Epithelial Polarity, but Essential for Muscular and Neuronal Function

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Abstract. The Caenorhabditis elegans genome encodes one α spectrin subunit, a β spectrin subunit (β-G), and a β-H spectrin subunit. Our experiments show that the phenotype resulting from the loss of the C. elegans α spectrin is reproduced by tandem depletion of both β-G and β-H spectrins. We propose that α spectrin combines with the β-G and β-H subunits to form α/β-G and α/β-H heteromers that perform the entire repertoire of spectrin function in the nematode. The expression patterns of nematode β-G spectrin and vertebrate β spectrins exhibit three striking parallels including: (1) β spectrins are associated with the sites of cell–cell contact in epithelial tissues; (2) the highest levels of β-G spectrin occur in the nervous system; and (3) β spectrin-G in striated muscle is associated with points of attachment of the myofilament apparatus to adjacent cells. Nematode β-G spectrin associates with plasma membranes at sites of cell–cell contact, beginning at the two-cell stage, and with a dramatic increase in intensity after gastrulation when most cell proliferation has been completed. Strikingly, depletion of nematode β-G spectrin by RNA-mediated interference to undetectable levels does not affect the establishment of structural and functional polarity in epidermis and intestine. Contrary to recent speculation, β-G spectrin is not associated with internal membranes and depletion of β-G spectrin was not associated with any detectable defects in secretion. Instead β-G spectrin-deficient nematodes arrest as early larvae with progressive defects in the musculature and nervous system. Therefore, C. elegans β-G spectrin is required for normal muscle and neuron function, but is dispensable for embryonic elongation and establishment of early epithelial polarity. We hypothesize that heteromeric spectrin evolved in metazoans in response to the needs of cells in the context of mechanically integrated tissues that can withstand the rigors imposed by an active organism.

Key words: membrane skeleton • unc-70 • RNAi • cell–cell contact

Introduction

Maintenance of tissue integrity, particularly in tissues that undergo constant stress, is critical to the survival of an organism. Components important in this process are likely to include cytoskeletal proteins. Protein localization and in vitro studies make spectrin a likely candidate. Members of the spectrin gene family and their associated proteins have been identified in diverse tissues of vertebrates, echinoderms, and Drosophila melanogaster, suggesting that spectrin-based membrane skeletal structures are a ubiquitous feature of metazoan animal cells (Bennett and Gilligan, 1993; Thomas et al., 1997; De Matteis and Morrow, 1998). A specialized version of one spectrin-based structure has been resolved in molecular detail in the human erythrocyte membrane, where this assembly of proteins is essential for the survival of erythrocytes in the circulation (Lux and Palek, 1995). Since most cell types contain additional transcellular cytoskeletal elements, paradigms derived from the red cell membrane skeleton are of limited use in studying functions of nonerythroid spectrin.

Current views of physiological functions of spectrin are derived from localization of spectrin in cells and tissues and interactions of spectrin with proteins in vitro assays. Spectrin has been implicated in the establishment and/or maintenance of epithelial polarity based on localization at sites of cell–cell contact and association in complexes with cadherins (Nelson and Veshnock, 1987; Nelson and Hammerton, 1989; Yeaman et al., 1999). Spectrin also has been proposed to participate in the establishment of membrane subdomains in striated muscle (Craig and Pardo, 1983; Bloch and Morrow, 1989), in lymphocyte capping (Black...
et al., 1988), and in the organization of synaptic vesicles at presynaptic nerve endings (Sikorski et al., 1991). More-
over, spectrin is thought to participate in axonal transport,
based on localization and dynamic behavior in axons (Le-
vine and Willard, 1981). Recently, β-spectrin has been
linked to Golgi function based on colocalization with Golgi membranes (Beck et al., 1994; Devarajan et al., 1997; Fath et al., 1997; Stankevich et al., 1998). These ob-
servations and other results (Holleran et al., 1996) have
led to speculation on possible roles of spectrin in maintain-
ing Golgi structure and in intracellular vesicular transport
(Beck and Nelson, 1998; De Matteis and Morrow, 1998;
Kiehart, 1994; Dubreuil et al., 1997; Thomas and
McKearn, 1994; Dubreuil et al., 1997; Thomas et al., 1998;
Zarnescu and Thomas, 1999). α and β spectrins, the addu-
cin-like hts product, and ankyrin all have been identified
as molecular components of Drosophila fusomes (Lin et
al., 1994; de Cuevas et al., 1996) and a related structure in
germline stem cell termed the spectrosome. The precise
function of spectrin in these structures has yet to be estab-
lished.

Caenorhabditis elegans is a simple metazoan with a de-

defined genome that is accessible to both genetics and cell bi-

ology. The C. elegans genome contains only three spectrin
genomes encoding an α subunit, a β-G subunit, and a β-H subunit (see Fig. 2). Mutations in each of these spectrin
subunits have been identified genetically. A mutation in the
α spectrin gene, spc-1(r2409), has been isolated in a
screen for lethal mutations that cause muscle defects (Nor-
man, K., and D. Moerman, personal communications).
Mutations in β-H spectrin encoded by the sma-1 gene
cause elongation and morphogenetic defects (M eckown
et al., 1998). Recently, H ammarlund et al. (2000) mapped
mutations in the unc-70 gene to β-G spectrin, resulting in
animals exhibiting multiple defects in the muscle and the
nervous system.

This study presents an overview of spectrins and, in par-


ticular, β-G spectrin in C. elegans, and critically examines
the proposed roles in intracellular membranes and in the
establishment of epithelial polarity. The expression pat-
ttern of the single β-G spectrin in all cells throughout the
life cycle of the nematode is described, and striking paral-
els are seen between this pattern and that previously char-
acterized for Drosophila and vertebrate orthologues of
spectrin. Further experiments critically examine the ef-


tects of depletion of β-G spectrin on epithelial polarity,
embryogenesis, and Golgi function. A surprising conclu-
sion is that β-G spectrin is not required for these pro-
cesses. Similar results have been obtained by analysis of
unc-70 β-G spectrin-null animals (H ammarlund et al.,
2000). Instead, the results support the hypothesis that het-
eromer spectrin evolved in metazoans in response to the
need for a multipotent adaptor that could help integrate
individual cells into complex tissues and organs.

Materials and Methods

Reagents

Abbreviations used in this paper: β-G, C. elegans β spectrin; β-H, β-H
2 spectrin; dsRNA, double-stranded RNA; ORF, open reading frame; PH, pleck-
strin homology; RNAi, RNA-mediated interference; ssRNA, single-
stranded RNA.

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ing JAM-1, mAb D M.5.6 recognizing mhcA, mAb B M H 25 against C. elegans β-integrin, and mAb B M H 5 against a hemidesmosomal component (all from Sigma Chemical Co.) was used to visualize DNA and was added along with secondary antibody. Immunofluorescent images were obtained using a laser scanning microscope. Phase-contrast images were obtained using excitation at 633 nm to minimize bleaching of GFP and FITC. For muscle, the freeze crack protocol for embryos and L1 larvae was modified by using the formaldehyde-methanol fixation method of Finney and Ruvkun (Epstein and Shakes, 1995) at −20°C. Biotin-X-phalloidin (Molecular Probes, Inc.) was used along with primary antibodies, and Cy5-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc.) at 1 μg/ml was added with secondary antibodies.

**Sequence Analysis**

Orthologs of C. elegans spectrin genes were identified using BLAST (A Itschul et al., 1997). Sequences accessed from GenBank for this paper are as follows: human nonerythroid α spectrin SPTA1 N (J05243), non-erythroid β-spectrin SPTBN1 (NM 000128), β1-2-spectrin SPTBN2 (A1899567); for Drosophila, β1 spectrin D RO BSPE C (M92288); and for C. elegans, α spectrin (KN12345), β-1 spectrin SMA-1 (A053496), and β-5 spectrin BG5-1 (A F166170 and A F166199). The SPC-1 open reading frame (ORF) was predicted using the Baylor College of Medicine GeneFinder program. Dot matrix plots were generated using M a c V e c t o r v6.0.1. Sequence alignments were made using Geneworks v2.5.1.

**Molecular Biology**

Standard cloning techniques were used (Sambrook et al., 1989). The ADD-2 polypeptide was identified because of partial homology to vertebrate adducin, and cloned by S. Moorthy. The (add-2:GFP) plasmid construct pSM 69.57 was made using plasmid pPD95.69 as parent vector, and and the cosm id F57F5 as the source of promotor sequence. The plasmid pSM 69.57 fuses 8.1 kb of upstream genomic sequence and a part of the ADD-2 ORF, in-frame with the GFP.

**Worm Culture**

C. elegans strains were maintained at 20°C using standard culture conditions (Sulston and Hodgkin, 1988), sma-1(r181) strain (M C K e w n et al., 1998) was a gift from J. A u s t i n (University of Chicago, Chicago, IL). Transgenic strains carrying the extrachromosomal arrays (cosmid T 2 C 12; pRF 4) and (pSM 69.57; pRF 4) (see above) were selected using the pRF 4 (rol-6(e1006)) reporter phenotype as a dominant selectable marker; the latter strain expresses GFP only in the gut cells.

**Nematode β-G Spectrin Antibody**

BGS-1 COOH-terminal residues, A 1851 to K 2257 of the shorter splice, was cloned into pG em e x-2 (P Omega) in-frame with viral Gene 10. Recombinant fusion protein was purified to near homogeneity from inclusion bodies (Li and Bennett, 1996), and was used to immunize male New Zealand rabbits. Serum from individual rabbits was preadsorbed over a Gene 10 column to eliminate antibodies recognizing Gene 10, and affinity-purified over an antigen fusion protein column. The final antibody, which recognizes both spectrin-spliced isoforms, showed a minimal cross-reaction with other C. elegans proteins or purified Gene 10.

**Immunoblots**

Synchronized nematode populations (Epstein and Shakes, 1995) at different developmental stages were harvested and directly dissolved in boiling SDS-PAGE buffer. Protein lysates were separated on a gradient PAGE gel as described (Li and B. Bennett, 1996). Electrophoresed proteins were transferred onto nitrocellulose and blotted with affinity-purified rabbit antibodies against β-5 spectrin. The blot was washed, incubated with [125I]-labeled protein A, washed again, and exposed on either film or a Phosphoimager screen to visualize the band that corresponds to β-5 spectrin.

**Immunofluorescence Microscopy**

Embryos, young larvae, and adults were fixed and stained for indirect immunofluorescence using standard protocols (Epstein and Shakes, 1995). In brief, the freeze crack methanol fixation method was used for embryos and young larvae, whereas adult worms (Korda et al., 1995) from adults were fixed using the formaldehyde-methanol freeze thaw method of Finney and Ruvkun (Epstein and Shakes, 1995). Specimens were preblocked with 10% normal goat serum in PBS containing 2% B SA and 0.1% T ween 20. Primary antibodies and sera were diluted (1:500 for β-5 spectrin, 1:1000 for M H 27, 1:250 for D M 5.6 and M H 25, and 1:100 for M H 5) and incubated with fixed specimens at 4°C overnight; followed by three PBS-Tween 20 washes at room temperature (15 min each for embryos, 1-h washes for adults). Incubation with the secondary antibody (1 μg/ml) was for 2 h at 4°C, followed by similar washes. H och e 153323 (Sigma Chemical Co.) was used to visualize DNA and was added along with secondary antibody. Immunofluorescent images were obtained using a laser scanning microscope. Phase-contrast images were obtained using excitation at 633 nm to minimize bleaching of GFP and FITC. For muscle, the freeze crack protocol for embryos and L1 larvae was modified by using the formaldehyde-methanol fixation method of Finney and Ruvkun (Epstein and Shakes, 1995) at −20°C. Biotin-X-phalloidin (Molecular Probes, Inc.) was added along with primary antibodies, and Cy5-conjugated streptavidin (J Jackson ImmunoResearch Laboratories Inc.) at 1 μg/ml was added with secondary antibodies.

**Visualization of Dye-filling Amphid Neurons**

Living L1 larvae were suspended in a 1–5 μm solution of dye in M 9 buffer for 1–2 h, and then washed three times for 10 min at room temperature (Hedgecock et al., 1985). A nimals were allowed to recover briefly on unseeded plates before being mounted on a soft agarose pad using 10 mM Na cl in M 9 buffer as an anesthetic. The pad was gently sealed with a No. 1.5 coverslip, using a small amount of mineral oil to prevent evaporation. A nimals were observed on the confocal microscope using rhodamine optics for D I I.

**Protein Depletion Using RNAi**

Single-stranded RNA (ssRNA) 1–2 kb in length was synthesized using the M eg a S c r i p t RNA Synthesis K it (A mbion, Inc.). Plasmids used as templates for RNA synthesis were the following: CE E SK 92, pSCSM-2 and pSCSM-II for C. elegans β-spectrin, yk919h and yk36c9 for spc-1, spectrin, and yk96e11 and yk148d4 for sma-1 β-spectrin. Sense/antisense ssRNA annealing to generate double-stranded RNA (dsRNA) was carried out in 1× S TO P buffer for 30 min, at 68°C (Fire et al., 1998). A nimals were microinjected into the syncytial gonad with each dsRNA at a concentration of 1 μg/ml or higher (Fire et al., 1998). If there were two or more genes, dsRNA preparations corresponding to each gene were concentrated and mixed to give a final concentration of 1 μg/ml with respect to each dsRNA. Survivors of the injection were pooled on a single plate for ~8 h and placed separately on individual seeded plates. The adult hermaphrodite was transferred to a fresh plate every 12–24 h, resulting in a semi-synchronous set of embryos being left behind at each transfer. Progeny in the 24–72 h interval after egg laying either were analyzed for phenotype or were killed for immunoblots to determine the efficiency of RNAi depletion of β-spectrin. A ll preparations corresponding to a given gene gave identical results. Usually ~90% of the F1 progeny of surviving animals displayed the same gene-specific phenotype. Injected adult worms did not seem affected by the RNAi and lived a normal life span. In all the RNAi experiments, the C. elegans N2 parental strain was used as the negative control.

**Live Imaging of C. elegans Embryos and Larvae**

G a v i d adult hermaphrodites were killed by cutting their uteri to release embryos into egg salts buffer (Epstein and Shakes, 1995). Embryos were mounted on a soft agarose pad in the same buffer, gently covered with a coverslip, and sealed with mineral oil. Development from zygote to hatching stage was observed using a 40× dry objective with Nomarski differential interference optics at 22–23°C, and also recorded on videotape. The developmental stage (1.5-fold, 2-fold, 3-fold, pretzel, cuticle expression, and pharyngeal pumping) of each embryo was recorded at 30-min intervals and later compared with the tape. E m b r y o s that arrested early during embryogenesis were eliminated from the analyses. E mbryonic lengths were measured off the video monitor using appropriate conversion factors.

**Phosphoimaging to Determine Efficiency of Protein Depletion by RNAi**

Immunoblots were exposed to a phosphoimage screen to quantitate the amount of β-G spectrin in RNAi and control progeny using the phosphoimage program, ImageQuaNT (M olecular D ynamics). The immunoblot background was subtracted from each set of phosphoimage data, and the result was divided by the number of animals used to make the lysates (250 for both control and bgs-1 RNAi). The resulting numbers were presented as arbitrary units of β-G spectrin per animal.

**Electron Microscopy**

A nimals were fixed and embedded for electron microscopy (Epstein and...
Shakes, 1995). Stained, dehydrated, and resin-infiltrated animals were cut into 90-nm sections, and the sections were placed on uncoated copper grids.

Results

Isolation of C. elegans β-G Spectrin (BGS-1) cDNAs

cDNA and reverse transcriptase-PCR clones corresponding to the C. elegans β-spectrin were isolated, the ORF was determined, and a functionally significant alternate splice isoform was identified. A BLAST search (see Materials and Methods) using the sequence of human β-G spectrin (SPTBN1) recognized a putative β-spectrin subunit distributed between adjacent cosmids K11C4 and T19F4 on C. elegans chromosome V (C. elegans Sequencing Consortium, 1998). The search also identified a 2.8-kb fragment (clone CEE5K92) from Y ui K ohar a’s expressed sequence tag library. Probes derived from CEE5K92 were used to screen a C. elegans multistaged cDNA library (see Materials and Methods: Barstead and Waterston, 1991). Multiple cDNAs covering ∼5.8 kb of a predicted 7.6-kb mRNA were obtained (Fig. 1 A). Reverse transcriptase-PCR and 5′RACE were used to extend this sequence further 5′. The very 5′ region of the ORF was not reached; however, based on homology with human β-G spectrin (Hu et al., 1992), results of the BLAST search, and the consensus sequence for C. elegans intron/exon borders (Blumenthal and Steward, 1997), a good candidate for the first exon was identified.

The final predicted nematode β-G spectrin cDNA encodes a protein made of 2,302 amino acids that is distributed over 13 exons (Fig. 1 B). The protein contains all domains typical of β spectrin including the following: an NH$_2$-terminal calponin homology region, which is a domain shown to be involved in the formation of a ternary complex between β spectrin, actin, and adducin (Li and Bennett, 1996) and for lateral association between NH$_2$-terminal calponin homology region, which is a domains typical of

Figure 1. Cloning of C. elegans β-G spectrin cDNAs, including two alternatively spliced variants. (A) A partial cDNA clone, CEE5K92, was used to screen a C. elegans mixed stage λZAP cDNA library. Positions of representative partial cDNAs (SCSM-II and SCSM-2) and the reverse transcriptase-PCR sequence corresponding to the β-G spectrin (BGS-1) mRNA are shown. (B) Bgs-1 exon distribution. The transcriptional unit is distributed over 13 exons, of which at least two are alternatively spliced (asterisks). The BGS-1-predicted ORFs code for two polypeptides of 2,302 and 2,257 amino acids (see below). The coding sequence is completely contained within the unsequenced cosmid T23C12, and distributed between sequenced cosmids K11C4 and T19F4. (C) An alternate splice. The two exons marked by asterisks in B are alternatively spliced in a small number of transcripts to introduce 45 novel amino acids before the PH domain. Only the region flanking the alternate splice (extending into the PH domain) was included in the alignment. (D) Genomic location of C. elegans spectrin genes. Sequencing of the genome has identified three candidate spectrin genes: an α subunit (spc-1), a β-G subunit (bgs-1/unc-70), and a β-H (heavy) subunit (sma-1). The chromosome number and genes mapped to the vicinity of each spectrin subunit are indicated. Bars: (A and B) 1 kb; (D) 0.5 kb.

loop regions that determine substrate specificity of the PH domain (Acias et al., 1994; Fig. 2 D).

To date, no other β-spectrin gene except for the β-H spectrin sma-1 has been identified. With 97% of the C. elegans genome already sequenced, it is unlikely that there would be another β spectrin. Hence, the nematode β spectrin is a C. elegans orthologue of the human β-spectrin family including SPTB (erythrocyte β spectrin), SPTB1 (β2 or β-G), and SPTB2 (β3; Fig. 2 C). C. elegans β spectrin is referred to in this paper as β-G or BGS-1 (β-G spectrin-1) to distinguish this protein from β-H, and to reflect the general expression in multiple tissues and throughout development (see below).
Moorthy et al. Development of Epithelial Polarity without β-G Spectrin

β-G Spectrin Associates with the Plasma Membrane at all Sites of Cell–Cell Contact throughout C. elegans Development

The expression pattern of C. elegans β-G spectrin during development was determined using an affinity-purified rabbit polyclonal antibody raised against the COOH-terminal 407 residues of the shorter spliceform (Fig. 1 C). This antibody recognized a single band at z225–235 kD (Fig. 3 A) in embryos (E), L2-L3 larvae (L), mixed populations (M), and Dauer larvae (D). The relative molecular mass of this band is lower than that predicted by the molecular mass of the BGS-1 protein (253,200 for the larger splice form), but this faster migration on SDS-PAGE gels is characteristic of other β-spectrins.

Indirect immunofluorescence staining of the embryos of a single gravid hermaphrodite showed that β-G spectrin levels were low but discernible in early embryos. β-G spectrin was localized to the plasma membrane at sites of cell–cell contact throughout development, starting at the first cell division (two-cell embryo; Fig. 3 C, i). β-G spectrin expression increased significantly after the gastrulation stage, a time when most cells are undergoing differentiation into various tissue types (Fig. 3, B and C). Embryos that are undergoing morphogenesis show significantly higher levels of β-G spectrin in many cells, including those of the primordial gut (Fig. 3 C, iii), the epidermis (Fig. 3 C, iv), the myoepithelia of the pharynx (ph), and the polarizing endothelia of the gut (gt; Fig. 3 C, v). Cytoplasmic staining was observed principally in early embryos; when present, it was diffuse and did not show any distinctive pattern.

In the adult (Fig. 3, D and E), β-G spectrin staining was detected in all cells including the epithelial seam cells (s), body-wall muscles (m), and the intestine (gut). Tissues that experience mechanical stress expressed higher levels of β-G spectrin; these included the pharynx (ph), the spermatheca (sp), and the vulva (not shown). The nervous system (the nerve ring [nr], nerve cords [vnc, ventral nerve cord], and the commissural axonal bundles [ca]) had the highest levels of β-G spectrin. The only cell lacking β-G spectrin is the mature amoeboid sperm cell in which spectrin was sequestered into the residual body along with actin (data not shown).

The RNAi Phenotype of the Single C. elegans α Spectrin Can Be Reproduced by Tandem Depletion of β-G and β-H Spectrin Subunits

One α spectrin and two β spectrin subunits comprise the currently known spectrin family in C. elegans (Fig. 1 D). Since vertebrate spectrins are known to function as α2β2 tetramers, a hypothesis was that C. elegans α spectrin assembles into separate α/β-G and α/β-H complexes, which...
together perform spectrin activities. This idea was tested by determining the phenotypes resulting from depleting the three spectrin genes, individually and in various combinations using the RNA-mediated interference (RNAi) technique (Guo and Kemphues, 1996; Fire et al., 1998). dsRNA was prepared using partial cDNAs corresponding to different regions of each of the three identified spectrin subunits and was microinjected at 1 μg/μl into the syncytial gonad of young adult hermaphrodites (Fire et al., 1998). The F1 progeny born in the 8–72-h period after injection was analyzed. In all cases, dsRNA which were prepared from different regions of each gene, gave identical phenotypes that were identical to those exhibited by genetic nulls of each gene (McKeown et al., 1998; Hammar...
In bgs-1(RNAi) embryos, β-G spectrin was reduced to undetectable levels at all stages of embryogenesis (Fig. 4 A, arrowheads indicate two-celled embryos). Immunoblots of bgs-1(RNAi) larvae indicated that RNAi was effective in reducing β-G spectrin by 98% (Fig. 4, C and D). Thus, as expected, bgs-1(RNAi) L1 larvae showed undetectable levels of β-G spectrin in all tissues with the exception of a low but reproducible level of staining in the nervous system (Fig. 5 G). β-G spectrin was present in normal levels in both sma-1(RNAi) and spc-1(RNAi) animals (Fig. 5, H and I) as predicted, which indicates that β-G spectrin depletion is specific to bgs-1 dsRNA treatment.

The bgs-1(RNAi) progeny arrested as lethal L1 larvae (also known as the Let phenotype) and exhibited uncoordinated (Unc) coiling and kinking movements that severely limited motility (Fig. 5 B). Sma-1(RNAi) offspring were viable but had small rounded heads (also known as the Sma phenotype; Fig. 5 C). Spc-1(RNAi) animals displayed an Sma, Unc, Let phenotype; i.e., they arrested as uncoordinated early larvae and, in addition, had morphogenetic defects similar to those of sma-1 L1 larvae (Fig. 5 D; McKewon et al., 1998). bgs-1(RNAi);sma-1(RNAi) double mutants arrest with an Sma, Unc, Let phenotype (Fig. 5 E) that was indistinguishable from that of spc-1(RNAi) animals. Hence, α-β-G and α-β-H complexes have separate and apparently complementary functions.
Finally, since the observed RNAi mutant phenotypes of the single α spectrin subunit can be reproduced by depletion of the two β subunits in tandem, it is likely that spc-1, bgs-1, and sma-1 together define the complete spectrin family in *C. elegans*.

**β-G Spectrin Is Not Required for Embryonic Morphogenesis**

As mentioned, morphogenetic defects were observed in sma-1, spc-1, and sma-1;bgs-1 (RNAi) animals. To examine the role of spectrins in morphogenesis, embryonic elongation in RNAi progeny of various spectrin genes was followed by light microscopy (see Materials and Methods). AII animals completed the initial proliferative stage of embryogenesis within the appropriate time interval (~350 min) and, in all cases, epidermal cells completed ventral closure normally (Fig. 6 B, bean stage). The body lengths of RNAi animals were measured (Fig. 6 A) at the start of morphogenesis (1.5-fold stage), through elongation (Fig. 6 B, middle row of ~2-fold embryos), and to the stage just before hatching (Fig. 6 B; Priess and Hirsh, 1986). bgs-1 (RNAi) embryos showed elongation kinetics close to that of the control embryos, whereas the other three RNAi animals (sma-1, spc-1, and bgs-1;sma-1) had slower rates of elongation, and usually failed to reach >50–80% of the length of control L1 larvae. These results indicate that β-G spectrin is not required for embryonic elongation.

**β-G Spectrin Is Not Required for the Establishment of Epithelial Polarity in *C. elegans* Embryos**

To determine if apical and basolateral domains of polarized epithelia were still physically segregated in the β-G spectrin RNAi mutants, adherens junctions were visualized using the MAb M H27. In control animals, β-G spectrin associates with only the lateral membranes of polarized epithelia, both in the epidermis (Fig. 7 A, arrowheads, left column; see seam cells in Fig. 3 D), and in the intestine (Fig. 7 A, arrows, left column, Di). Depletion of either β-G spectrin (Fig. 7 A, middle column) or both β-G and β-H spectrin subunits (Fig. 7 A, right column) does not affect localization of the MH27 antigen to the apicolateral domains of epidermal (arrowheads) or gut cells (arrows), which indicates that at least some adherens junction structures are assembled normally.

We determined the distribution of two proteins that normally distribute to the lateral domains of epidermal and intestinal epithelia: the *C. elegans* orthologue of vertebrate ankyrin, UNC-44 (Otsuka et al., 1995), and the ankyrin-binding protein, L1-related cell adhesion molecule LAD-1 (Chen, L., and V. Bennett, unpublished data). Each protein showed a normal polarized distribution in bgs-1(RNAI) animals to the lateral domains of epidermis (arrowheads) and gut cells (arrows), which indicates that at some level these molecules are retained in the lateral domains. Further, in the apical domain, indicating that the signals required to recruit and retain these molecules at the lateral domains were independent of β-G spectrin.

Polarity and structural integrity of epithelia was confirmed at the ultrastructural level using electron microscopy of bgs-1(RNAI)-arrested larvae (Fig. 8 B; see Materials and Methods). A transverse section through the middle region showed that the L1 larvae in bgs-1(RNAI) animals had normal cuticle and alae (al), which are collagen-containing structures produced by the secretory activity of
seam cells. Polarized secretory functions that were associated with epidermal cells, therefore, were unaffected in these mutants. Further evidence of normal Golgi function is that storage granules (y) were clearly visible in the cytoplasm of gut cells. The intestinal microvilli (mv) had distinct terminal webs as expected, since the BGS-1 protein does not localize to the apical domain. The adherens junctions (aj) between the two intestinal cells were normal in structure and located at the apicolateral domain. Lateral membranes (lm) were distinct and intact, as was the basement membrane encasing the gut (note the coelomocyte (Cc) situated in the pseudocoelomic cavity). These results indicate that β-G spectrin is not required for the establishment of embryonic epithelial polarity in C. elegans.

spc-1(RNAi) larvae have normal levels of β-G spectrin (Figs. 7 C and 51). Because of severe morphogenetic defects in spc-1(RNAi) larvae, the intestinal cells, which normally twist 180° along the length of the animal (Sulston et al., 1983), instead were squashed and not properly aligned. However, it was clear that β-G spectrin continued to distribute to only the lateral domains of epithelia (portion of the gut enlarged). β-G spectrin staining is also restricted to the lateral domains in the intestinal epithelia (arrows) of sma-1(ru18) mutant adults (Fig. 7 D, arrows in ii). These data suggest that β-G spectrin subunits may be expressed and targeted to the membrane independently, but both α and β subunits are needed to form a functional spectrin molecule. Together, these results indicate that the establishment of embryonic epithelial polarity in C. elegans does not require spectrin function.

**Depletion of β-G Spectrin by RNAi Leads to Progressive Defects in the Organization of the Myofilament Lattice of Body-wall Muscles**

β-G spectrin is associated with the plasma membranes of the spindle-shaped body-wall muscle cells (m) from the embryonic stage (not shown) into adulthood (Fig. 3 D). Immunofluorescence of the adult muscle labeled for β-G spectrin identified two patterns of staining at two very close but distinct focal planes near the sarcolemma. When a thin optical section was centered closer to the myofilament lattice, staining adjacent to the sarcolemma appeared mainly as longitudinal striations (Fig. 9 A, L).

![Figure 6. RNAi embryos depleted of β-G spectrin complete normal morphogenesis, unlike those α or β-H spectrin RNAi progeny. RNAi experiments were performed with various dsRNAs, and the microinjected mothers were killed on the second day. F1 progeny embryos were observed using differential interference optics and simultaneously videotaped (see Materials and Methods). Morphogenesis was considered to begin when the animal reached the 1.5-fold stage (~65 μm). The developmental stage of each animal was noted at regular intervals thereafter. (A) Elongation kinetics. Average length of each RNAi mutant strain was plotted against time starting at the 1.5-fold stage. bgs-1(RNAi) animals show kinetics of elongation close to that of control animals, unlike the remaining three strains that elongate more slowly and do not achieve normal length. Error bars indicate the range of lengths at the final time point. (B) Phase micrographs. Examples of three time points during embryonic development. Elongation is completed by 600 min, though animals were followed until they showed increased refractivity in the terminal bulb, which caused by secretion of the pharyngeal cuticle (bottom row, embryos at ~720 min).
When the optical section was centered closer to the epidermal cell border, \(\beta\)-G spectrin staining at the sarcolemma formed a grid pattern (Fig. 9 A, S). Double labeling experiments using antibodies against \(\beta\)-G spectrin and specific muscle components (Francis and Waterston, 1985; Hresko et al., 1994) revealed that \(\beta\)-G spectrin is associated with the sarcolemma adjacent to the I-bands (Fig. 9). When muscle sarcolemma was double labeled (Fig. 9 B) for \(\beta\)-G spectrin (BGS-1) and pat-3, which encodes \(\beta\)-integrin (MH25; Gettner et al., 1995), the two proteins colocalized (arrowheads) at the base of dense bodies, nematode homologues of Z discs. The two staining patterns did not completely overlap since BGS-1 staining extended more continuously along the I-bands. Conversely, at muscle–muscle cell boundaries, \(\beta\)-integrin staining was relatively more intense. When muscle cells were double labeled (Fig. 9 C) for BGS-1 and the myosin heavy chain A (mhcA), the broader longitudinal stripes of BGS-1 staining (arrowheads) lay between the stripes defined by mhcA (arrows), which is confined to the center of A-bands (Waterston, 1989).

It was not possible to determine whether the BGS-1 staining pattern that runs perpendicular to the long axis of the muscle cell (Fig. 9 A, S) represented \(\beta\)-G spectrin that was associated with the muscle sarcolemma or the epidermal membrane cytoskeleton. This was because the microscope’s limit of resolution in the z axis is \(\sim 1\) \(\mu\)m, whereas the hypodermal cell itself is only \(\sim 50\) \(\mathrm{nm}\) thick in the region overlaying the muscle cell, and the plasma membranes of the two cells are separated only by a basement...
membrane. The BGS-1 staining pattern was similar (Fig. 9 D) but distinct from the pattern obtained by staining with mA b M H5, which recognizes a component of hemidesmosomes. Finally, we detected low but distinct BGS-1 staining at the base of the M-lines, in a pattern that colocalized with β-integrin (Fig. 9, small vertical arrows in B and C).

These results suggest that β-G spectrin is associated with the sarcolemma in regions where it is attached to adjacent cells. At the level of individual sarcomeres, β-G spectrin localizes to the region adjacent to the sarcolemma, adjoining I-bands, dense bodies, and to the base of the M-line; the latter two are regions of attachment of the muscle cells to the overlying epidermal cell (Hresko et al., 1994). Spectrin further localizes in a pattern mimicking that of hemidesmosomes, components of the fibrous bodies that link together muscle sarcolemma, epidermis, and cuticle, and help transmit the force generated by muscle contraction to the cuticle during movement.

Consequences of depletion of β-G spectrin for organization of actin filaments and myosin thick filaments were evaluated in L1 larvae (Fig. 10). bgs-1(RNAi) L1-arrested larvae showed a perturbation in the distribution of actin microfilaments in muscle tissue, compared with wild type (Fig. 10 A). This disarray in arrangement of F-actin filaments was observed in both body-wall muscle and the myoepithelia of the pharynx. Freshly arrested larvae appeared to have fewer defects, whereas larvae that had survived on the plate for longer periods accumulated more disorganized foci. When body-wall muscles were stained for the thick filament component mhcA (Fig. 10 B), the myofilament lattice of animals depleted of β-G spectrin often displayed an unraveled appearance of A-bands. This progressive dystrophy of muscle tissue in bgs-1(RNAi) larvae suggests the hypothesis that β-G spectrin provides essential strength and elasticity to the sarcolemmal membrane during activity of C. elegans body-wall muscles.

**Depletion of β-G Spectrin in Early Larvae by RNAi Leads to Multiple Defects in the Nervous System**

A subset of chemosensory neurons in the head concentrate fluorescent dye when nematodes are suspended in a solution containing the cationic membrane tracer dye DiI, allowing visualization of their neurite processes in live animals (see Materials and Methods; Hedgecock et al., 1985). When these neurons were observed in bgs-1(RNAi) animals, they usually displayed defects in the morphology of the neurite processes (Fig. 11). These lesions included apparent vacuolation in the dendritic processes of some amphid neurons (Fig. 11 B, white arrowhead), enlarged neuronal cell bodies (Fig. 11 B, thick arrow in ii), and occasional ectopic displacement of cell bodies (Fig. 11 B, thin arrow in ii). These data suggest that β-G spectrin plays a structural role in stabilizing neurite membranes, but do not exclude additional roles in membrane recycling and synaptic transmission.

**Discussion**

This paper presents a global analysis of C. elegans spectrins, and in particular β-G spectrin, the single orthologue of vertebrate β spectrins. This analysis includes the developmental expression, cellular localization, as well as consequences of depletion of β-G spectrin alone and in combination with other members of the C. elegans spectrin family via RNAi. RNAi has proven to be an effective approach in depleting C. elegans of many gene products (Fire et al., 1998). Indeed, our data show that spectrin is successfully removed with RNAi. Immunoblots indicate that at least 98% of spectrin is removed in RNAi-treated animals, and immunolabeling of RNAi-treated animals reveals es-
sentially undetectable amounts of spectrin. Moreover, the RNAi of each spectrin gene results in phenotypes identical to those of the spectrin-null alleles (McKeown et al., 1998; Hammarlund et al., 2000; Norman, K., and D. Moerman, personal communication). The results of this study indicate that spectrin is not required for embryonic morphogenesis or the establishment of epithelial polarity, but it is important for proper body-wall muscle and nervous system development.

Several previous observations have established a role for spectrin in the maintenance of epithelial polarity, but could not directly address a possible role in its establishment. β Spectrin is recruited to the cytoplasmic surface of the basolateral plasma membranes of epithelial cells and to fibroblasts transfected with cadherins (McNeil et al., 1990). Spectrin also participates in complexes with cadherins and ankyrin (Nelson and Hammerton, 1989; Nelson et al., 1990). When overexpressed in cell lines, functional domains of human β-G spectrin disrupt membrane skeleton organization and cell polarity (Hu et al., 1995). In Drosophila, α spectrin is essential for larval survival and development, and spectrin plays essential roles in the maintenance of cell shape and cell–cell interactions (Lee et al., 1993, 1997). These studies support some role of spectrin in epithelial polarity, but do not address the consequences of the absence of spectrin from the onset of differentiation. In this study, the RNAi technique was successful in depleting β-G spectrin at a very early stage in embryogenesis (Fig. 4), before zygotic expression or tissue differentiation takes place. Surprisingly, depletion of the nematode β-G spectrin by RNAi, to essentially undetectable levels, does not affect enclosure of the embryo by migrating epithelia (Fig. 6 B, bean stage embryo), nor does it prevent embryonic elongation that depends on the epidermis (Fig. 6). Moreover, the spectrin-binding protein, ankyrin, and the ankyrin-
binding cell adhesion molecule, LAD-1, continue to target to lateral membranes and are excluded from the apical domain (Fig. 7). The epithelial cells retain integrity and function, and are ultrastructurally normal (Fig. 8). These data indicate that β-G spectrin is not required for the establishment of polarity in C. elegans epithelia; instead, they support a model in which cell adhesion molecules direct assembly of the spectrin-based membrane skeleton to the sites of cell–cell contact (Yeaman et al., 1999), and where this cytoskeleton plays a subsequent role in the maintenance of integrity of the epithelial monolayer.

While we cannot formally rule out the possibility that the remaining 2% of β-G spectrin, which is not depleted by RNAi, may play a role in establishing early epithelial polarity, it is extremely unlikely that such a small amount of a structural protein would be sufficient to carry out such a process. Moreover, the epithelial polarity in unc-70-null mutants is normal (Hammarlund et al., 2000). A further, albeit remote, possibility is that β-H spectrin may be performing redundant functions as β-G spectrin in the epithelia. However, β-H and β-G spectrins expression patterns do not overlap (apical [McKeeown et al., 1998] versus basolateral, respectively). Moreover, the loss of both β-H and β-G spectrins does not affect epithelial polarity as indicated by the presence of adherens junctions and proper localization of basolateral markers in the epithelium (Fig. 7 A).

The RNAi phenotype of the single C. elegans α-spectrin can be reproduced by tandem depletion of both β-G and β-H spectrin subunits. This data combined with a lack of other spectrin genes in the C. elegans genome suggests that α spectrin associates with β-G and β-H subunits to form α/β-G and α/β-H heteromers, which perform the entire repertoire of spectrin function in the nematode. The loss of either α spectrin or both β spectrins (β-G and β-H), thus, results in animals with no functional spectrin. Removal of functional spectrin by the depletion of either the spc-1 α spectrin or the sma-1 β-H spectrin also did not affect epithelial polarity. Hence, spectrin is not required for the establishment of epithelial polarity.

The concept of a widespread Golgi-centric spectrin-ankyrin skeleton is exciting (Beck and Nelson, 1998; D Matteis and Morrow, 1998; Holleran and Holzbaur, 1998), but the precise Golgi spectrin isoforms have yet to be de-
fined. The newly cloned β-spectrin III, SPTBN2, (Ohara et al., 1998), which was reported as a Golgi-associated spectrin (Stankwich et al., 1998) but has a restricted tissue expression, is most highly expressed in the brain and stains dendrites more strongly than cell bodies (Ohara et al., 1998). In this study, an affinity-purified antibody against the only C. elegans β-G spectrin did not recognize any specific intracellular organelle. No distinctive cytoplasmic staining was evident in early founder cells (Fig. 3 C, i and ii), in differentiated gut or hypodermal cells (Fig. 3 C, iii–viii, and Fig. 7) or in any other examined cell type. RNAi depletion of β-G spectrin to levels below the threshold of detection did not prevent C. elegans embryos from developing from a one-cell zygote to a morphologically normal L1 larva of ~550 cells. Further, several developmental processes that require Golgi-dependent secretory functions proceeded normally; these included the secretion of cuticle by the epidermal cells, the formation of collagen-derived alae by the seam cells, and the generation of the various basement membranes. These data do not support a role for β spectrin in Golgi function and intracellular transport in C. elegans. Several caveats should be considered in extrapolating the lack of a C. elegans spectrin to vertebrates. It is conceivable that C. elegans spectrin did not appear till later in evolution, or that C. elegans lost the requirement for this proposed function of the spectrin cytoskeleton. Since ~3% of the genome remains to be sequenced, it is also formally possible but highly unlikely that a third β-spectrin and a second α spectrin gene remain to be identified in C. elegans that may localize to Golgi membranes.

The expression patterns of both nematode β-G spectrin and vertebrate β spectrins exhibit striking parallels. In both cases, β spectrins are associated with sites of cell–cell contact in epithelial tissues; the highest levels of β spectrin occur in the nervous system; and β spectrin in striated muscle is associated with points of attachment of the myofilament apparatus to adjacent cells. Spectrin in a vertebrate-striated muscle is organized in a two-dimensional lattice at the sarcolemma, with longitudinal and transverse components. The transverse bands repeat in register with I-bands of the adjacent lattice (Craig and Pardo, 1983). Visualization of β-G spectrin localization in C. elegans muscle reveals a strikingly similar lattice pattern, but the origins of the two patterns are distinct, since there is no costamere equivalent in the nematode. In addition, the I-bands run in longitudinal stripes, with the Z-disc equivalents (dense bodies) no longer in register but interspersed as discrete structures at the center of the stripes (Frances and Waterston, 1985). Thus, in the C. elegans β-G spectrin–staining lattice, the longitudinal bands arise from subsarcomemal β-G spectrin adjacent to I-bands in a series of sarcomeres that are slightly offset from each other. The transverse elements in the C. elegans β-G spectrin lattice may actually define a different structure, one that seems to mimic the distribution of hemidesmosomes in the overlying epidermis. One important similarity between the two muscle types is the occurrence of β-spectrin at regions where the myofilament apparatus is attached to the sarcolemmal membrane. In C. elegans, these sites also help to anchor the muscle to the overlying hypodermis, and through it to the cuticle, allowing direct transmission of force from the contractile apparatus to the exoskeleton. The progressive disorganization of muscle seen in L1 larvae depleted of β-G spectrin may be due to an activity-based detachment of the myofilament apparatus from the sarcolemma, indicating a structural role for spectrin at these sites.

Spectrin and its associated proteins show robust expression in the C. elegans nervous system, along with the spectrin-binding protein ankyrin (UNC-44; Ötsuka et al., 1995), the ankyrin-binding protein LA D-1 (Chen, L., and V. Bennett, unpublished data), and the spectrin assembly factor adducin, A D-1 (M orthy, S., and V. Bennett, unpublished data). Spectrin has been suggested to have a role in axonal transport (Levine and Willard, 1981), neurite extension (Sobue and Kanda, 1989; Sihag et al., 1996), and organization of synaptic vesicles (Sikorski et al., 1991; Sakaguchi et al., 1998). The defects accumulated by C. elegans neurons depleted of β spectrin may reflect a disruption of these activities as well as a role in stabilizing plasma membranes of axonal and dendritic processes.

Spectrin has not been detected in yeast, and while spectrin-related proteins have been reported in unicellular organisms (Pollard, 1984; Bennett and Condeelis, 1988), a spectrin α–β heteromer has been reported only in metazoans (for review see Thomas et al., 1997). The remarkable similarity of spectrins from organisms as diverse as C. elegans, Drosophila melanogaster, and Homo sapiens suggests that many functions can be expected to be conserved among these species. One hypothesis is that heteromeric spectrin evolved in metazoan animal cells in response to the need for formation of mechanically integrated tissues that can withstand the rigors imposed by an active organism. Such a role would preclude participation of spectrin in fundamental cellular processes, but would account for the wide range of spectrin isoforms observed in vertebrates. Consistent with this hypothesis is the expression pattern of spectrin, which is first observed at the plasma membrane of a two-cell embryo. Spectrin levels remain low but distinct through gastrulation, when most cell proliferation has been completed (Sulston et al., 1983). However, spectrin levels dramatically increase after gastrulation, when most cells are undergoing differentiation. Spectrin levels are particularly high in the nervous system, and are high in tissues that experience mechanical stress such as the bodywall muscles, the pharynx, and the spermatheca. Such a dramatic increase in spectrin levels after gastrulation similarly is seen during Drosophila development (Pesacreta et al., 1989). The spectrin heteromer could perform functions ranging from that of a simple plasma membrane scaffold providing elasticity and strength to the plasma membrane, as in erythrocytes, to that of a component that integrates extracellular signal transduction with cytoplasmic responses. This paper has helped define the range of cell types in which spectrin may play an essential role in a relatively simple metazoan. With genetic mutations now available in all three spectrin genes C. elegans promises to become an important model system for the study of the spectrin-based membrane cytoskeleton.

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