Mutations in β-Spectrin Disrupt Axon Outgrowth and Sarcomere Structure

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Abstract. β-Spectrin is a major component of the membrane skeleton, a structure found at the plasma membrane of most animal cells. β-Spectrin and the membrane skeleton have been proposed to stabilize cell membranes, generate cell polarity, or localize specific membrane proteins. We demonstrate that the Caeenorhabditis elegans homologue of β-spectrin is encoded by the unc-70 gene. unc-70 null mutants develop slowly, and the adults are paralyzed and dumpy. However, the membrane integrity is not impaired in unc-70 animals, nor is cell polarity affected. Thus, β-spectrin is not essential for general membrane integrity or for cell polarity. However, β-spectrin is required for a subset of processes at cell membranes. In neurons, the loss of β-spectrin leads to abnormal axon outgrowth. In muscles, a loss of β-spectrin leads to disorganization of the myofilament lattice, discontinuities in the dense bodies, and a reduction or loss of the sarcoplasmic reticulum. These defects are consistent with β-spectrin function in anchoring proteins at cell membranes.

Key words: unc-70 • Caeenorhabditis elegans • cytoskeleton • neurons • muscles

Introduction

β-Spectrin is an essential component of the membrane skeleton, a dense protein mesh that is associated with the intracellular surface of plasma membranes. The membrane skeleton is primarily formed from αβ-spectrin tetramers, each composed of two α-spectrin and two β-spectrin subunits (for reviews see Bennett, 1990; Bennett and Gilligan, 1993). Each spectrin subunit is a long, rod-shaped protein consisting mainly of tandem triple helical spectrin repeats (Yan et al., 1993). α-Spectrin has 21 spectrin repeats, an SH3 domain, and a COOH-terminal EF hand, whereas β-spectrin has 17 spectrin repeats, an NH2-terminal actin-binding domain, and a COOH-terminal pleckstrin homology (PH) domain. αβ2 spectrin tetramers nucleate around short actin filaments to form the membrane skeleton. The membrane skeleton is linked to the plasma membrane by direct interactions between the PH domain in β-spectrin and membrane phospholipids (Davis and Bennett, 1994) and indirect interactions between β-spectrin and integral membrane proteins via the linker protein ankyrin (Bennett and Stenbuck, 1979). Since association of the membrane skeleton with plasma membranes is mediated by β-spectrin, membrane skeleton function at plasma membranes requires β-spectrin.

Spectrin was first identified in erythrocytes (for review see Lux and Palek, 1995). In these cells, detergent extraction leaves behind a ghost, which is a dense protein mesh that follows the contours of the plasma membrane. Erythrocytes are resilient cells that are capable of withstanding high shear forces caused by squeezing through narrow capillaries. Mutations in human erythrocyte spectrin genes cause hereditary anemias, and the erythrocytes from these patients are fragile and exhibit membrane defects such as herniation. These data indicate that β-spectrin and the membrane skeleton control the shape and elasticity of erythrocyte plasma membranes.

More recently, α- and β-spectrins have been found in most metazoan cells and tissue types that have been examined, including most nonerythrocyte tissues of vertebrates. In particular, nonerythrocyte spectrins are abundant in neurons, muscle, and polarized epithelial cells. Two genes for α-spectrin and three for β-spectrin have been identified to date in both mice and humans, each of which is alternatively spliced to produce multiple spectrin isoforms (for review see Morrow et al., 1997). The expression of isoforms is regulated in a complex, tissue- and time-specific manner in a variety of cells. Additionally, spectrin isoforms are not evenly distributed at the plasma membrane, as observed in erythrocytes. For example, in skeletal muscle, spectrin is localized to the Z and M lines, which are the myofilament attachment structures for actin and myosin.
In cultured MDCK epithelial cells, β-spectrin is associated with the basolateral domain and not the apical domain (Nelson and Veshnock, 1986). This uneven distribution of spectrin isoforms suggests that the membrane skeleton is performing specialized functions at specific regions of the plasma membrane rather than playing a general role in membrane elasticity.

Invertebrates also have a membrane skeleton. Caenorhabditis elegans has single genes for α- and β-spectrin (Norman, K., and D. M. Omeran, personal communication) and β-spectrin (this work), and also a gene for βH-spectrin, which is a very large spectrin with limited expression (M. K. Ewen et al., 1998). The Drosophila genome also contains single genes for α- and β-spectrin (A. Dams et al., 2000) as well as for βH-spectrin (T. Thomas et al., 1998). Data from these organisms suggest that their membrane skeleton is functionally similar to that of vertebrates. Most strikingly, the distribution of β-spectrins in C. elegans closely parallels the distribution of vertebrate β-spectrin (M. Orth et al., 2000 [this issue]). For example, C. elegans β-spectrin is polarized to the basolateral domain in the gut epithelium, and is concentrated at the myofilament attachment structures in muscle.

These results support three models for the role of the membrane skeleton in both vertebrates and invertebrates (Lee et al., 1997). First, the spectrin-based membrane skeleton might function to maintain cell shape and membrane integrity, as proposed for erythrocytes (Lux and Palek, 1995). Second, the membrane skeleton might function to recruit or stabilize interacting proteins to specific regions of the cell membrane (M. Orrow et al., 1997). Third, the membrane skeleton might play a role in the maintenance of cell polarity (for review see D. Rubin and N. Nelson, 1996).

One way to distinguish among these models is to analyze defects found in mutants lacking spectrin. In the present work, we characterize the unc-70 locus, which encodes the C. elegans homologue of β-spectrin. unc-70 is the first mutation characterized in any species in nonerythrocyte β-spectrin. The first mutant alleles of unc-70 to be identified were dominant mutations that caused an uncoordinated phenotype (Brenner, 1974; Park and Horvitz, 1986). Recessive lethal alleles of the unc-70 locus were obtained by reversion of the dominant alleles (Park and Horvitz, 1986), in a screen for lethal mutations (J. Johnsen and Baillie, 1991). Of these alleles, only uncoordinated was lethal in all cells.

Materials and Methods

Strains and Genetics

Strains were grown at room temperature and maintained as described (Brenner, 1974). unc-70 null mutants are subviable and are usually maintained as balanced heterozygotes. We found that we could maintain homozygous unc-70 animals by growing them on fresh plates seeded with HB101 and transferring them every 2 or 3 d. The following strains were used: M T2590, +/et1; dpy-11(e224) unc-70(n493n1171)/et1 (Park and Horvitz, 1986), E G194: +/et1; unc-70(n794)/et1. E G 194 was constructed from strain TR 184: smg-1, et1; unc-70(n794)/et1 (+) (Calvi and Anderson, 1998). E G 198 was constructed from strain BC2303: dpy-18(e364)/et1; unc-46(e177) unc-70(s1502)/et1 (J. Johnsen and Baillie, 1991), and E G 198 was constructed from strain BC 2440: dpy-18(e364)/et1; unc-46(e177) unc-70(s1639)/et1 (J. Johnsen and Baillie, 1991).

Genetic Mapping

unc-70 was previously mapped to chromosome V (Brenner, 1974) to the right of dpy-11 (Park and Horvitz, 1986) and to the left of unc-68. We mapped unc-70(n493n1171) to the interval between snb-1 and unc-68. From the progeny of dpy-11(e224) unc-70(n493n1171)/snb-1(md247), we isolated nine Dpy non-UNC animals. Four of these nine animals segregated the snb-1 phenotype, indicating that unc-70 is to the right of snb-1.

unc-70 Rescue

Cosmids from the snb-1 to unc-68 interval were injected into MT2590 and scored for rescue of unc-70. The cosmids T19F4 was capable of complete rescue. To identify the open reading frame (ORF) corresponding to unc-70, individual ORFs from the T19F4 cosmid were amplified using the Expand Long Template PCR system (Boehringer Mannheim); the fragments were gel-purified and recovered using a QIAquick column (Qiagen), injected into M T2590, and assayed for rescue. A 12.9-kb PCR product containing the spectrin ORF was capable of complete rescue. This fragment extended 2.6 kb, 5' of the predicted start, and 1.3 kb, 3' of the predicted stop. All injections were performed essentially as described (Mello et al., 1991). Injection mixtures for the rescue contained either 20 ng/pg of the plasmid pRF4, which contains the dominant roller mutation rol-6(su1006), as a transformation marker (Mello et al., 1993). For both T19F4 and PCR fragment rescue, three of three stable roller lines rescued the mutant phenotype.

cDNA Sequence

Most of the unc-70 cDNA sequence was obtained by spliced leader sequence PCR (K. Rouse, 1995) and reverse transcriptase-PCR. For these experiments, the total C. elegans RNA was prepared essentially as described (A. Nders and Thummler, 1994). Reverse transcription and PCR were performed by standard methods using the following primer sets, which yielded a sequence extending from the SL-splice in exon 1 through the 3' end of exon 7: RT: M H134, PCR: SL1 and M H144, RT: M H141, PCR: M H106 and M H142, M H103 and M H104; RT: M H138, PCR: M H139 and M H140, M H107 and M H108; and RT: M H137, PCR: M H113 and M H114, M H111 and M H112. PCR products were purified and all bands were sequenced. These experiments yielded the CeS51 isoform; no alternative splicing was detected. However, the results from Genefinder predictions (Baylor College of Medicine) suggested that there might be alternative exon usage. Therefore, we repeated this experiment using the following primer set: RT: M H143, PCR: M H163 and M H144. A second isoform was detected corresponding to CeS52.

The remaining cDNA sequence 3' of exon 7 was obtained in two ways. First, a cDNA library obtained from P. Okkama (University of Illinois at Chicago, Chicago, IL) was probed with a 10.4-kb EcoRI-SpeI genomic fragment from T19F4, and one clone was isolated. Second, two cDNA clones corresponding to unc-70, yk14463, and yk16464, were obtained from Y. Kohara (Genome Biology Lab, National Institute of Genetics, Mishima, Japan). All three clones were completely sequenced and no alternative splicing was detected. The Okkama clone contained a poly(A)" sequence downstream of a strong consensus polyadenylation signal. Sequence alignments were performed with ClustalW (Thompson et al., 1994).
unc-70 Mutant Sequence
For each allele, the overlapping fragments covering the entire unc-70 genomic region were amplified from homozygous unc-70 animals. The sequences of all exons were determined using an Applied Biosystems automated DNA sequencing apparatus at the Sequencing Core Facility (University of Utah).

unc-70::GFP Reporters
To determine in which cells the two unc-70 splice variants (Ce51 and Ce52) are expressed, we used reporter constructs expressing green fluorescent protein (GFP; Chalfie et al., 1994). Reporter constructs, including a 4-kb construct upstream of the predicted ATG codon, were prepared by amplifying fragments from the T19F4 cosmid using the Ex pand Long Template PCR system (Boehringer Mannheim); primers were tagged with a PstI site at the 5′ end and an SacI site at the 3′ end. These fragments were cloned into the pPD9.67 GFP vector (1995 Fire vector kit) in the PstI–SacI sites. The resulting constructs contain the unc-70 promoter and differing lengths of unc-70 coding sequence in-frame with the nuclear localization sequence and GFP.

For Ce51 expression, we fused the GFP ORF to the second exon of unc-70 (Ce51::GFP). Conceptual translation of Ce51::GFP predicts that it encodes the 22 NH2-terminal amino acids of Ce51 linked to GFP. For Ce52 expression, we fused the GFP ORF to the first-in-frame A T G in the third exon of unc-70 (Ce52::GFP). We also fused GFP to exons 4, 5, and 6, which are common to both Ce51 and Ce52. GFP expression from exons 4, 5, and 6 was identical to Ce51::GFP. These constructs are not expressed in the gut, even though they should include expression from the Ce52 splice form. However, gut expression has been verified by immunofluorescence (Morothy et al., 2000 [this issue]).

Primer pairs used for construction of GFP fusions were as follows: for Ce51::GFP, MH149 and MH148; for Ce52::GFP, MH123 and MH122; for exon5::GFP, MH149 and MH147; for exon6::GFP, MH149 and MH180; and for exon4::GFP, MH149 and MH182. Each construct was injected into 20 ng/ml lin-15(n765ts) animals, together with the lin-15(aac; and SL1 ggtttaattacccaagtttgag). Primer pairs used for construction of GFP fusions were as follows: for Ce51::GFP, MH149 and MH148; for Ce52::GFP, MH123 and MH122; for exon5::GFP, MH149 and MH147; for exon6::GFP, MH149 and MH180; and for exon4::GFP, MH149 and MH182. Each construct was injected into 20 ng/ml lin-15(n765ts) animals, together with the lin-15(aac; and SL1 ggtttaattacccaagtttgag).

For muscle staining, progeny of homozygous unc-70 animals expressing GFP, were prepared for electron microscopy as previously described (McIntire et al., 1997). We injected cosmids containing a single predicted ORF was amplified by PCR, reverse transcription PCR, and from cDNA libraries. An unpaired t test was used to compare data sets and a two-tailed P value was calculated.

Results
Molecular Characterization of unc-70
R ecessive unc-70 mutants are shorter than the wild type, are paralyzed, and are nearly inviable (Fig. 1). To determine the protein encoded by unc-70, we characterized the gene. We mapped unc-70 to a narrow interval flanked by snb-1 and unc-68 and obtained the complete rescue of unc-70(n493n1171) with the single cosmid T19F4. A 12.9-kb region of this cosmid containing a single predicted ORF was amplified using PCR and was capable of completely rescuing unc-70(n493n1170) (Fig. 2 B).

A nalysis of the rescuing ORF indicated that it encodes the Ce933 elegans homologue of vertebrate β-spectrin (Fig. 3). We determined the structure of the unc-70 mRNAs by examining cDNA isolated by split leader sequence PCR, reverse transcription PCR, and from cDNA libraries. These experiments identified two splice variants of the unc-70 gene that differ only at their NH2 termini (Fig. 2 C).

Electron Microscopy
A dult progeny of homozygous unc-70(s1639), unc-70(s1502), and unc-70(r974) hermaphrodites were prepared for electron microscopy as previously described (Richard et al., 1999). Ribbons of ultrathin sections (~35 nm) were collected and examined on a Hitachi H-7100 transmission electron microscope (Hitachi Ltd.) equipped with a Gatan slow-scan digital camera (Gatan, Inc.). Images were adjusted for brightness, contrast, and size with a dobe Photoshop 5.0. M orphometry was analyzed using the public domain software package NIH Image.

For quantification of the synaptic vesicle number and localization, we examined 500 serial sections from two animals of the wild type, 650 sections from three animals of unc-70(s1639), and 100 sections from two animals of unc-70(s1502). A synapse was defined as all adjacent serial sections containing a higher than average number of vesicles surrounding a presynaptic density. To obtain the percentage of vesicles found at the plasma membrane, we counted all vesicles in each synapse that were touching or within one vesicle diameter of the plasma membrane, divided by the total number of vesicles per synapse. Similar percentages were obtai
Expression of unc-70

The severe uncoordinated phenotype of unc-70 suggested that unc-70 was likely to be expressed in the motor system, either in muscles or neurons. We determined the expression pattern of the unc-70 gene using GFP reporter constructs. GFP expression for the predominant isoform (CeS1::GFP, see Materials and Methods) was first detected in the embryo in all cells except the intestine; by hatching, expression was confined mainly to neurons and muscles. In the adult, the strongest expression was found in neurons, all or nearly all of which expressed this isoform (Fig. 4, A and C). This correlates well with the high levels of UNC-70 protein observed by immunofluorescence in neurons (Mohr et al., 2000 [this issue]). Robust expression was also observed in all muscles, including the pharyngeal (Fig. 4 A), vulval, uterine, enteric (Fig. 4 B), and the body wall (Fig. 4 C) muscles. Again, UNC-70 protein is found in these cells (Mohr et al., 2000 [this issue]). The spermatheca also expressed the predominant isoform (Fig. 4 D), and low levels of expression were observed in the hypoderm. The gut and gonad did not express visible levels of the predominant isoform. Expression of the second isoform (CeS2::GFP; see Materials and Methods) was also detected in embryos, in all larval stages, and in adults. However, expression of this isoform was confined to the gut from at least hatching onwards (Fig. 4, E and F). Expression of the unc-70 gene in the gut observed by immunofluorescence, which reveals UNC-70 protein in the gut at all developmental stages (Mohr et al., 2000 [this issue]). Neurons and muscles that expressed high levels of the predominant isoform did not express visible levels of the gut-specific isoform. Together, these results demonstrated that β-spectrin is expressed in most tissues of C. elegans throughout its life span.

unc-70 Mutations

To ensure that the phenotype observed in unc-70 animals represents the null phenotype for this locus, we identified a molecular null in the unc-70 gene by sequencing recessive alleles. unc-70(s1639) is likely to be a functional and a molecular null for three reasons. First, the mutation introduces a stop codon that truncates the predicted protein after 48 residues and, therefore, lacks all of the functional domains of the β-spectrin. This mutation is in an exon

Domain-by-domain comparison of the percent identity between CeS1, HsS1, and Drosophila β-spectrin. The NH2-terminal domain, spectrin repeats, and COOH-terminal domains are indicated below.

Figure 2. Genetic and molecular characterization of unc-70. (A) Genetic map of the unc-70 region. (B) Rescue of unc-70. The cosmid T19F4 rescued the unc-70 phenotype in three out of three lines. A 11.9-kb PCR fragment, only containing the spectrin gene, also rescued the unc-70 phenotype in three out of three lines. Predicted ORFs are shown below. (C) unc-70 cDNA s. Exons are shown as closed boxes and introns as lines. Two splice variants, CeS1 and CeS2, are shown. CeS1 is trans-spliced to the SL1 sequence. The 5‘ end of CeS2 was not determined. (D) cDNA showing the exon boundaries and the positions of three nonsense alleles induced by ethylmethane sulfonate. unc-70(s1639) is a G to A transition that changes Gln576 to a UAA codon; and unc-70(r707) is a G to A transition that changes Trp927 to a UGA codon. (bottom) Domains of the protein. (E) Domain-by-domain comparison of the percent identity between CeS1, HsS1, and Drosophila β-spectrin. The NH2-terminal domain, spectrin repeats, and COOH-terminal domains are indicated below.

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common to both the Ceβ51 and the Ceβ52 isoforms. Second, it is as severe as any other unc-70 allele; in fact, all alleles are indistinguishable as homozygotes. Third, unc-70(s1639) homozygous animals show no immunoreactivity to a β-spectrin antibody (data not shown; antibody provided by S. Moorthy and V. Bennett). We also identified two other alleles, s1502 and r974, that truncate the predicted protein after 575 and 927 residues respectively; these mutations are also in exons common to the two isoforms (Fig. 2 D). We observed no phenotypic differences among these three alleles. Thus, we used these presumptive null alleles to infer the function of β-spectrin.

unc-70 is the only β-spectrin in the C. elegans genome. Further, while βH-spectrin is similar to β-spectrin in some respects, it is unlikely to substitute functionally for the loss of β-spectrin. βH-Spectrin is expressed primarily during embryogenesis, and is found mainly in epithelial tissues (McKeown et al., 1998). Even in epithelial tissues that express both β-spectrin and βH-spectrin, these proteins appear to play separate roles since β-spectrin is located at the basolateral membrane, whereas βH-spectrin is found at the apical membrane (Thomas and Kiehart, 1994). Therefore, null mutations in unc-70 are likely to result in a complete loss of β-spectrin function.

β-Spectrin Is Not Required for Cell Polarity or Membrane Integrity

One hypothesized role for β-spectrin is that it functions in the generation of cell polarity (Drubin and Nelson, 1996). To test whether the loss of β-spectrin affects cell polarity, we compared the polarized epithelial cells of the C. elegans gut in unc-70(r974) to those of the wild type (White, 1988). We found no evidence of a loss of polarity in unc-70(r974) animals by the ultrastructural criteria (n = 6 worms). Both the apical (Fig. 5, A and C) and the basolateral (Fig. 5, B and D) domains of the intestine were normal in unc-70(r974) mutants. Specifically, the microvilli and electron-dense structures underlying the apical membrane were indistinguishable from those of the wild type (Fig. 5, A and C, arrowheads). Belt desmosomes were also present in unc-70(r974) (Fig. 5, A and C, arrows). The basolateral membrane of unc-70(r974) was also indistinguishable from the wild type (Fig. 5 B and D, arrow). Similar results were found in unc-70(s1502) and unc-70(s1639). Together, these results show that β-spectrin is not required for the generation or maintenance of cell polarity in the gut epithelium. To test whether this was true in other tissue types, we examined unc-70(r974), unc-70(s1502), and unc-70(s1639) neurons and muscles for cell
polarity defects (see below). While both of these tissues exhibited severe defects in unc-70 mutants, we found no evidence that cell polarity was defective. Thus, \( \beta \)-spectrin is not a general determinant of cell polarity.

\( \beta \)-Spectrin also has been proposed to function in the general membrane integrity (Lux and Palek, 1995). We inspected the plasma membrane of unc-70(r974), unc-70(s1502), and unc-70(s1639) for the presence of general membrane defects such as herniation, invagination, vesiculation, or the loss of cell-cell contact. We found no general membrane defects in any of the tissues examined, which included the gut epithelia (Fig. 5), neurons (see Fig. 7), and muscles (see Fig. 9). Thus, neither epithelial cells, neurons, nor muscles rely on \( \beta \)-spectrin to maintain the integrity and shape of their plasma membranes.

**\( \beta \)-Spectrin Is Required for Axonal Outgrowth**

Animals homozygous for null alleles of unc-70 exhibit phenotypes, such as paralysis, which could be attributed to abnormal neural function. To assess potential defects in the nervous system of unc-70 homozygous animals, we visualized the \( \text{GABA} \) nervous system of wild-type and unc-70(s1639) animals using a GFP reporter construct (Fig. 6, A–D). To test whether the lack of \( \beta \)-spectrin affects neurogenesis or neuronal cell differentiation, we assayed whether unc-70(s1639) animals have the normal number of \( \text{GABA} \) neurons. We found that unc-70(s1639) does not result in a defect in the number of neurons; all worms examined had the normal complement of 19 \( \text{GABA} \) motor neurons in the ventral nerve cord (Fig. 6, A–D). Moreover, while spacing of the neuronal cell bodies is not as regular in unc-70(s1639) as in the wild type, in general these cell bodies are positioned along the ventral side of the animal and are distributed along the anterior–posterior axis. Thus, \( \beta \)-spectrin does not appear to play an essential role in neurogenesis, neuronal cell identity, or migration, at least in the \( \text{GABA} \) nervous system.
motor neurons. Similar results were obtained with unc-70(s1502).

To determine the role of β-spectrin in axon outgrowth, we examined three structures in unc-70 animals: the ventral nerve cord, the commissures that extend from the ventral to the dorsal cord, and the dorsal cord. We found defects in all of these structures, demonstrating that unc-70 has an important role in axon outgrowth. Specifically, the ventral nerve cords in all unc-70(s1639) animals examined (n = 5 worms) were defasciculated, and in most animals, segments of the ventral nerve cord appeared to be composed of a single process (n = 5 worms; Fig. 6 D, arrow).

The number of commissures that reached the dorsal cord. Only commissures that reached the dorsal surface were counted. Our analysis may slightly underestimate the number of commissures since the actual number in the wild type is 19 (White et al., 1986).
Despite these defects, however, all animals had regions of their ventral cord that appeared similar to the wild type, suggesting that some axon outgrowth along the ventral cord occurs even in the absence of β-spectrin.

Al µ-unc-70(s1639) animals examined had severe defects in their commissural outgrowth (Fig. 6 E). Only 2.4 (± 0.5, n = 5) commissures reached the dorsal cord in unc-70(s1639) animals, compared with 17.4 (± 0.2, n = 5; P < 0.0001) identified in wild type. The few commissures present in unc-70 animals, whether or not they reached the dorsal cord, displayed a variety of aberrant morphologies (Fig. 6 D, arrowheads). Many wandered, were branched, and often gave rise to large, complex elaborations elongated along the anterior–posterior axis. Other commissures terminated prematurely, sometimes ending with a large terminal expansion similar in appearance to a growth cone. We also observed commissures extending anterior of the GABA-expressing RME neurons, where none are normally present (Fig. 6 B, arrowheads).

Compared with these severe defects in commissural outgrowth, outgrowth along the dorsal cord was relatively unaffected. Specifically, of the commissures that reached the dorsal cord in the unc-70(s1639) animals, most (7/12 commissures) extended along the anterior–posterior axis to form a short segment of dorsal cord (Fig. 6 B, arrow). Thus, extension along the dorsal cord appears to be more normal (58%, 7/12) than the extension from the ventral to dorsal cord (14%, 12/87) in unc-70 mutants. Growth cone migration from the ventral to the dorsal cord may be specifically difficult because of the absence of previously formed axons with which a growth cone can fasciculate or because of an increased number of physical obstacles that the growth cone must negotiate (Knobel et al., 1999).

**β-Spectrin Is Not Required for Synaptogenesis or Synaptic Vesicle Localization**

Because UNC-70 is highly expressed in mature as well as in developing neurons, *C. elegans* β-spectrin could play a role in neuronal function in addition to its requirement during axon outgrowth. To determine whether spectrin is required for the structure of the synapse, we analyzed the ultrastructure of neuromuscular junctions in unc-70(s1639) and unc-70(s1502) animals. Surprisingly, we found no significant difference between wild-type and unc-70 animals (Fig. 7, A and B). Neuronal membranes appeared identical in wild-type and unc-70 animals; as described above, they showed no membrane defects such as herniation, invagination, vesiculation, or loss of cell–cell contact. Presynaptic specializations also appeared normal in unc-70 animals (arrows).

To identify the potential defects in synaptic vesicle production, localization, release, or endocytosis, we quantified the distribution of synaptic vesicles in unc-70(s1639) and wild-type synapses (Fig. 7, C). The average number of synaptic vesicles per synapse did not differ significantly between wild-type and unc-70(s1639) animals (wild type = 297 ± 30, n = 19; unc-70(s1639) = 386 ± 74, n = 16; P = 0.25). Furthermore, we found no significant difference in the localization of these synaptic vesicles, both for the proportion of the vesicles at the plasma membrane (wild type = 34 ± 3%, n = 17; unc-70(s1639) = 35 ± 3%, n = 15; P = 0.73) and for the proportion of vesicles at the presynaptic specialization (wild type = 11 ± 1%; unc-70(s1639) = 9 ± 1%; P = 0.24). Although these data do not preclude a functional role for spectrin at the synapse, they do suggest that spectrin is not playing a structural role. In addition, the presence of synaptic vesicles and other synaptic components at their normal locations suggests that neuronal cell polarity does not require β-spectrin.

**β-Spectrin Is Required for Normal Sarcomere Structure**

Because β-spectrin is expressed in muscles at all developmental stages, it is possible that the paralyzed phenotype of unc-70 mutants is caused in part by the loss of spectrin in muscles. To visualize sarcomere structure, we stained thick filaments using an antibody against paramyosin (Goh and Bogaert, 1991). Because *C. elegans* body wall...
muscles are obliquely striated, the myosin- and paramyosin-containing thick filaments (A bands) are arranged in regular bands almost parallel to the axis of contraction (Fig. 8 A). These brightly staining bands are separated by thick nonstaining bands, which are the actin-containing thin filaments (I bands, arrow), and by thin nonstaining bands, which are the myosin attachment structures (M lines, arrowhead). To determine whether unc-70 mutations affect the overall shape of muscle cells, we compared the length and width of these cells to those of the wild type (Fig. 8 C). We found that on average, unc-70(s1639) muscles were shorter than wild-type muscles, which is consistent with the short overall body size of the unc-70 mutants (wild type = 135 ± 3.7 μm, n = 4; unc-70(s1639) = 74 ± 2.6 μm, n = 8; P < 0.0001). The width of unc-70(s1639) muscles was not significantly different than wild type (wild type = 14.6 ± 0.7 μm, n = 5; unc-70(s1639) = 14.0 ± 0.5 μm, n = 9; P = 0.5276). In addition to the reduced length of muscles in the mutants, the arrangement of sarcomeres was disrupted when compared with those of the wild type. Fewer sarcomeres were found in unc-70(s1639) muscles than in the wild type (wild type = 9.3 ± 0.3, n = 4; unc-70(s1639) = 6.2 ± 0.2, n = 9; P < 0.0001). On average, each unc-70(s1639) sarcomere was wider than in the wild type (wild type = 1.57 μm, unc-70(s1639) = 2.26 μm). However, it was readily apparent that this average increase in sarcomere width in unc-70 muscles was unevenly distributed (Fig. 8 B). The width of individual sarcomeres often varied along their length in unc-70 mutants; variable widths were never observed in the wild type. Further, the relationship between adjacent sarcomeres was disrupted in unc-70, such that in some areas, large gaps appeared between A bands (arrow), whereas in other areas, adjacent A bands appeared to overlap (arrowhead). However, most muscles also had small regions in which the banding pattern appeared normal (double arrowhead). These data suggested that unc-70 is required for normal myofilament organization.

These defects in sarcomere structure were confirmed at the ultrastructural level (Fig. 9). In transverse sections of wild-type specimens, the alternating bands of thick and thin filaments (A and I bands, respectively) were clearly defined. Rarely, we observed unc-70(s1639) sections that lacked thick filaments; these sections likely correspond to the gaps between sarcomeres discussed above. In most unc-70(s1639) sections, a distinct band of thin filaments was not visible; instead, thick filaments were found throughout the section, often clustered around the dense bodies. These phenotypes appear to correspond to the enlargement of brightly staining thick filament-containing areas that we observed with paramyosin staining. In addition to the mislocalization of thick filaments, these filaments were not clustered in evenly spaced columns as they are in the wild type. Finally, M lines were reduced or absent.

In addition to defects in the arrangement of the myofilament lattice, unc-70 animals had defects in dense body and sarcoplasmic reticulum morphology. Dense bodies in mutant specimens were narrower than the wild type and often observed to taper sharply, whereas in the wild type, they appeared uniformly thick. Discontinuities in the electron-dense portion of the dense bodies, never observed in the wild type, are often present in unc-70 muscles (Fig. 9 B, arrow). In the wild type, the sarcoplasmic reticulum is a series of elongated vesicles closely associated with the dense bodies and with the plasma membrane beneath the myofilament lattice (Fig. 9 A, arrowheads). These structures were reduced in number or absent in unc-70 muscles. When present, their distribution was restricted to the plasma membrane, never extending along the sides of the dense bodies as observed in the wild type.

Despite the severe defects observed in unc-70 sarcomere organization, the overall muscle organization was normal, with the myofilaments and dense bodies polarized to the hypodermal side of muscle cells. Also, the sarcomeres were correctly oriented parallel to the body axis. This sug-
gests that β-spectrin is not required for overall cell polarity in muscles.

The structural defects seen in unc-70 muscles could be due to the β-spectrin function in the development of the sarcomeres or to the β-spectrin function in maintenance of sarcomeres during muscle contraction. To distinguish between these possibilities, we tested whether the unc-70 phenotype was suppressed by unc-54(e1092). unc-54 encodes the predominant myosin in the body muscles, and unc-54 mutant animals are capable of only weak muscle contractions (Dibb et al., 1985). We found that unc-54(e1092); unc-70(r974) animals were significantly longer than unc-70(r974) animals, although not as long as the wild type (Fig. 10). These data indicate that muscle contraction causes at least part of the length defect in unc-70, and suggest that an important function of β-spectrin in muscle is during muscle contraction.

Discussion
The results presented in this study are the first characterization of adult animals lacking β-spectrin, a component of the membrane skeleton that is found in most tissues in vertebrates and C. elegans. The unc-70 gene encodes the C. elegans homologue of β-spectrin. A null allele of unc-70 that have been previously characterized as lethal mutations can survive and propagate under certain conditions. These animals have two striking phenotypes: they are paralyzed and dumpy. To determine which cellular processes are disrupted in unc-70 animals, we examined three tissues in which β-spectrin is expressed: gut epithelia, neurons, and muscles.

In erythrocytes and in some other tissues, the loss of β-spectrin leads to general defects of the plasma membrane such as herniation and the loss of cell adhesion (for review see Lux and Palek, 1995). Thus, one possible func-

Figure 9. Ultrastructural defects in unc-70 muscles. Sections show a representative region of the adult wild-type (A) and unc-70(s1502) (B) body wall muscle. Each panel shows three dense bodies (db) and the neighboring myofilament lattice; the hypodermis is at the bottom. The I and A bands and the M line are indicated in the wild-type muscle; no similar organization is present in unc-70(s1502). Arrowheads indicate sarcoplasmic reticulum, which is not present in unc-70 animals.

Figure 10. Suppression of unc-70 length defects by a mutation in the body wall myosin. A duet progeny of homozygous animals were mounted in 10 mM sodium azide and photographed. Length at the midline was measured using NIH Image. Data are shown as mean ± SD, n = 5. unc-54(e1092); unc-70(r974) is significantly different from unc-70(r974) and from the wild type (P < 0.001).
tion for β-spectrin is to support general membrane integrity and adhesion. However, we did not observe any general membrane defects in unc-70 animals. Plasma membranes and cell–cell contacts appeared normal in muscles and gut epithelia. In neurons, we also failed to observe any general membrane defects, although the defect we observed in axonal architecture could be due to membrane defects in growth cones. We conclude that C. elegans β-spectrin is not required for general membrane integrity or for cell adhesion.

β-Spectrin displays a differential localization in some polarized cells, such as MDCK cells and vertebrate neurons (for review see Morrow et al., 1997). This suggests that β-spectrin may be a determinant of cell polarity. However, we observed no polarity defects in any of the tissues we analyzed. In the gut epithelia, the apical and basolateral domains retained their normal identity in unc-70 animals by a number of ultrastructural criteria. In muscles, we found the myofilament lattice to be polarized to the lateral surface and correctly oriented relative to the body axis in unc-70 animals. In neurons, we found that the ultrastructure of neuromuscular junctions of unc-70 animals was normal. Together, these data suggest that β-spectrin is not a determinant of cell polarity in C. elegans.

Although we failed to find evidence for β-spectrin function in processes of cell polarity, we did find abundant defects in unc-70 animals, which indicate that β-spectrin is essential for specific processes at cell membranes. One process that requires β-spectrin is axon outgrowth. While unc-70 animals appeared to have normal complement of neurons, these neurons did not extend axons to their targets. These data suggest that β-spectrin is required in growth cones during axon extension. A role for spectrin in growth cone function is supported by the observed distribution of spectrin in cultured vertebrate neurons (Sobue and Kanda, 1989; Sobue, 1990). In these cells, spectrin is found localized to the sites of membrane–substratum adhesion in growth cones. In addition, neurite extension in cultured neuroblastoma cells is inhibited by the injection of the NH2-terminal β-spectrin peptides, which presumably disrupt the spectrin–actin interactions (Sihag et al., 1996). Together with our results, these data suggest that functional β-spectrin is required in growth cones during neuronal outgrowth, potentially at adhesion sites. Alternatively, β-spectrin may be required in the substrate across which growth cones migrate.

β-Spectrin is also expressed at high levels in the mature nervous system of C. elegans, which suggests that it plays a role in neuronal function after development is complete (Sikorski et al., 1999). However, we did not observe any defects that suggested a role for β-spectrin in mature neurons. Our results suggest that β-spectrin does not function in axonal transport or synaptic vesicle localization. We observed no decrease in synaptic vesicle number, as would be expected for a defect in axonal transport of vesicles (Hall and Hedgecock, 1991). We also observed no defects in the distribution of vesicles at the synapse, as would be expected if unc-70 was involved in synaptic vesicle localization. It has been proposed that β-spectrin is a component of the 100-nm filaments that appear to anchor synaptic vesicles to the presynaptic density (Goodman et al., 1995). We did not directly address the presence or absence of these filaments in animals lacking β-spectrin, since it was difficult to observe them even in our wild-type sections. However, our data suggest that if β-spectrin is a component of these 100-nm filaments, then either the filaments are not required for synaptic vesicle localization at the resolution of our electron micrographs, or a compensatory mechanism exists. Finally, β-spectrin does not appear to be an essential component of the presynaptic density, since this structure appears normal in electron micrographs.

It is possible that spectrin plays a role in synaptic vesicle exocytosis, rather than in the organization of the synapse. A vertebrate β-spectrin isoform has been shown to interact with the vertebrate UNC-13 protein, which is an essential component of the presynaptic vesicle release machinery (Ohara et al., 1998; Sakaguchi et al., 1998). Mutations in the unc-13 gene in C. elegans prevent synaptic vesicle fusion and result in a large accumulation of vesicles at the synapse (Richmond et al., 1999). We did not observe a statistically significant accumulation of vesicles in unc-70, suggesting that spectrin is not required for synaptic vesicle exocytosis. Unfortunately, a more detailed analysis of such a role is not possible because of the severe developmental defects in the unc-70 nervous system.

In addition to its role in neuronal development, we also identified a requirement for β-spectrin in muscles. Our analysis of muscle structure in animals lacking β-spectrin demonstrated that the sarcoplasmic reticulum was absent from around the dense bodies, and that the thin filament attachment structures (dissociation and assembly of actin) were disrupted. β-Spectrin is specifically found at thin and thick filament attachment structures in both C. elegans (Morony et al., 2000 [this issue]) and vertebrate muscle (Nelson and Lazarian, 1984), and the regular, striated arrangement of myofilaments is blurred in unc-70 mutant animals. These data suggest that β-spectrin may function to link the sarcoplasmic reticulum and the thin filaments to the dense bodies, and the M lines to the cell membrane. In the absence of β-spectrin, the myofilament lattice detaches from its anchors and becomes disorganized. Alternatively, β-spectrin could be playing a more direct role in muscle contraction. Specifically, we found that the dumpy phenotype of β-spectrin mutants was partially suppressed by a mutation that reduces muscle contraction. Moreover, in animals lacking β-spectrin, thick filaments invaded the I bands, suggesting that these muscles are hypercontracted. Thus, β-spectrin and the membrane skeleton may function as a compressive spring in muscle, acting to return muscle cells to their expanded state after contraction.

Our analysis of β-spectrin in C. elegans suggests that β-spectrin’s function in nonerythrocyte cells is not for general membrane support since we observed no defects in membrane integrity in any of the tissues we examined. β-Spectrin does not appear to be essential for the generation or maintenance of cell polarity since we found no cell polarity defects in any tissue. We conclude that β-spectrin has specific but essential functions in a variety of tissues. The link that unites these functions is likely to be the membrane skeleton’s ability to anchor proteins at or near the plasma membranes. While sarcomere stabilization and neuronal outgrowth appear to be dissimilar processes,
their requirement for β-spectrin suggests that muscles and neurons use a common mechanism to control protein localization at membranes, although the specific target proteins may differ.

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