  S  L  M  S  P  S  K  I  W  L  D  S  T  S  I  S  R  S     540
1621  TGTCGCACGCCATTGGCAAGTGTTCAGAGCCAAAACAGCGACTTAACGCTAGTCAACCAG   1680
541   C  R  T  P  L  A  S  V  Q  S  Q  N  S  D  L  T  L  V  N  Q     560
1681  CAGGCCCAATCGTTGATCCGCCAGGCGGACGCTCGCAATCGCCAGCTGATCGAGCAGGAT   1740
561   Q  A  Q  S  L  I  R  Q  A  D  A  R  N  R  Q  L  I  E  Q  D     580
1741  AACGCCGGACTGAATAGATCTTGGCAGGATCTGGTGCGCACGCTGGAACAGCGACGCGAC   1800
581   N  A  G  L  N  R  S  W  Q  D  L  V  R  T  L  E  Q  R  R  D     600
1801  AACCTGCAACAGTTGGCCGAACATTGGGACGGCTTCGAGAACAGCCTGCACGCCTGGGAA   1860
601   N  L  Q  Q  L  A  E  H  W  D  G  F  E  N  S  L  H  A  W  E     620
1861  AAGGCACTTGGTCGACTGGAGGACAAGTTCCGCAATGTCGATCCGACTGTAAGATCCCGA   1920
621   K  A  L  G  R  L  E  D  K  F  R  N  V  D  P  T  V  R  S  R     640
1921  CGTCACTTGGAAGATACGAAGAATGCCATTCAGGAACTGCGTGAAGAATCAAATCAACTT   1980
641   R  H  L  E  D  T  K  N  A  I  Q  E  L  R  E  E  S  N  Q  L     660
1981  AAATCATCACATAAAGAAATCGAGGCGCTCTCTAAATCCATCCTCACATTCCTTGGGGAA   2040
661   K  S  S  H  K  E  I  E  A  L  S  K  S  I  L  T  F  L  G  E     680
2041  GTACACAAACCCTCGGCGGAGGCCATACAGGCCAAAGTGGATAAGTTAGTTGAACAGCAG   2100
681   V  H  K  P  S  A  E  A  I  Q  A  K  V  D  K  L  V  E  Q  Q     700
2101  GCCAAATTGAACGACACTCTGCGCGATAAGGAGCAACAGGTTAGCAAGGATCTCGAGGAG   2160
701   A  K  L  N  D  T  L  R  D  K  E  Q  Q  V  S  K  D  L  E  E     720
2161  ATCGAGCAAGTCTTCCGTCGCATCTCGCAGCTGCAGGACAAGCTAAACGCGCTTCACGAG   2220
721   I  E  Q  V  F  R  R  I  S  Q  L  Q  D  K  L  N  A  L  H  E     740
2221  CAACTACAATCGGTTCACGTCTACGACGAGCACATAGCCCAAACGGAACAGCTCCTCATC   2280
741   Q  L  Q  S  V  H  V  Y  D  E  H  I  A  Q  T  E  Q  L  L  I     760
2281  ACATTGAACAGCCAGGTGCAGCAGGCCGCCGAGGAGAGCAAATTACTGGTGGCCCAGACA   2340
761   T  L  N  S  Q  V  Q  Q  A  A  E  E  S  K  L  L  V  A  Q  T     780
2341  ACGGCCCACTACCAGGCCAAACAGAATCAGCTGCCCAGCGATATTGCCCAGGAGTTTACG   2400
781   T  A  H  Y  Q  A  K  Q  N  Q  L  P  S  D  I  A  Q  E  F  T     800
2401  GCTCTTGAGCTACTTGCCGAACGCGTCCAAGTGACCATGGAGACCAAAGAGAAGGATTTC   2460
801   A  L  E  L  L  A  E  R  V  Q  V  T  M  E  T  K  E  K  D  F     820
2461  AAGCGGGCCAAGACCGTGCGCACCGAATATGTGGACGGTGTGGACGAGGTGCAGCGTTGG   2520
821   K  R  A  K  T  V  R  T  E  Y  V  D  G  V  D  E  V  Q  R  W     840
2521  CTGCTCCAGGCAGAGGTGCAGGTGCAGGAGAGAAGCCTCACACCCACACAGATGAAGGAG   2580
841   L  L  Q  A  E  V  Q  V  Q  E  R  S  L  T  P  T  Q  M  K  E     860
2581  CTGCTGCAGCGCATTAACCACGAGATCACCGCCATCTACGAACGCTTCACCCTGGTCAAG   2640
861   L  L  Q  R  I  N  H  E  I  T  A  I  Y  E  R  F  T  L  V  K     880
2641  ACCAACGGTCAGCTGATCATCGAGAACTGTCGCAACAGCGAGGAGAAGACGCTGGTGCAG   2700
881   T  N  G  Q  L  I  I  E  N  C  R  N  S  E  E  K  T  L  V  Q     900
2701  ACGACCATCGATCAGTTGGCCGCATCGCTGGCCCAGGTACGCGGTTGGCTGGATGAGAAG   2760
901   T  T  I  D  Q  L  A  A  S  L  A  Q  V  R  G  W  L  D  E  K     920
2761  AAGCAGGCGGTGGGCGATAGTCTGGACGCGTCCACGAGGTTCATGAACCTCTACCAGATC   2820
921   K  Q  A  V  G  D  S  L  D  A  S  T  R  F  M  N  L  Y  Q  I     940
2821  GTGATGTCGTGGGCATCCGAGAAGCGCAACTTCACTGACCAGACCATCGAGCTGCGCACC   2880
941   V  M  S  W  A  S  E  K  R  N  F  T  D  Q  T  I  E  L  R  T     960
2881  CTGCCCGAGGCACGCAACAAATTGAACGACTATGTGACGTCTGTGAAGAGTATTAAACCG   2940
961   L  P  E  A  R  N  K  L  N  D  Y  V  T  S  V  K  S  I  K  P     980
2941  ATTGTGAAGCATCTGAGCGAAATGGACAAGGAACTGGAGCACATTGGCCAAGTGACGACT   3000
981   I  V  K  H  L  S  E  M  D  K  E  L  E  H  I  G  Q  V  T  T     1000
3001  GTGGGCGATCTCAAGGACAAACTGCAGGAGGCCGAGGATGCGAAGATATCCGTGGAAGCG   3060
1001  V  G  D  L  K  D  K  L  Q  E  A  E  D  A  K  I  S  V  E  A     1020
3061  GTGCTGCTAGAGAGGAACTCCCTGCTGCAAGAGGCCTGCGAGGAATGGGACCAATGTGAA   3120
1021  V  L  L  E  R  N  S  L  L  Q  E  A  C  E  E  W  D  Q  C  E     1040
3121  CGCAAGATTAAGGATATACGCTCCTGGCACGAAAAGACGAAGCAGGGACTGGACTCCTCG   3180
1041  R  K  I  K  D  I  R  S  W  H  E  K  T  K  Q  G  L  D  S  S     1060
3181  CAGCAGCAGAAGAAACCGCTGCGCGATCAGCTCGGTTTCTGTGAGAAGACCCTGGCCGAT   3240
1061  Q  Q  Q  K  K  P  L  R  D  Q  L  G  F  C  E  K  T  L  A  D     1080
3241  ATCAATGTGCAGAAAACGAAACTGAGACTGTCCATCGAAAAACTGGAGGTTCATTTCCGC   3300
1081  I  N  V  Q  K  T  K  L  R  L  S  I  E  K  L  E  V  H  F  R     1100
3301  AATGGCATGGGCGGTGATCCGCGCCTGAGCGAGAACGTCGATGATCTGGTGCGCGTGCTC   3360
1101  N  G  M  G  G  D  P  R  L  S  E  N  V  D  D  L  V  R  V  L     1120
3361  GACGGCCTCGGCGAATTGGTCAAGGCCAAGTCGCAGAGCCTCGAGCAGACGCTGGCCCAG   3420
1141  I  D  V  Y  Q  Q  Q  M  Q  S  L  R  Q  R  I  I  Q  E  E  Q     1160
3481  CACGTCCGCCTGGTGATGGCGCCCACGTACTTGCCGCACGATGCGAGCGCGCATTAGCCG   3540
1161  H  V  R  L  V  M  A  P  T  Y  L  P  H  D  A  S  A  H  *        1180
3541  AGCAACAGGCATGCCGGGAACGTGTCAAAAATCTGCATTCGAAAATAACTGCACGTAATG   3600
3601  AGCGCATCAAGTTGTTAATACATCGCGGCAAGCCGGACGATGCCAAGCTAGAGATTTAGG   3660
3661  ATGCCACAACCTTAACCATAAGATAAAGCCAATCTAGGAGTACATCAAAGCCAAAGCAGC   3720
3721  CACCCCGGCGTCTTCCGTTTGCCCCTGCCACCGATTTCCTTCTCCCAGGAACGGTGGCTA   3780
3781  CGGTACAACTGTCAACTGCCGTCATTACTCTAATCAATGAAACATGACCCGTGTTTTCGA   3840
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Fig. 2. Nucleotide and amino acid sequence of the entire 3.9 kb cDNA clone.

separate experiments: first, a study of the interrelationships between MSP-300 and the microfilament system in primary cultured myotubes was carried out. Myoblasts were allowed to undergo fusion and to differentiate in vitro. The cells were fixed, permeabilized and double labeled for MSP-300 and actin. Prominent staining with the MSP-300 antibodies was noticed at three subcellular locations in the myotubes. (1) A dot-like staining was noticed along the actin microfilament system (Fig. 5(1)A and B). This staining was prominent only in muscle cells but not in any other cell type, such as epithelial colonies or nerve processes. (2) Following two days in culture, some of the myotubes develop sarcomeric architecture in which MSP-300 was associated with Z discs (Fig. 5(1) C and D). (3) In migrating myotubes, the staining of MSP-300 was concentrated at the leading edge of the cell (as in Fig. 5(2) B). The staining pattern at this region consisted of small radial, regularly spaced spikes. Non-migrating myotubes expressed MSP-300 circumferentially in small ordered spikes at the cell edges (Fig. 5(2)A).

A biochemical approach was also taken to test the association of MSP-300 with actin filaments. A detergent-soluble extract of the embryonic membrane fraction was obtained. This extract was mixed with rabbit skeletal muscle G actin, and the actin was allowed to polymerize in a polymerization buffer. The actin filaments formed were precipitated and the pellet was analyzed by western blot for the presence of MSP-300. As shown in Fig. 6, MSP-300 was present in the filamentous actin-containing fraction, but was not found in a pellet that did not contain actin. This experiment indicates that MSP-300 is either associated directly or indirectly with actin filaments. Thus, the subcellular localization of MSP-300 and its biochemical association with rabbit actin filaments strongly suggest that MSP-300 is an actin-binding protein.

Developmental distribution and subcellular localization of MSP-300 during embryogenesis

The embryonic distribution of MSP-300 was studied by examining whole-mount-stained embryos at various developmental stages. The subcellular localization of MSP-300 was further investigated by microscopic analysis of semithin stained sections. The protein is initially expressed towards the end of germ band retraction (stage 12) in a small subset of muscle precursors in each segment at ventral, lateral and dorsal locations (Fig. 7A). No significant differences in the intensity of staining have been noticed among these three locations. However, the staining pattern of MSP-300 as revealed by whole-mount embryonic staining, is elongated rather than patch-like, suggesting a polar concentration of the protein at the edge of the muscle precursors. Visceral muscles also express MSP-300 at this stage of development (Fig. 7B). No polarized staining has been observed in the visceral mesoderm. Analysis at a higher level of resolution using semithin sections (~2 μm) of these stained embryos, reveals prominent membranal staining specifically enriched at the attachment sites between the muscle precursors and the ectoderm (Fig. 8A).

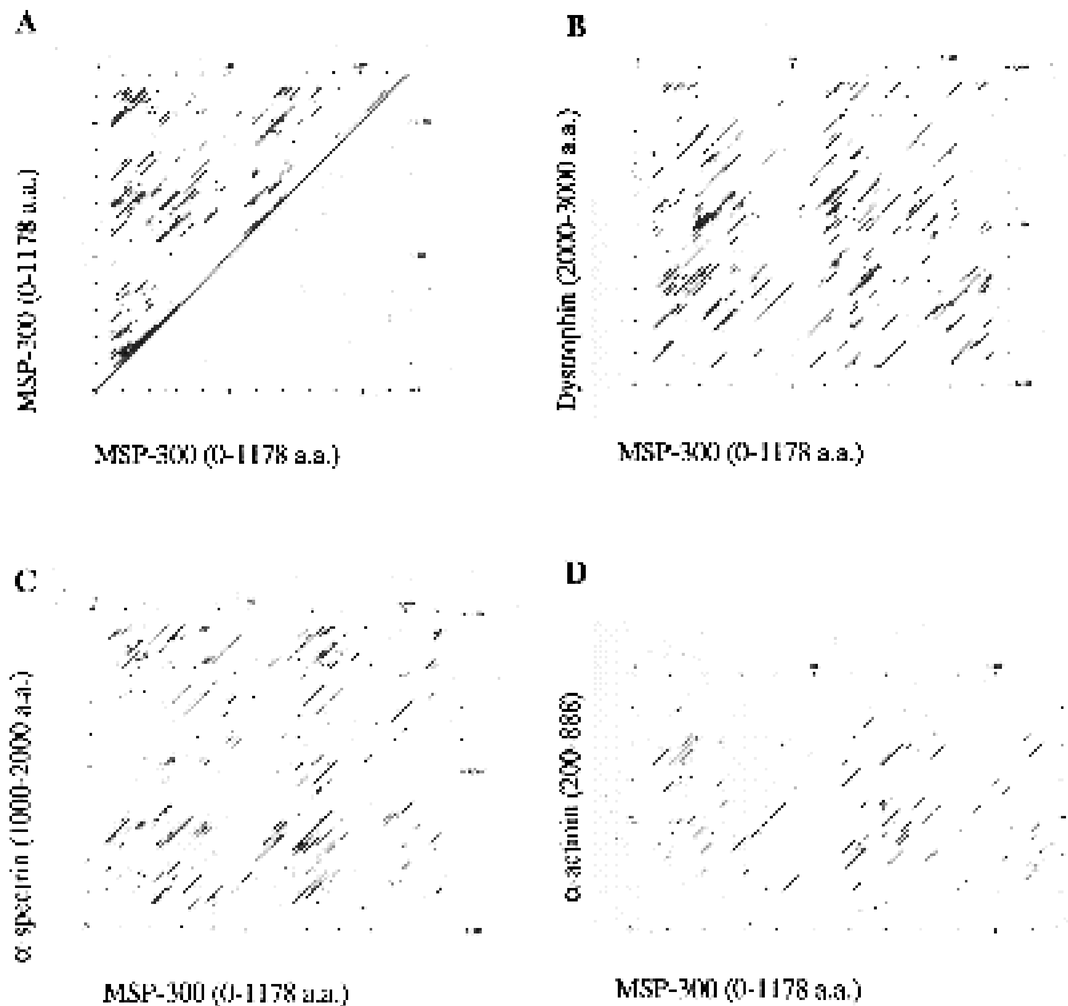

Fig. 3. Dotplot comparisons of MSP-300 with sequences of MSP-300, dystrophin, α-spectrin and α-actinin. The predicted polypeptide sequence of the 3.9kb MSP-300 cDNA fragment was compared to itself to identify repeat-containing sequences (A), to the corresponding regions of human dystrophin (B), to the corresponding regions of chick α-spectrin (C), and to the full-length chick α-actinin (D) at a stringency of 35 identical matches/100 residue window. Notice that repetitive sequences that are found between residues no. 68 to 460, and between residues 587 to 812 in MSP-300 are also identified in comparisons with the sequences of the other proteins.

As development continues, attachments are formed between myotubes and the ectoderm and between neighboring myotubes. At this stage, the protein is expressed in all the muscle cells, where it is almost exclusively localized at muscle-muscle and muscle-ectoderm attachments (see Fig. 7C,D and Fig. 8A,B). Towards the end of embryogenesis, the staining of the protein becomes prominent not only along the membranes but also inside the cytoplasm of the elongated muscle cells. This can be seen in whole-mount staining (Fig. 7E,F) and also in stained semithin sections (Fig. 8C). The prominent staining at the attachment sites is no longer visible at this stage, suggesting a second function for the protein at this stage of development.

MSP-300 co-localizes with integrin at focal adhesion and cell-cell contact sites

All the three proteins that belong to the spectrin superfamily, namely spectrin, α-actinin and dystrophin, were shown to be associated with an integral membrane protein, which is tightly associated with the extracellular matrix (Cohen and Branton, 1979; Bennett and Stenbuck, 1979; Campbell and Kahl, 1989; Otey et al., 1990; Ibraghimov-Beskrovnaya et al., 1992). The possible association of MSP-300 with receptors of the integrin family was examined. For this purpose, the co-localization of MSP-300 and integrin in primary cultured myotubes was analyzed. Primary cultures were obtained as described above, fixed and double labeled for MSP-300 and β-integrin. As shown in Fig. 9 (A,B), the staining of both proteins is partially overlapping. While most of the cell types in the culture exhibit β-integrin staining, only the muscle cells were labeled for MSP-300. Careful comparison of the staining pattern of both proteins in the same cells reveals that they partially co-localized at small patches which may represent focal adhesion plaques. In some cases, MSP-300 was identified at intercellular contacts between myotubes and non muscle cells. However, there were many muscle-non muscle cell contact sites in which integrin staining was prominent and no MSP-300 staining was identified, suggesting that MSP-300 may be only transiently expressed at the contact sites.

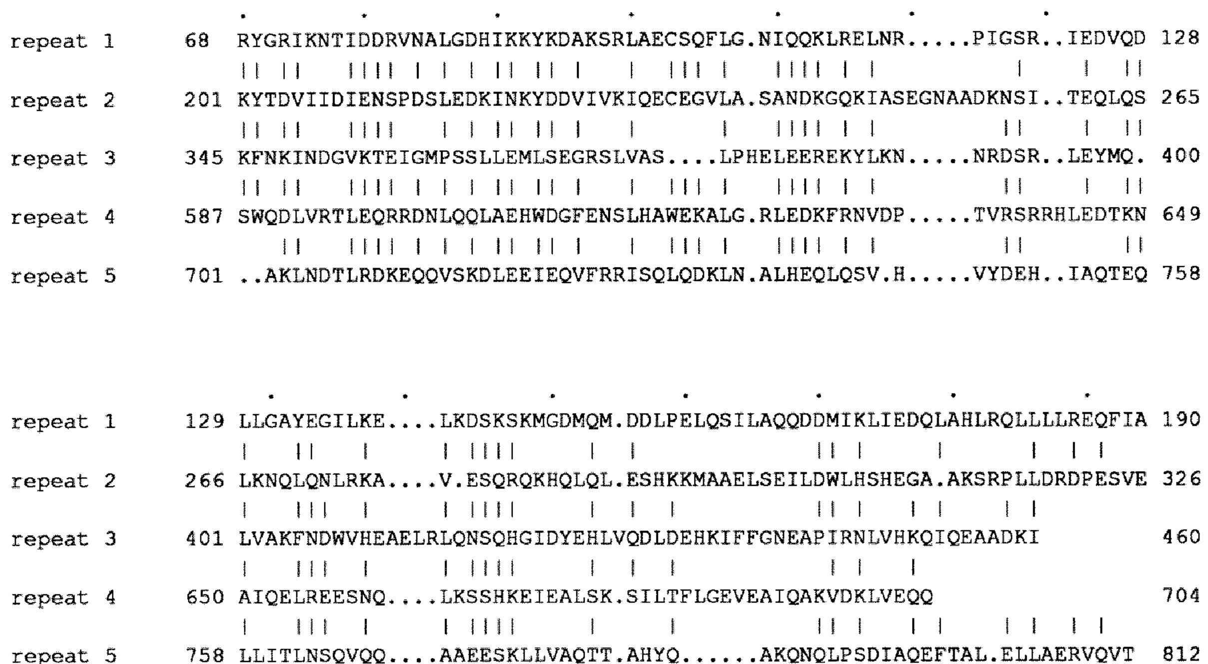

Fig. 4. Repeated sequences identified in the amino acid sequence of MSP-300. The repetitive sequences identified by the dotplot comparisons were aligned in register using the BESTFIT program. Five repeated sequences were identified; each repeat contained ~120-130 residues. Alignment was carried out according to the following grouping: hydrophobic (L,I,V,M,W,Y,F,H,C,A,T), hydrophilic (D,E,K,H,R,N,Q,S,T) and aromatic (W,Y,F,H).

Changes in MSP-300 subcellular localization in somatic muscle cells deficient for integrin

Since both proteins, MSP-300 and integrin, partially co-localize in cultured myotubes, it was important to examine further their interrelationship. We wished to determine whether the membranal localization of MSP-300 that was detected in the muscle precursors was determined by the distribution of integrin. To answer this question, a detailed study of the distribution of MSP-300 in *myospheroid* muscle cells was performed. Embryos from the *myospheroid* XG43 line were first stained for integrin. The homozygous mutant embryos in which integrin was not found were restained for MSP-300. As shown in Fig. 10 (A,B), the characteristic membranal localization of MSP-300 and its concentration at the attachment sites is not prominent under these conditions. These experiments suggest that the distribution of MSP-300 in cells is partially determined by that of integrin, and that its membranal localization in myotubes in vivo is related to integrin expression in these cells.

Discussion

This paper describes the cloning and characterization of a new *Drosophila* embryonic muscle-specific protein, MSP-300. The localization of this protein in embryonic muscles, as well as the structural homology found between MSP-300 and proteins from the spectrin superfamily, suggest an important role for this protein in muscle morphogenesis.

MSP-300 belongs to the spectrin superfamily

Proteins from the spectrin superfamily share several functional and structural properties. These include: binding to filamentous actin, association with the membrane skeleton through interactions with specific integral membrane protein complexes, a protein structure that displays a long central rod-like α-helical domain exhibiting internal repeated sequences, and a more variable C-terminal region (Cohen and Branton, 1979; Bennett and Stenbuck, 1979; Hammond, 1987; Mimura and Asano, 1987; Baron et al., 1987; Wasenius et al., 1987; Noegel et al., 1987; Koenig et al., 1988; Arimura et al., 1988; Davison and Critchley, 1988; Wasenius et al., 1989; Byers et al., 1989; Campbell and Kahl, 1989; Dubreuil et al., 1989; Otey et al., 1990; Ibraghimov-Beskrovnaya et al., 1992).

Studies in this paper suggest that MSP-300 shares many of these properties with the spectrin superfamily. The partial sequence data of the C-terminal region shows a limited but significant level of homology to the sequences of dystrophin, α-actinin and α-spectrin. Five internal repeats of ~120-130 residues were identified in the sequence of MSP-300, which are the most homologous to those of dystrophin. Since the N-terminal sequence of MSP-300 has not been completed, it is not known whether the protein contains an actin-binding domain. However, immunolocalization of MSP-300 shows that it decorates actin filaments and it has been localized to three different sites where actin filaments are associated with the membrane. These include the leading edge of migrating myotubes, at sites of intercellular contacts, and along Z-discs in the forming myotube. Association of MSP-300 with the sedimented actin filaments in the actin sedimentation assay further supports the notion that MSP-300 is associated with actin filaments.

Both the partial co-localization of MSP-300 with inte-

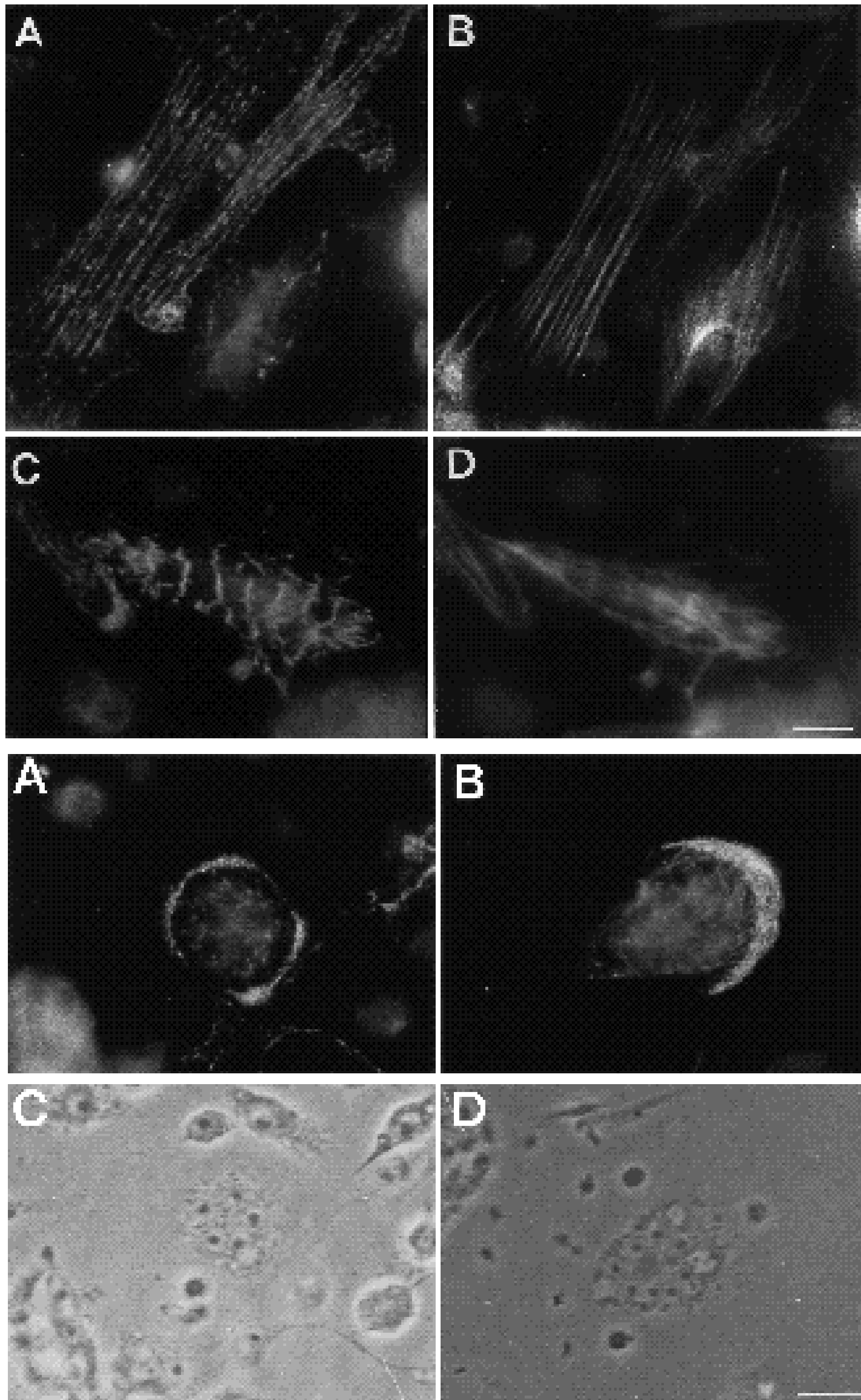

Fig. 5. (1) Co-localization of MSP-300 and actin in primary cultures of *Drosophila* cells. Embryos were dissociated and cultured on laminin-coated coverslips for 16 hours (A,B) or 48 hours (C,D). Cells were fixed, permeabilized and double immunofluorescently labeled for MSP-300 (A,C) and actin (B,D). Notice that MSP-300 decorates the microfilaments in the fused myotubes. In cells that developed sarcomeric architecture, most of MSP-300 staining is noted along the Z-discs. Bar indicates 10 μm. **(2)** Differential staining for MSP-300 in migrating and non-migrating myotube. Embryonic primary cultured myotubes labeled for MSP-300 (A and B) and their corresponding phase-contrast images (C and D). Prominent staining along the leading edge of migrating myotube is evident in B while the staining in a resting myotube is expressed circumferentially in small ordered spikes as in A. Bar indicates 10 μm.

grin in the cultured cell system, as well as the study that indicates that the membranal localization of MSP-300 depends on the presence of functional integrin at the surfaces of the myotubes link MSP-300 to integrin. Proteins from the spectrin superfamily have been shown to be associated with an integral membrane protein complex. For example, spectrin is tightly associated with ankyrin and band 3 in the erythrocyte membrane skeleton (Cohen and Branton, 1979; Bennet and Stenbuck, 1979; Elgsaeter et al., 1986). Dystrophin was recently shown to be associated with a glycoprotein integral membrane complexes (Campbell and Kahl, 1989; Ibraghimov-Beskrovnaya et al., 1992), which bind to laminin at their external surfaces, and α-actinin was found to interact directly with integrin receptors (Otey et al., 1990). Therefore, integrin may be part of the integral membrane complex with which MSP-300 is associated. However, since integrin is not present at the leading edge of migrating cells, it is possible that MSP-300 is associated with other integral membrane proteins as well.

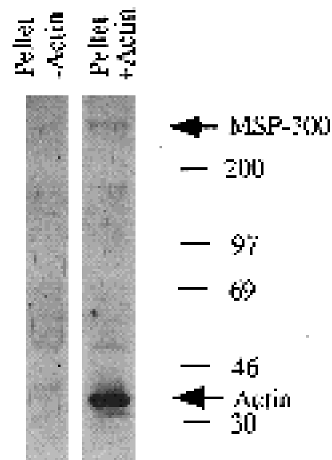

Fig. 6. Actin sedimentation assay with MSP-300. Rabbit skeletal muscle G-actin was mixed with a detergent soluble extract of embryonic membranes. Actin was allowed to polymerize in a polymerization buffer and the filaments were precipitated. MSP-300 content was analyzed by western blot analysis with anti-MSP-300 antibodies, in the pelleted fraction that did not contain F-actin (pellet–actin) and compared to the content in the F-actin-containing pelleted fraction (pellet+actin). Actin was visualized on the same blot by reacting it with anti-actin monoclonal antibodies (Sigma, Israel). Molecular masses are shown.

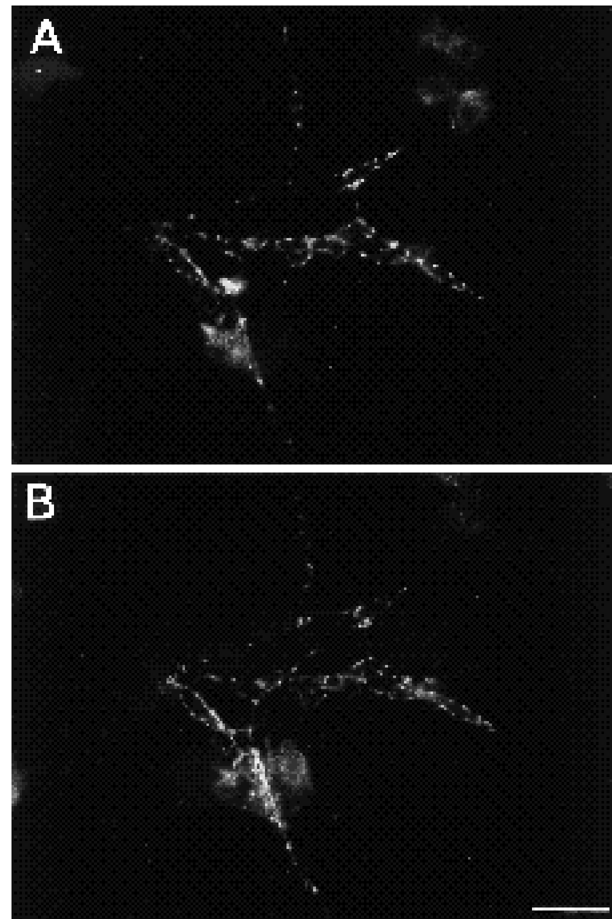

Fig. 9. Co-localization of MSP-300 and β-integrin in primary cultures of *Drosophila* cells. Embryos were dissociated and cultured on laminin-coated coverslips for 16 hours. Cells were fixed, permeabilized and double immunofluorescently labeled for MSP-300 (A) and β-integrin (B). Note that many of the integrin-positive patches are also positive for MSP-300. Bar indicates 10 μm.

The β_H form of spectrin was described by Dubreuil et al. (1990). This spectrin form is a high molecular weight protein, which was proposed by the authors to be homologous to dystrophin. Our data suggest that MSP-300 is not similar to this spectrin form. Spectrin-β_H is not a muscle-specific protein and its chromosomal localization differs from that of MSP-300.

Thus, while MSP-300 belongs to the spectrin superfamily, it is not identical to any previously reported spectrin species. The large size of MSP-300, together with the homology found in the repeated sequences of MSP-300 with those of dystrophin, as visualized by the dotplot comparison, support the notion that MSP-300 is more homologous to dystrophin than to α-actinin or spectrin.

The possible involvement of MSP-300 in myotube migration on the ectoderm

By following dye-injected myotubes, Bate (1990) showed that newly formed myotubes send their lamellipodia over the ectoderm towards various locations. The molecular mechanism enabling this process is not understood. The subcellular localization of MSP-300 in the cultured myotubes suggests that it may be involved in lamellipodia migration. Analyzing the subcellular localization of MSP-300 in primary cultured myotubes indicates that MSP-300 is located at sites where microfilaments are connected to the plasma membrane (such as the leading edge of migrating cells, adhesion plaques etc.). Additionally, it is shown that, while in resting myotubes MSP-300 is localized in small patches of cell-matrix adhesions at the cell edges, in migrating myotubes it is concentrated at their leading edge.

Several experiments in vertebrate cell cultures indicated that actin-binding proteins (ABPs) directly affect cell motility. Elimination of ABP expression in cell lines derived from human malignant melanomas induced impaired locomotion and displaced circumferential blebbing of the plasma membrane. When these cells were transfected with ABP, they regained their motile properties (Cuningham et al., 1992). Overexpression of gelsolin (an F-actin severing protein) in NIH 3T3 fibroblasts induces enhanced motility (Cunningham et al., 1991), while overexpression of vinculin, a protein that is associated with the linkage of microfilaments to the plasma membrane, suppresses cell motility in BALB/c 3T3 cells (Rodriguez Fernandez et al., 1992). Overexpression of α-actinin in these cells promotes cellular paralysis (Glueck and Ben-Ze'ev, personal communication). In light of these results, it is possible that MSP-300 is actively involved in the directional migration of the myotube lamellipodia, transducing specific external signals to cytoskeletal reorganization.

The possible involvement of MSP-300 in the formation of muscle-ectoderm attachments

The studies of Bate (1990) show that newly formed myotubes interact with both the ectoderm and other nascent myotubes. At the muscle attachment sites, similar interactions are taking place. In primary cultured myotubes, MSP-300 staining is localized to some of the muscle-muscle and muscle-non muscle contact sites. In the embryo, MSP-300 is also prominent at muscle ectoderm and muscle-muscle adhesion sites. Thus, MSP-300 might participate in the cytoskeletal response to these intercellular interactions, enabling the formation of cytoskeletal associated stable attachment sites. The observed interactions between myotubes raises the possibility that while some of the myotubes attach to the ectoderm at very specific sites, as dictated by the ectoderm, other myotubes are guided to these sites by contacts with such already attached myotubes. The expression of MSP-300 at these contact regions during this stage of development suggests that MSP-300 might participate in this type of interaction, as well.

A better understanding of the developmental role of MSP-300 will help reveal the mechanism of muscle patterning in *Drosophila*, and may disclose important functional properties of proteins from the spectrin superfamily.

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Fig. 7. Developmental distribution of MSP-300 in the *Drosophila* embryo. Whole-mount staining was carried out using the anti-MSP-300 antibodies in embryos at stage 12 (A,B); stage 14 (C,D); stage 16 (E,F) (stages were determined according to Campos-Ortega and Hartenstein, 1985). Note that initially the protein is expressed in a polar fashion in the muscle precursors, later it is prominent along the myotubes membranes as well as at the muscle-ectoderm attachment sites (C,D) and at an even later stage (E,F) it is prominent in all the muscles. Bar indicates 50 μm.

Fig. 8. Subcellular localization of MSP-300 in the developing *Drosophila* embryo. Semi-thin longitudinal sections (2 μm thickness) were obtained from stained embryos with anti-MSP-300 antibodies at stage 12 (A), stage 14 (B) and stage 16 (C) (stages were determined according to Campos-Ortega and Hartenstein, 1985). Muscle insertions are indicated by arrowheads in B and C. The ectoderm is designated by **e**. Notice the prominent staining at the muscle-ectoderm attachments in A and the significant membranous staining of MSP-300 at the earlier stages compared to the more cytoplasmic staining at stage 16. Bar indicates 100 μm.

Fig. 10. The subcellular distribution of MSP-300 in *myospheroid* embryos deficient of β-integrin. Semithin sections (2 μm thickness) were obtained from stained *myospheroid* embryos at stage 13 (A) and at stage 16 (B) with anti-MSP-300 antibodies. The staining is no longer membranal as was noticed in the wild-type embryos at this stage. Bar indicates 100 μm.