α spectrin is essential for morphogenesis and body wall muscle formation in Caenorhabditis elegans

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A common feature of multicellular animals is the ubiquitous presence of the spectrin cytoskeleton. Although discovered over 30 yr ago, the function of spectrin in nonerythrocytes has remained elusive. We have found that the spc-1 gene encodes the only α spectrin gene in the Caenorhabditis elegans genome. During embryogenesis, α spectrin localizes to the cell membrane in most if not all cells, starting at the first cell stage. Interestingly, this localization is dependent on β spectrin but not βheavy spectrin. Furthermore, analysis of spc-1 mutants indicates that β spectrin requires α spectrin to be stably recruited to the cell membrane. Animals lacking functional α spectrin fail to complete embryonic elongation and die just after hatching. These mutant animals have defects in the organization of the hypodermal apical actin cytoskeleton that is required for elongation. In addition, we find that the process of elongation is required for the proper differentiation of the body wall muscle. Specifically, when compared with myofilaments in wild-type animals the myofilaments of the body wall muscle in mutant animals are abnormally oriented relative to the longitudinal axis of the embryo, and the body wall muscle cells do not undergo normal cell shape changes.

Introduction

The spectrin-based membrane cytoskeleton is a ubiquitous structure found in all metazoans examined. Members of the spectrin gene family and their associated proteins are widely distributed in most tissues in both vertebrates and invertebrates (Bennett and Baines, 2001). The spectrin molecule is a rod-shaped tetramer composed of two α and two β subunits (Bennett and Baines, 2001). Each subunit consists of a series of homologous 106 amino acid spectrin repeats, and nonrepetitive sequences are found in each subunit that could direct additional protein interactions. For example, the α spectrin molecule contains an SH3 domain and two EF hand Ca²⁺ binding domains. β spectrin contains an actin binding domain and a pleckstrin homology domain.

The spectrin-based membrane cytoskeleton was first characterized in erythrocytes and has subsequently been studied in great detail. In the erythrocyte, spectrin forms a submembrane cytoskeletal network that is important for maintaining the structural integrity of the membrane and maintaining cell shape (Lux and Palek, 1995). However, the role of the spectrin-based membrane cytoskeleton in nonerythrocytes has remained elusive. Studies in Drosophila and Caenorhabditis elegans are providing new information on the function of the spectrin cytoskeleton in nonerythrocytes. In Drosophila and C. elegans, three spectrin genes have been identified. One gene encodes an α spectrin, and the other two genes encode β spectrins. The α and one of the β spectrins are highly similar to the nonerythroid spectrins (Dubreuil et al., 1987; Moorthy et al., 2000). However, the other β spectrin, βheavy (βH) is unusual in that it encodes a very large spectrin molecule (~400 kD) (Dubreuil et al., 1990; Thomas and Kiehart, 1994; McKeown et al., 1998) compared with the nonerythroid β spectrin, which is ~220 kD (Dubreuil et al., 1987). In Drosophila, α and β spectrin are widely expressed in most tissues (Pescacreta et al., 1989). Conversely, the βH isoform is restricted to certain tissues, such as the epithelia (Pescacreta et al., 1989; Thomas and Kiehart, 1994). Similarly, C. elegans β spectrin is found in most tissues (Moorthy et al., 2000), whereas the C. elegans βH spectrin is restricted to the epidermis (hypodermis), pharynx, and intestine (McKeown et al., 1998). In Drosophila epithelial tissues, two spectrin isoforms associate with different membrane domains: the (αβ)2 isoform localizes to the lateral and basal membrane, and the (αβH)2 isoform localizes to the apical membrane (Dubreuil et al., 1997).

Examination of Drosophila α spectrin mutants reveals several developmental defects, which implicates spectrin in the...
Figure 1.  spc-1 encodes α spectrin. (A) The genomic structure of the spc-1 gene and the encoded protein structure. The exons are indicated by the black boxes, and the introns are indicated by the thin black lines. The nonsense mutation is shown (ra409), and the Tc1 insert is shown by the inverted triangle in the 10th exon. The cDNA clones used for generating dsRNA are shown above the genomic structure. Spectrin repeats are illustrated by ovals. The nonrepetitive domains are indicated by boxes. † and * indicate where the spc-1(ra409) mutation and the Tc1 introduce premature stop codons, respectively. (B) Deduced amino acid sequence of C. elegans α spectrin aligned to Drosophila and human nonerythroid α spectrin. The C. elegans α spectrin (Ce-α; sequence data available from GenBank/EMBL/DDBJ under accession no. AAB53876) is 61% identical and 76% similar to Drosophila α spectrin (Dm-α; sequence data available from GenBank/EMBL/DDBJ under accession no. P13395), and it is 57% identical and 72% similar to the human nonerythroid α spectrin (Hs-α; sequence data available from GenBank/EMBL/DDBJ under accession no. NP_003118). Identity is boxed, and similarity is shaded.
processes of cell growth, differentiation, and specification (de Cuevas et al., 1996; Lee et al., 1997a; Thomas et al., 1998). Examination of β spectrin mutant animals in Drosophila and C. elegans implicate this molecule in body wall muscle function, axon pathfinding, synapse function, and the maintenance of protein localization at the membrane (Dubreuil et al., 2000; Hammarlund et al., 2000; Moorthy et al., 2000; Featherstone et al., 2001). βII mutants have defects in cell morphological events including embryonic elongation of C. elegans embryos (McKeown et al., 1998) and epithelial morphogenesis in Drosophila oocyte development (Zarnescu and Thomas, 1999).

We have identified a mutation in the C. elegans α spectrin gene, spc-1. C. elegans α spectrin localizes to cell membranes in all tissues examined during embryogenesis. Phenotypic characterization of the spc-1 mutant embryos reveals a defect in embryonic elongation and defects in body wall muscle differentiation. spc-1 mutants have a slow rate of elongation and fail to elongate beyond twofold in length. This defect in elongation results from the failure of the apical actin cytoskeleton within the hypodermis to organize properly. Also, the body wall muscle displays an abnormal arrangement of myofilaments, and the body wall muscle quadrants are twofold wider than normal muscle quadrants. This wider partitioning is mirrored by the underlying basement membrane and in the hypodermal body wall muscle attachment structures. These results indicate that the spectrin-based membrane cytoskeleton is required for the normal development of these two tissues in C. elegans.

Results

C. elegans α spectrin is widely expressed throughout embryonic development

The C. elegans genome contains one α spectrin gene encoded by spc-1 (C. elegans Sequencing Consortium, 1998). spc-1 is composed of 13 exons (Fig. 1 A) and encodes a putative 2,421 amino acid protein containing 22 spectrin repeats, a central SH3 domain, and two EF hands located at the COOH terminus (Fig. 1 A). The C. elegans α spectrin is 61% identical and 76% similar to Drosophila α spectrin and 57% identical and 72% similar to the human nonerythroid α spectrin (Fig. 1 B).

To determine the localization of α spectrin in the developing embryo, a rabbit polyclonal antiserum was produced, designated AS1 (see Materials and methods). Wild-type embryos were stained with AS1 and examined by immunofluorescence microscopy. In early embryos, α spectrin is found...
at or near the cell membrane in most if not all cells (Fig. 2) starting at the earliest stage of embryogenesis, indicating that $\alpha$ spectrin is maternally supplied (Fig. 2 A). After morphogenesis and organogenesis initiates, $\alpha$ spectrin localizes to the body wall muscle, hypodermis, pharynx, intestine, and developing nervous system (Fig. 2). In epithelial tissues, $\alpha$ spectrin is found at both the apical and basolateral regions of the membrane (Fig. 2 B). This is unlike the localization pattern observed for $\beta$ spectrin, which is found in only the basolateral region of the membrane (Fig. 2 and Fig. 3 C) (Moorthy et al., 2000). With the exception of the apical regions, the localization of $\alpha$ spectrin overlaps with the distribution of $\beta$ spectrin (Fig. 2 and Fig. 3 C) (Moorthy et al., 2000).

To determine specificity of AS1 antiserum, a putative $\alpha$ spectrin–null mutant (spc-1[ra409]) [see below] was stained with AS1. No signal was detected in spc-1(ra409) embryos except for faint immunofluorescence in the nervous system (unpublished data). Since $\alpha$ spectrin is maternally supplied, this faint labeling in the nervous system could be the result of maternal $\alpha$ spectrin. Using RNA-mediated interference (RNAi) of spc-1 to disrupt maternal and zygotic $\alpha$ spectrin (Fire et al., 1998), $\alpha$ spectrin immunofluorescence was eliminated in early embryos, and no signal was detected in older embryos including the nervous system (unpublished data). Furthermore, AS1 immunofluorescence was eliminated by preincubating the AS1 antiserum with the GST–$\alpha$ spectrin fusion protein but not with control fusion proteins (unpublished data). Additionally, Western blot analysis of a mixed population of wild-type worm extracts revealed a single band at $\sim$240 kD (Fig. 2 A’). This band was not detected when AS1 antiserum was preincubated with the GST–$\alpha$ spectrin fusion protein (Fig. 2 A’).

To determine if $\beta$ spectrin is required for the localization of $\alpha$ spectrin, the localization of $\alpha$ spectrin was examined in $\beta$ spectrin– and $\beta_H$ spectrin–null mutants, unc-70(s1639) and sma-1(ru18), respectively (McKeown et al., 1998; Hammarlund et al., 2000). In $\beta$ spectrin mutants, $\alpha$ spectrin immunofluorescence is not observed in any tissues except for bright immunofluorescence in the apical membrane region of the intestine and hypodermis (Fig. 2 B and Fig. 3 A). Faint immunofluorescence is also observed in the nerve ring (unpublished data). Interestingly, this indicates that $\beta$ spectrin is required for the localization of $\alpha$ spectrin in all tissues except the apical membrane in the hypodermis and intestine. Conversely, $\beta_H$ spectrin was not required for the localization of $\alpha$ spectrin. In all tissues, $\alpha$ spectrin localized normally including the apical membrane region of the hypodermis and intestine (Fig. 3 B). To complement this analysis, $\alpha$ spectrin mutant embryos (see below) were stained
with β spectrin antisera (Moorthy et al. 2000). In α spectrin mutants, β spectrin localized to the membrane; however, the staining was weak in all tissues except for the nervous system (Fig. 3 D). Surprisingly, this indicates that α spectrin is required for the stability but not the localization of β spectrin at the membrane.

Loss of α spectrin leads to a failure to complete embryonic elongation and lethality

In a mutagenesis screen designed to identify mutations in genes involved in body wall muscle development, an ethyl-nethanesulfanate-induced mutation in the C. elegans α spectrin gene was isolated. This mutation, spc-1(ra409), was genetically mapped to the left arm of the X chromosome between two cloned genes, unc-2 and unc-20. Several approaches were used to determine that the spc-1(ra409) mutation is in the α spectrin gene. First, the spc-1(ra409) mutant phenotype was rescued by transformation rescue with a cosmid containing the full-length α spectrin gene (M01F12). Second, since this cosmid contained other genes, the spc-1(ra409) phenotype was rescued with a subclone of M01F12 containing only the α spectrin gene. Third, injection of double-stranded RNA (dsRNA) specific to the α spectrin coding region was found to phenocopy spc-1(ra409) mutants. Fourth, a transposon-induced allele of spc-1(ra417::Tc1) (Fig. 1 A) fails to complement spc-1(ra409). Finally, by sequencing the entire α spectrin gene in spc-1(ra409) homozygotes the molecular lesion was identified, which introduces a premature stop at amino acid position 2111 (Fig. 1 A). To determine if spc-1(ra409) is a null mutation, it was crossed into a strain carrying a deficiency that deletes the spc-1 locus (syDf1), and the heterozygous progeny were examined. spc-1(ra409)/syDf1 progeny exhibited the same phenotype (described below) as spc-1(ra409) homozygotes, suggesting that spc-1(ra409) is a null allele of the spc-1 locus.

To determine the role of α spectrin in embryogenesis, the phenotype of homozygous spc-1(ra409) mutant animals was analyzed using light microscopy. spc-1(ra409) mutant embryos undergo embryogenesis without any obvious defects until the embryo begins to elongate. Elongation converts the embryo from a ball of cells (lima bean stage) into the elongated threefold vermiform shape. Embryonic elongation is composed of two phases: (a) extension from lima bean to twofold, which is thought to occur by cell changes in the hypodermis, and (b) progressing from two to threefold, which is thought to be driven by body wall muscle contraction (Chin-Sang and Chisholm, 2000). spc-1(ra409) mutant embryos undergo elongation at a slower rate than wild-type animals and fail to complete elongation. Arrows indicate the abnormal morphology of the pharynx. Bar, 10 µM. (D) Lengths of N2 (n = 7), spc-1 (n = 5), egl-19 (n = 4), sma-1 (n = 6), let-502 (n = 5), and mlc-4 (n = 4) embryos measured every 15 min from first cleavage at 24°C. All the embryos initiated elongation at the same time. N2 and egl-19 continue at the same rate until twofold in length where egl-19 ceases elongation. spc-1, sma-1, let-502, and mlc-4 undergo elongation at a slower rate. Additionally, body wall muscle contraction commenced at the same time (∼400 min) in all the embryos except egl-19, which failed to display muscle contraction. Data is shown as the mean ± SD.

Minutes after first cleavage
in α spectrin mutants is disorganized (Fig. 5 B). For example, several breaks along the length of the actin bundles can be observed, and the bundles are no longer held in a parallel arrangement (Fig. 5 B). To further investigate the role of the spectrin cytoskeleton in the organization of the hypodermal apical actin cytoskeleton, the organization of the actin cytoskeleton in the β spectrin mutants was investigated. sma-1(ru18) βH spectrin and unc-70(s1639) β spectrin mutants were stained with fluorescent-labeled phalloidin and analyzed by confocal microscopy. The actin bundles are disorganized in βH spectrin mutant animals (Fig. 5 C). As is seen with α spectrin mutants, there are several gaps in the actin filaments and they are not tightly organized in parallel bundles (Fig. 5 C). βH spectrin mutants also have defects in elongation (McKeown et al., 1998). Although elongation is slower than normal in βH spectrin mutants, these animals elongate further than α spectrin mutants (Fig. 4 D). Conversely, analysis of the unc-70 β spectrin mutants revealed no defects in the hypodermal apical actin cytoskeleton (unpublished data). This indicates that β spectrin has no direct role in maintaining the integrity of the hypodermal apical actin cytoskeleton. Unlike the α spectrin and β3 spectrin mutants, β spectrin mutants do not have elongation defects. Therefore, it appears that (αβH)2 and not (αβ)2 spectrin is required for the organization and function of the hypodermal apical actin cytoskeleton during embryonic elongation.

A disorganized hypodermal apical actin cytoskeleton in α and βH spectrin mutants can be explained by one of two possibilities. Either the disorganization of the apical actin cytoskeleton results in slow embryonic elongation or the lack of normal elongation results in the abnormal organization of the hypodermal apical actin cytoskeleton. To investigate the possibility of the latter, two mutants, let-502(h738) and mle-4(or253), that have a similar slow elongation phenotype (Fig. 4 D) were stained with fluorescent-labeled phalloidin and analyzed by confocal microscopy. let-502 encodes a rho kinase, and mle-4 encodes a myosin light chain (Wissmann et al., 1997; Shelton et al., 1999). Both of these proteins have a putative role in the regulation of the contraction event required for embryonic elongation but not in actin assembly itself (Chin-Sang and Chisholm, 2000). The hypodermal apical actin cytoskeleton appears normal in both let-502 and mle-4 mutants (Fig. 5 D and E). Interestingly, the spacing between the parallel actin bundles in let-502(h738) embryos appears irregular (Fig. 5 D). However, this is caused by the folding and wrinkling of the hypodermis in these mutants and not the structure of the actin bundles. Therefore, unlike the α and βH spectrin mutants, let-502 and mle-4 mutants display normal actin cytoskeleton organization. This suggests that the disorganization of the hypodermal apical actin cytoskeleton in α and βH spectrin mutants is a direct result of the loss of the (αβH)2 spectrin cytoskeleton and is not due to a defect in elongation.

**Elongation is required for correct morphogenesis of the body wall muscle**

During the analysis of the hypodermal cytoskeleton, abnormalities in the distribution of the thin filaments were observed in the body wall muscle of α spectrin mutant animals. This defect is not only associated with the thin filaments,
since similar defects are seen when the thick filaments of \( \alpha \) spectrin mutants are examined by immunofluorescence microscopy with a monoclonal antibody specific to body wall muscle myosin. Specifically, the muscle quadrants of these mutants appear twofold wider than normal (Fig. 6, B and E). In addition, the myofilaments are abnormally oriented to the longitudinal axis. Normally, the sarcomeres are arranged at an oblique angle (6°) to the longitudinal axis of the nematode (Fig. 6 A) (Waterston, 1988). In \( \alpha \) spectrin mutants, this oblique striation is exaggerated to an almost 20° angle. This deflection of the sarcomeres is particularly prevalent in the anterior muscle cells (Fig. 6 B). Additionally, there is a large gap between the myofilament lattice in neighboring muscle cells within the same muscle quadrant that is not observed in mus-
cle quadrants of wild-type animals (Fig. 5 B and Fig. 6 B). In wild-type animals, the myofilament lattice in neighboring cells is closely associated (Fig. 5 A and Fig. 6 A). In \( /H9251\) spectrin mutant animals, the myofilament lattice is not tightly associated, and there is a 1–2 micron gap between the myofilament lattice in neighboring cells (Fig. 5 B and Fig. 6 B). This defect is not the result of the failure of the muscle cells to polarize, since the myofilaments are normally located against the basal membrane (unpublished data).

There are three possible causes for the morphological defects observed in the body wall muscle cells of \( /H9251\) spectrin mutant animals: (a) loss of the spectrin cytoskeleton in muscle, (b) termination of elongation of the embryo at the twofold stage of development, or (c) a slow rate of elongation up to twofold. Since \( \alpha \) and \( \beta \) spectrin are both expressed in body wall muscle and most likely form a complex, the myofilament lattice in \( \beta \) spectrin, \( unc-70(s1639)\), mutant embryos was analyzed by immunofluorescence microscopy. From this analysis, the myofilament lattice of \( \beta \) spectrin mutants was indistinguishable from wild-type animals (unpublished data). This indicates that \( \beta \) spectrin does not have a role in myofilament organization in the \( C. elegans \) embryo. Additionally, since \( \alpha \) spectrin fails to associate with the membrane in the absence of \( \beta \) spectrin, this suggests that the alteration in body wall muscle cells of \( \alpha \) spectrin mutants is not a direct result of loosing the muscle spectrin cytoskeleton. To investigate whether termination of elongation at twofold is itself the cause of the altered muscle, the paralyzed and arrested at the twofold stage (Pat) mutant, \( egl-19(st556)\), was analyzed (Williams and Waterston, 1994). This mutant is defective for an L-type voltage-activated Ca\(^{2+}\) channel that is required for muscle contraction (Lee et al., 1997b). \( egl-19\) mutants initiate and undergo elongation normally until they reach twofold in length where elongation ceases (Fig. 4 D). Examination of the myofilaments in \( egl-19\) mutant embryos indicates that the myofilaments are normally assembled and organized (Fig. 6 D). Importantly, the width of the body wall muscle quadrants is identical to that of wild-type animals (Fig. 6 E). This demonstrates that the point where elongation ceases is not critical for the development of the body wall muscle. Interestingly, analysis of the slow elongation mutants, \( sma-\),
Body wall muscle cells fail to undergo cell shape changes in the slow elongation mutants. Representative wild-type (A and B), let-502(h738) (C), and egl-19(st556) (D) embryos double labeled with myosin antisera (red) and β spectrin antisera (green). In wild-type embryos, the body wall muscle cells change shape from round to spindle-shaped cells. (A) Comma stage wild-type embryo showing the initial shape of the body wall muscle cells at the start of embryonic elongation. (B) Threefold wild-type embryo indicating the cell shape change that the body wall muscle cells undergo during embryonic elongation. (C) In the slow elongation mutants (let-502), the body wall muscle cells fail to undergo normal cell shape changes and remain similar to the body wall muscle cells in the wild-type comma stage embryo (C compared with A and B). (D) Normal cell shape changes occur in the Pat mutant, egl-19. Arrowheads indicate cell width. Bar, 10 μM.

The basement membrane and hypodermal attachment structures are broader in slow elongation mutants
The body wall muscle is anchored to the cuticle via the basement membrane and hemidesmosomal-like components in the hypodermis (Moerman and Fire, 1997). Since the slow elongation mutants exhibit wider muscle quadrants, it is possible that the basement membrane and the hemidesmosomal-like components exhibit defects also. To determine if any defects have arisen in the basement membrane, the distribution of the basement membrane protein perlecan was analyzed. The basement membrane of wild-type, α spectrin, βIII spectrin, let-502, and mlc-4 mutant embryos was assessed by perlecan immunofluorescence microscopy. Perlecan is an essential component of the basement membrane that is required to transmit the force of body wall muscle contraction to the hypodermis (Rogalski et al., 1993). The distribution of perlecan in α spectrin, βIII spectrin, and let-502 mutant embryos matched the expansion of the body wall muscle quadrants and results in a wider basement membrane (Fig. 8, A–C). Aside from the larger size of the basement membrane, the distribution of perlecan appears wild type, and perlecan is evenly distributed under the muscle quadrants. The distribution of perlecan in egl-19(st556) was very similar to wild type, indicating that the basement membrane has not expanded in these mutants (Fig. 8 D).

To determine if similar alterations occur in the hemidesmosomal-like structures of the hypodermis, the organization of these attachment structures was investigated by using MH4 immunofluorescence. The MH4 antiserum recognizes intermediate filaments that are associated with hemidesmosomal-like structure in the hypodermis that assemble adjacent to the body wall muscle cells in the dorsal and ventral hypodermis (Francis and Waterston, 1991). In wild type, these hemidesmosomal-like structures resemble the spatial and temporal distribution of the muscle components and are tightly associated with the hypodermal membrane adjacent to the muscle cells where the myofilament lattice is positioned (Fig. 8 E) (Hresko et al., 1994). Although the spacing and organization of the hypodermal attachment structures appeared normal, MH4 immunofluorescence examination of the slow elongation mutants reveals that these structures closely mimic the expansion of the muscle cells (Fig. 8, F and G). This indicates...
that the complete linkage from muscle to the cuticle is affected in these mutants. Analysis of MH4 distribution in egl-19 reveals a wild-type pattern (Fig. 8 H).

**Discussion**

The *sma-1* locus of *C. elegans* encodes the ortholog of human nonerythroid α spectrin. During *C. elegans* embryogenesis, α spectrin localizes near the plasma membrane of most, if not all, cell types beginning at the first cell stage. During later stages of embryogenesis, α spectrin localizes to all tissues. This localization of α spectrin overlaps with β spectrin except in the apical membrane region of epithelial tissues. In the absence of β spectrin, α spectrin only localizes to the apical membrane region of epithelia, and small amounts are detected in the nerve ring. Conversely, although β spectrin appears to localize to the membrane in the absence of α spectrin, it is not stably localized. However, we did observe higher levels of β spectrin in the nervous system of *spe-1(ra409)* mutants, which is probably due to maternal contribution of α spectrin. Using a different experimental procedure, Moorthy et al. (2000) found normal levels of β spectrin in *spe-1(RNAi)* embryos (Moorthy et al., 2000). In our study, the genetic null was characterized, whereas Moorthy et al. (2000) examined RNAi-induced embryos. It is possible that RNAi produces a broad range of penetrance. In support of this hypothesis, when we used the same cDNA templates that Moorthy et al. (2000) used for dsRNA production we found that this resulted in a very mild defect, and only a small percentage of embryos phenocopied the *spe-1*-null phenotype. Therefore, it is possible that in their analysis α spectrin was present, albeit in lower quantities, and allowed for normal β spectrin localization.

βH, spectrin has been reported to localize to the apical region of epithelial tissues in both *C. elegans* and *Drosophila* (Thomas, 2001) and therefore may be required for the apical localization of α spectrin. However, examination of α spectrin localization in βH spectrin (*sma-1*) mutants reveals a normal distribution of α spectrin, indicating that βH spectrin is not required for α spectrin localization in *C. elegans*. This suggests that the mechanisms that localize α spectrin to the apical versus the basolateral membrane are distinct.

The localization pattern of the different spectrin isoforms and the examination of α and β spectrin localization in the reciprocal mutants suggests that the spectrin cytoskeleton is organized into two populations: an *(αβ)1* spectrin that localizes to all tissues except the apical membrane in epithelia and an *(αβH)2* spectrin that localizes to the epithelial apical membrane. This model is further corroborated by the analysis of the double mutants (this study; Moorthy et al., 2000). Double mutants constructed with null alleles of β and βH spectrin resulted in a phenotype identical to that of the single loss of function allele of α spectrin.

**The *(αβH)2* spectrin cytoskeleton is essential for embryonic morphogenesis**

Time-lapse video recording of α spectrin (*spe-1*) mutants during embryogenesis indicates that these mutants have a...
slow rate of elongation resulting in L1 larvae that are half the size of wild-type L1 larvae. Circumferential contraction of the apically localized actin cytoskeleton in the hypodermis is the driving force behind the process of elongation during morphogenesis (Priess and Hirsh, 1986; Costa et al., 1997). Examination of this cytoskeleton in α spectrin mutants reveals that the actin filaments are discontinuous and disorganized. The same defect is observed in β⁺ spectrin mutant animals. Also, β⁺ spectrin mutants have defects in elongation, whereas β spectrin mutants do not. These data indicate that the (αβ)₂ spectrin cytoskeleton, and not (αβ)₃, is essential for the proper organization and function of the apical actin cytoskeleton in the hypodermis and is a key component involved in epithelial morphogenesis. The importance of the spectrin cytoskeleton in epithelial morphogenic events has been documented recently in Drosophila. Zarnescu and Thomas (1999) have shown that β⁺ spectrin is required for normal epithelial morphogenesis in Drosophila follicle cells during oocyte development. Additionally, spectrin has been implicated in morphogenic events that occur during mouse neural tube formation (Sadler et al., 1986) and during gastrulation in sea urchin embryos (Wessel and Chen, 1993). These observations together with our data suggest that the spectrin cytoskeleton may function globally in coordinating the cytoskeleton within epithelial tissues during morphogenic events.

The process of elongation occurs in two steps. First, the hypodermal apical actin cytoskeleton is required to extend the embryo to twofold, and second, body wall muscle contraction induces hypodermal cell changes that extend the embryo to threefold (Chin-Sang and Chisholm, 2000). Unlike Pat mutants, the elongation mutants described here (spe-1, sma-1, let-502, and mlc-4) are capable of muscle contraction and have a slow rate of elongation from lima bean to twofold. Pat mutants elongate to twofold normally, but they fail to elongate beyond twofold because their lack of muscle contraction fails to induce changes in the hypodermis. spe-1, sma-1, let-502, and mlc-4 have defects in the hypodermal actin cytoskeleton and in extending to twofold. However, these mutants are capable of body wall muscle contraction. Presumably, the induction of hypodermal changes by body wall muscle contraction fails or is inefficient in these mutants because the completion of this induction event requires an intact hypodermal actin cytoskeleton, which is absent in these mutants.

**Hypodermal-mediated contraction determines the shape of body wall muscle cells**

Two lines of evidence indicate that the expression and function of α spectrin in the hypodermis, as opposed to spectrin expression within body wall muscle, is critical for determining the shape of the developing muscle cells. First, unlike α spectrin mutants, the β spectrin mutant, unc-70, does not have any defects in embryonic elongation or body wall muscle development. In β spectrin mutants, α spectrin fails to localize to the muscle cell membrane. These observations imply that spectrin expression within muscle is not important for determining muscle cell shape. Since unc-70 mutants do not impede elongation, it appears that the presence of spectrin within the hypodermis is sufficient for embryonic elongation to occur. A second line of evidence implicating the hypodermis in influencing muscle cell shape comes from the study of genes regulating the apical actin cytoskeleton. The genes we examined that influence the apical cytoskeleton with the exception of spe-1, including let-502, sma-1, and mlc-4, are expressed in the hypodermis but not in muscle (Wissmann et al., 1997; McKeown et al., 1998; Shelton et al., 1999). Mutants in all three genes elongate to twofold more slowly than wild-type embryos, and all three have broader muscles than wild-type embryos.

Embryonic elongation leads to a change in shape of the body wall muscle in wild-type embryos. A slower rate of elongation in the phase leading to a twofold embryo leads to a muscle cell with greater surface area in contact with the underlying hypodermis. Why a slower elongation rate leads to this final muscle cell shape is not clear. However, the consequences, a twofold increase in basement membrane in contact with the basal face of the muscle and a wider hemidesmosomal complex within the hypodermis, are quite dramatic. This is perhaps the most striking phenotype of these mutants and is what first drew our attention to the spe-1 mutants.

**During C. elegans embryogenesis, the myofilaments of muscle and the hypodermal hemidesmosomes assemble concurrently and have the same spatial distribution (Hresko et al., 1994). How the complementary patterning of hemidesmosomes and myofilament attachment sites is achieved is an intriguing problem in development. Does the pattern within muscle determine the pattern within the hypodermis, or is it the other way around?** The hypodermis could direct the formation of the body wall muscle via the hemidesmosomal-like components that anchor the body wall muscle to the cuticle. Specifically, the hypodermal cytoskeletal rearrangements that are required for elongation could also be required to restrict hemidesmosomal placement. If these rearrangements are perturbed, this could lead to a wider distribution of the hemidesmosomes and result in wider muscle quadrants. Conversely, it is possible that the body wall muscle cells determine where the basement membrane and hypodermal hemidesmosomal-like structures are assembled. Laser ablation of body wall muscle precursors can lead to a gap in a muscle quadrant. Within this gap, no perlecan accumulates, and within the hypodermis adjacent to the gap, no hemidesmosomes accumulate (Moerman et al., 1996; Hresko et al., 1999). One interpretation of these observations is that muscle is essential for organizing hemidesmosomal complex assembly within the hypodermis. If muscle is acting as an inducer, wider muscle cells may therefore lead to a wider hemidesmosomal complex within the hypodermis.

Our data cannot distinguish between these two models. What our observations do underscore is that during embryogenesis there is a dynamic interaction between muscle and hypodermis to ensure that force transmission from muscle is distributed evenly across the apposing face of the hypodermis. We have shown that spectrin has a critical role in this interaction. Future studies using the power of C. elegans and the spectrin mutants may enable us to describe the function of the spectrin cytoskeleton during development in still greater detail.
Materials and methods

Strains, maintenance, and positional cloning of spe-1

C. elegans strains were maintained and cultured as described by Brenner (1974). The N2 Bristol strain was used as the control wild-type strain. Unless otherwise specified, all genetic experiments were performed at 20°C. The following mutant alleles and chromosomal rearrangements were used in this study: dpy-3(e27), egf-19(a556), let-502/7(h378), lon-2(e678), mlc-4(or253), sma-1(ra18), unc-2(e553), unc-20(e1128s), unc-70(/493:n1771), unc-70(/61639), and syDF1.

spe-1(r4409) was isolated in a mutant screen designed to identify mutations in genes required for muscle development (IRN and DCM [unpublished data]). The spe-1(r4409) mutation mapped to the X chromosome. To further localize spe-1, three factor mapping was conducted. unc-2 + lon-2+/ spe-1 + and dpy-3 + unc-20+/ spe-1 + animals were generated and allowed to self. 12/48 Unc-2 non-Lon-2 and 6/51 Unc-20 non-Dpy-3 recombinants were mutant for spe-1, placing spe-1 between unc-2 and unc-20. Additionally, spe-1(r4409) mutants failed to complement the deficiency syDF1.

Transgenic strains were generated as described by Mello and Fire (1995). N2 animals were injected with two different cocktails of DNA. The first cocktail consisted of 80 µg/ml of pRF4 (dominant rol-6 mutation; rol-6(su1006dmi) and 10 µg/ml of MO1F12. The second cocktail consisted of 80 µg/ml of pRF4 and 20 µg/ml of DM2#14 (subclone of MO1F12 containing only the α spectrin ORF).

Molecular biology

PCR was conducted as described by Barstead et al. (1991). DNA sequencing reactions were performed directly on PCR amplified genomic DNA and cDNA clones by using the BRL dsDNA Cycle Sequencing System as described by Rogalski et al. (1993) or by the Nucleic Acid/Protein Service unit at the University of British Columbia. Sequence alignments and comparisons were performed using BLAST (National Center for Biotechnology Information server [Altschul et al., 1997]) and ClustalW (Mac Vector). Exon/intron boundaries were determined by sequencing cDNAs yk2(2053), yk4(441), (gifts from Y. Kohara, National Institute of Genetics, Mishima, Japan), cm9a10, DM2#455, and PCR-amplified fragments from a cDNA library (a gift from R. Barstead, Oklahoma Medical Research Center, Oklahoma City, OK).

General molecular biological techniques were performed as described in Sambrook et al. (1989). The bacterial strains DH5α (BRL) and XL1-Blue (Stratagene) were used for subcloning and fusion protein expression. To generate the full-length α spectrin clone, a 4.7-kb Avr II SpeI fragment from the cosmid MO1F12 (a gift from A. Coulson, Sanger Center, Hinxton, England) was subcloned into SpeI-digested fBluescript (Stratagene) to produce DM2#131. Subsequently, a 9.1-kb SpeI fragment from the cosmid MO1F12 was subcloned into the SpeI site of DM2#131, producing a clone (DM2#141) that contains the full-length C. elegans α spectrin gene including 2-kb upstream and 1-kb downstream.

To generate a fusion protein encoding part of the C. elegans α spectrin gene, the complete third spectrin repeat plus part of the second and fourth spectrin repeat (amino acids 208–449) encoded by exon 4 was cloned into pGEX 4T-1 to produce DM2#151. Specifically, PCR was performed to amplify a 957 base pair region of the fourth exon from genomic DNA. Subsequently, the clone DM2#151 was produced by digesting this PCR product with ApoI, and the fragment was subcloned into the EcoR1 site of the pGEX 4T-1 vector (Amersham Pharmacia Biotech).

The production of dsRNA and RNAs were conducted as described in Norman and Moerman (2000). The template used for RNA production was the GST clone yk2(053) and DM2#455.

Antibody production

The GST α spectrin fusion protein was produced and purified by the procedure described previously (Smith and Johnson, 1988). To generate polyclonal antisera, two New Zealand white rabbits were injected subcutaneous-ly with purified fusion protein emulsified in Freund’s complete adjuvant (0.3 mg protein/rabbit). Rabbits were boosted by intramuscular injections at 4–6-wk intervals with fusion protein emulsified in Freund’s incomplete adjuvant (0.3 mg protein/rabbit), and blood samples were taken 10–12 d after injection. Immune response was monitored by Western blotting of purified fusion proteins and wound extracts and by immuno-fluorescent staining of nematodes. Antibodies were affinity purified according to Miller and Shakes (1995). Antibodies were further purified with a bacterial-GST aceton powder that removed all GST reactivity.

Immunofluorescence and microscopy

Embryo fixation, antibody staining, and microscopy were performed as described in Norman and Moerman (2000). Fluorescence microscopy was conducted on a ZEISS Axiovert equipped with the Radiance 2000 confocal system (Bio-Rad Laboratories, Inc). A minimum of 10 animals was examined for each genotype. Animals were staged according to pharyngeal development. The antibodies used in this study were a mouse monoclonal antibody to myosin heavy chain A (DM5.6 [Miller et al., 1983]), a mouse monoclonal antibody to nematode intermediate filaments (MH4 [Francis and Waterston, 1991]), a rabbit polyclonal to nematode perlecan (GM1 [Moerman et al., 1996]), a rabbit polyclonal to nematode β spectrin (Moorthy et al., 2000), DM5.6, GM1, and the AS1 were diluted 1:50; MH4 was diluted 1:100; the β spectrin antibody was diluted 1:500. The secondary antibodies were FITC-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and were diluted 1:200. For control, spe-1(rna1) embryos, by using yk2053 and DM#455 for dsRNA production, were labeled with AS1 to test for specificity. Additionally, AS1 antisem was incubated over night at 4°C with the spectrin–GST fusion protein that was used to generate the AS1 antisem and strain wild-type embryos. No immunofluorescence signal was detected in these experiments. Embryos for FITC-phalloidin staining were prepared as described by Costa et al. (1997). Differential interference contrast images were collected on a ZEISS Axiopt microscope equipped with differential interference contrast optics. The images were collected using a Dage-MTI CCD-100 digital camera and Scion image software. Adobe Photoshop 4.0® was used to present images. Time-lapse video recording was performed as described by McKeown et al. (1998) except embryos were imaged every 5 s.

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