The *C. elegans* septin genes, *unc-59* and *unc-61*, are required for normal postembryonic cytokineses and morphogenesis but have no essential function in embryogenesis

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Accepted 28 August; published on WWW 17 October 2000

SUMMARY

Septins have been shown to play important roles in cytokinesis in diverse organisms ranging from yeast to mammals. In this study, we show that both the *unc-59* and *unc-61* loci encode *Caenorhabditis elegans* septins. Genomic database searches indicate that *unc-59* and *unc-61* are probably the only septin genes in the *C. elegans* genome. UNC-59 and UNC-61 localize to the leading edge of cleavage furrows and eventually reside at the midbody. Analysis of *unc-59* and *unc-61* mutants revealed that each septin requires the presence of the other for localization to the cytokinetic furrow. Surprisingly, *unc-59* and *unc-61* mutants generally have normal embryonic development; however, defects were observed in post-embryonic development affecting the morphogenesis of the vulva, male tail, gonad, and sensory neurons. These defects can be at least partially attributed to failures in post-embryonic cytokineses although our data also suggest other possible roles for septins. *unc-59* and *unc-61* double mutants show similar defects to each of the single mutants.

Key words: Cytokinesis, Septin, Cell division, Male tail, Vulva

INTRODUCTION

Cytokinesis is the act of cleaving a mother cell into two daughters and, as such, is one of the most fundamental of cellular processes. The basic process of cytokinesis consists of separating the cell constituents into two regions and pinching off and resealing the plasma membranes. There are two general strategies for cytokinesis in different organisms: progressive constriction of a contractile ring (Rappaport, 1996), or formation of a septum in the equatorial plane (Staehelin and Hepler, 1996). The contractile ring of animal cells is composed of actin filaments associated with many other proteins. The interaction of actin filaments and bipolar non-muscle myosin II generates a force that drives furrow ingression ultimately separating the cell into two independent entities (Cao and Wang, 1990; Satterwhite and Pollard, 1992; Sanders and Field, 1994). In contrast, although *Saccharomyces cerevisiae* has a contractile actomyosin ring (Lippincott and Li, 1998; Bi et al., 1998), it is not essential for cytokinesis; cells lacking F-actin can still divide (Ayscough et al., 1997). In *S. cerevisiae*, a bud emerges from the mother cell and enlarges whilst the mother cell maintains its volume. Vesicles fuse at the neck region to form new plasma membrane and the enlarging bud eventually separates from the mother cell by the deposition of chitin into the mother-bud neck to form a new cell wall (reviewed by Sanders and Field, 1994; Longtine et al., 1996).

Although these two processes of cytokinesis appear to be quite different, they both require a family of proteins called septins. The septin genes, *CDC3*, *CDC10*, *CDC11* and *CDC12*, were first identified in *S. cerevisiae* for conferring defects in cytokinesis when mutated (Hartwell, 1971). The four gene products may form the highly ordered ~10 nm diameter neck filaments organized in a ring-like structure at the mother bud junction as previously described (Byers and Goetsch, 1976a) as these filaments are not seen in septin mutants (Byers and Groetsch, 1976b) and septins have been shown to form filaments in vitro (Field et al., 1996; Frazier et al., 1998). By immunofluorescence, septins first appear at the onset of G¹ and persist for some time after cytokinesis. Temperature sensitive mutants of *CDC3*, *CDC10*, *CDC11* and *CDC12* cannot execute cytokinesis at the restrictive temperature and fail to localize septins at the mother bud neck. There is also a concomitant mislocalization of chitin deposition and failure to assemble a contractile ring (Haarer and Pringle, 1987; Kim et al., 1991; Ford and Pringle, 1991; DeMarini et al., 1997; Bi et al., 1998).

Septins have been found in several other organisms since they were first identified in *S. cerevisiae* such as the yeast *Candida albicans* (DiDomenico et al., 1994) and the fungus
Aspergillus nidulans (Momany and Hamer, 1997). The first animal septin gene, peanut (pnut), was identified and cloned in Drosophila. pnut mutants die after pupation due to cytokinesis failures in several larval tissues resulting in multinucleate cells. Pnubt protein localizes to the leading edge of the cleavage furrow of dividing cells and resides in the midbody (Neufeld and Rubin, 1994). Since this discovery, septins have been identified in mammals (Nottenburg et al., 1990; Kato, 1990; Nakatsuru et al., 1994; Kinoshita et al., 1997; Hsu et al., 1998). Injection of antibody raised against a murine septin, Ned5, resulted in cleavage furrow regression in SiHa cells (Kinoshita et al., 1997), suggesting that septins play a key role in cytokinesis of animal cells.

The predicted septin proteins are at least 26% conserved in amino acid sequence over their entire length across species (reviewed by Longtine et al., 1996). At the structural level, all septins contain a putative P loop nucleoside triphosphate-binding motif at the N terminus of the protein. Most septins have a C terminus coiled-coil domain that has been generally thought to facilitate protein-protein interactions (Saraste et al., 1990; reviewed by Sanders and Field, 1994; Longtine et al., 1996). The P-loop has been speculated to play a role in regulating filament assembly. Field et al. (1996) showed that Drosophila Peanut, Sep-1 and Sep-2 have GTPase activity; these proteins form hetero-meric filaments of 7-9 nm diameter and ~26 nm length in vitro. Subsequently, Frazier et al. (1998) performed similar experiments in yeast showing that septins form filaments 7-9 nm in diameter with the majority of the filaments being 32 nm in length. However, it is not known whether filament assembly is necessary for septin function. Indeed, CDC10Δ mutants of S. cerevisiae do not form neck filaments or septin filaments in vitro extracts, yet can localize proteins in the mother/bud neck and undergo cytokinesis (Frazier et al., 1998). Further support for the notion that septins have GTPase activity comes from other studies (Kinoshita et al., 1997) on Ned5. In cells microinjected with GTPyS, septin filaments failed to assemble. Furthermore, they showed that Ned5-GFP fusion proteins lacking the GTP binding domain interfered with the filamentous distribution of Ned5.

In this paper, we present molecular, immunolocalization, and phenotypic analyses of unc-59 (e261 and e1005) and unc-61 (e228 and n3169). We show that the unc-59 and unc-61 loci encode two C. elegans septins. Both unc-59 and unc-61 mutants show normal cytokinesis during embryogenesis, yet exhibit failures in postembryonic cytokinesis. Double mutant analysis and immunolocalization data suggest that these two proteins require each other for proper localization and function. Furthermore, we have obtained evidence that suggests that C. elegans septins are required for the proper formation and structural integrity of the somatic gonad.

MATERIALS AND METHODS

Strains and alleles

All C. elegans strains were cultured using standard techniques, and genetic manipulations were carried out as described (Brenner, 1974). The following strains were used in this study: wild-type N2, unc-59 (e261 and e1005), unc-61 (e228 and n3169), HSI [unc-61(n3169) V, him-5(e1490) V], HSS [unc-61(n3169) V, him-5(e1490) V], MT9145 [unc-61(e228); him-8(e1489)] and zyg-9(b244). A double mutant strain was generated using unc-59(e261) and unc-61(e228). Phenotypic analyses of the male tail came from HS5 and MT 9145. A double mutant [unc-59(e1005); him-1(e879)] and [unc-59(e261)] was generated using unc-59(e1005), unc-59(e261) and him-1(e879). The unc-59(e261), unc-59(e1005) and unc-59(e1005) alleles were isolated by Brenner (Brenner, 1974); the unc-61(n3169) was initially identified in a screen for male tail and phasmid defective mutants (H. Sawa, unpublished data).

Phenotypic characterization of unc-59 (e261 and e1005) and unc-61 (e228 and n3169)

Single L1/L2 larvae were placed on an individually seeded plate. Two days later, these animals were scored for following phenotypes: egg-laying defects, gonad extrusion, and vulva protrusion. As a control, wild-type embryos were scored side-by-side. All analysis was done at 20°C. Mutant animals were observed using Nikon Nomarski x60 or x100 optics. Images were captured using a Hamamatsu C2400 video camera and processed using Adobe Photoshhop 4.0.

Identification of the EST and 5' end of unc-59

unc-59 was first identified as an Expressed Sequence Tag (EST). Using the Drosophila peanut amino acid sequence, we performed Blast algorithm searches using the NCBI GeneBank to identify a C. elegans EST cm3a7 (Altschul et al., 1990). The 5’ end of the C. elegans unc-59 gene was determined by using reverse transcription-polymerase chain reaction (RT-PCR). We used the AMV reverse transcriptase kit (Boehringer Mannheim, Indianapolis, IN) with the following modifications: we used 5 μg of poly(A)* mRNA (see below for RNA isolation) and 1 μM of the specific primer to our EST. The reaction mixture containing poly(A)* mRNA was heated for 5 minutes at 65°C, then allowed to cool to 37°C. Ten units of AMV reverse transcriptase was then added, and the reaction was incubated at 37°C for two hours. To purify cDNA, we used Boehringer Mannheim’s High Pure PCR Purification Kit. The purified cDNA was subsequently subjected to PCR using the C. elegans trans-spliced leader sequence SL-1 and another primer specific from within the EST that is different from the first. The PCR reactions were done using Tff1 DNA Polymerase, XL (Perkin Elmer, Foster City, CA). The conditions for reaction were 4.5 μM of MgCl2, 40 pmole of each primer, 1 mM of dNTPs. The reaction was then denatured for 5 minutes at 95°C followed by 30 cycles of 95°C for 1 minute, 45°C for 30 seconds, and 72°C for 30 seconds. This regime was then followed by a period of 72°C for 10 minutes.

Cloning and molecular characterization of unc-59

A C. elegans cDNA library (Barstead and Waterson, 1989) was screened by plaque hybridization. All probes were made with Prime-a-Gene Kit (Promega, Madison, WI) and Redivue deoxycytidine 5’ a P-32 triphosphate (Amersham, Arlington, IL). Approximately 1.8x106 pfu were screened with the following hybridization conditions: 5x SSC, 10x Denhardt’s, 1% SDS, and 100 μg/ml of Herring Sperm DNA (Boehringer Mannheim, Indianapolis, IN) for primary, secondary and tertiary screens. All hybridizations were done at 65°C for at least 12 hours. This was followed by high stringency washes: 0.2x SSC and 0.5% SDS, 4 times for 30 minutes at 65°C. All plaques were inoculated in SM buffer (Sambrook et al., 1989) with a drop of chloroform added. Plaques were purified and excised from lambda ZAP as follows: RB4E cells were grown in 2YT along with 10 mM MgSO4 and 0.2% maltose until the culture reached OD=1.0. Cell cultures were spun down and resuspended in 10 mM MgSO4. 100 μl of the concentrated RB4E cells were combined with 1 μl of purified phage in SM and chloroform. This reaction was then incubated at 37°C for 30 minutes and plated out on ampicillin plates for selection. The identity of the excised plasmid was further confirmed with a Southern gel digested with BglII (Promega, Madison, WI). Sambrook et al., 1989). We isolated two overlapping clones called pBB-TN5 and pBB-TN8.
For mutant analysis, genomic DNA was isolated from the wild-type and mutant alleles as previously described (Epstein and Shakes, 1995). Primers flanking the coding region were generated. PCR was done according to the protocol in Boehringer Mannheim’s Expand PCR Kit (Boehringer Mannheim, Indianapolis, IN). Two independent clones from separate PCRs were obtained and subsequently subcloned into pGEM-T Easy Vector separately (Promega, Madison, WI). All sequencing was performed by the University of Wisconsin-Madison Biotechnology Center’s automated sequencing facility using ABI Dye Terminator Sequencing (Perkin Elmer, Foster City, CA).

Unincorporated dyes and nucleotides were removed using AutoSeq G-50 spin column (Pharmacia, Piscataway, NJ). Sequence analysis was done using the DNA Star (DNASTAR, Madison, WI) and DNA Strider programs.

Rescuing of unc-59 mutant alleles was done using cosmid W09C5 (10 ng/μl) that contains the full-length unc-59 sequence. Transformation was done as previously described (Epstein and Shakes, 1995).

Cloning and molecular characterization of unc-61

A cosmid (C18G4) that covers the unc-61 region of the genetic map contains the full-length genomic sequence. Furthermore, cosmid C18G4 and its subclones were used to rescue unc-61 mutants using the methods described (Epstein and Shakes, 1995). In addition, unc-61 cDNA was identified as an Expressed Sequence Tag (EST) with homology to unc-59 using the Blast algorithm. We identified three overlapping cDNA clones: yk109fl, yk411f2, and yk92R that give rise to a full-length cDNA (courtesy of Y. Kohara). We used the C. elegans Genomic Sequencing Server for our search.

For sequence analysis, genomic DNA was isolated from the wild-type and unc-61(e228) and unc-61(n3169) strains as previously described (Epstein and Shakes, 1995). Primers flanking the coding region were generated. The following forward and reverse primers were used to amplify genomic DNA for sequencing: the forward primer was 5’TATTCTACCCAAAGATTCAAT3’ and the reverse primer was 5’CGAATATAATGGGATAACGCA3’. PCR analysis was done according to Boehringer Mannheim’s Expand and High Fidelity Kits (Boehringer Mannheim, Indianapolis, IN). Subcloning and sequencing analysis were done as described above.

Preparation of C. elegans UNC-59 and UNC-61 antigen and antibody

Polyclonal peptide antibodies against C. elegans UNC-59 and UNC-61 were generated. Peptides of 15-18 amino acids at the N-termini of the proteins were selected due to their predicted antigenicity and lack of conservation relative to other known septins. Both peptides were modified with acetylation at the N terminus and a cysteine was added to the C terminus to facilitate coupling (UNC-59: acetyl-SRNDESLRTGKHKPNFYC-amide and UNC-61: (acetyl-SDIHEN-KLQHILPPHQPC-amide). Polyclonal antisera were generated in rabbits for UNC-59 and in a goat for UNC-61. Both were affinity purified by Quality Controlled Biotechnology (Boston, MA).

Western analysis was done using affinity purified UNC-59 and UNC-61 antibody. Wild-type N2 and mutants animals were grown on 60 mm peptone plates. Mixed stages of animals were washed with M9 buffer as possible, worms were resuspended in 500 μl of lysis buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA) containing protease inhibitor: 1 mM PMSF, and 10 μg/ml of pepstatin, aprotinin, and leupeptin. Worms were then homogenized with a pestle fitting the Eppendorf tube through a series of freezings and thawings. 250 μl of 2x sample buffer (Owl Scientific Inc., Woburn, MA) was added to the homogenized mixture. The sample was boiled for 10 minutes and cooled for 5 minutes on ice. The sample was then resolved by SDS-PAGE and immunoblotted onto nitrocellulose transfer membrane (MSI, Westborough, MA) as described (Sambrook et al., 1989). For UNC-59 immunoblots, the following conditions were used: The immunoblotted nitrocellulose membrane was blocked overnight at room temperature in 100 ml of 1x Tris-buffered saline (TBS), pH 7.5, 10% of non-fat dry milk, and an anti rabbit serum (1:1,000). Purified primary UNC-59 antibody was diluted 1:500 in TBS (0.5% Tween-20) at pH 7.5. The blots were incubated overnight at room temperature on a shaker, then washed three times for 10 minutes in TBS, pH 7.5. Blots were incubated with the secondary antibody (Amersham’s horseradish peroxidase-conjugated, goat anti-rabbit IgG diluted to 1:3000) in TBS, pH 7.5, for 2.5 hours at room temperature on a shaker. This was followed by 3 rinses in TBS, pH 7.5, for 10 minutes each. Detection was done according to ECL Kit protocols (Amersham, Arlington, IL). For UNC-61, the membrane was blocked in 5% non-fat dry milk and 0.3% Tween-20 in TBS, pH 7.5, overnight on a shaker at room temperature. The primary antibody was diluted 1:500 in 5% non-fat milk, 0.3% Tween-20 and 5% rabbit serum in TBS at pH 7.5 for 6 hours. This was followed by three washes of 15 minutes each in buffer identical to the incubation buffer without the rabbit serum. Blots were incubated in secondary antibody (Amersham HRP-conjugated anti-goat IgG) diluted 1:25,000 in the same buffer as the wash buffer overnight in the cold room. This was followed by two 15 minute washes with the same buffer as above, but at a pH of 9.5. A final wash was performed for 15 minutes using the pH 7.5 buffer. Detection was accomplished by using the ECL Kit protocols (Pharmacia-Amersham, Arlington, IL). Both the secondary antibody and rabbit serum were purchased through Jackson Immunoresearch Laboratory (West Grove, PA).

Indirect immunofluorescence

Embryos were stained using a modification of the protocol as described previously (Albertson, 1984; Skop and White, 1998). For the actin/septin double staining, embryos were fixed in 75% methanol, 4% EM grade formaldehyde, 0.5x PBS, and 0.1 mM EDTA for 12 minutes. All primary antibodies were diluted using PBSBT (PBS, 0.5% BSA and 0.25% Tween) and allowed to react overnight at 4°C in a damp chamber. The embryos were then washed 3 times for 10 minutes with PBSBT before adding 1:200 goat anti-rabbit FITC or/and goat anti-mouse rhodamine (Jackson Immunoresearch) for 2.5-3 hours at room temperature. The embryos were then washed 2 times for 10 minutes each with PBSBT followed by 1:1,000 dilution of 1 μg/ml of DAPI in PBST as a final wash. After 10 minutes, the embryos were mounted with 8 μl of mounting media containing Prolong Antifade (Molecular Probes, Eugene, OR). Antibodies and concentrations were as follows: UNC-59: 1:500, UNC-61: 1:200, actin (monoclonal mAbC4, ICN) 1:200 and NMY-2 (a gift from K. Kemphues) 1:200.

Fluorescence microscopy and image processing

All confocal microscopy was done using a Bio-Rad 1024 confocal microscope. Serial optical sections were collected using ×63 optics and an additional 2.5x software magnification, 0.5 μm increments and about 20-30 confocal sections. Data were converted into TIFF files or rendered as 3-D projections using NIH image. Image processing was done using Adobe Photoshop 4.0.

RNA-mediated interference

For unc-59, an RNA mediated interference (RNAi) construct was made by digesting pBB-TN8 with XbaI and EcoRI (Promega, Madison, WI) that was missing 56 bp of the 5’ end of which 51 bp were from the 3’ UTR. The 1.2 kb XbaI/EcoRI fragment was subsequently subcloned into BlueScript (Stratagene) to generate pBS-TN (XbaI-R) unc-59 (Stratagene, La Jolla, CA). The pBS-TN (XbaI-R) unc-59 plasmid was linearized with XbaI or EcoRI. We followed the protocol as described in Stratagene’s mCAP mRNA Capping Kit to make our sense and antisense RNA with the following exceptions: we doubled the quantities of everything that was prescribed except for the mCAP analog. We used 3.0 μl of 5 mM CAP analog instead of 2.5 μl. The final reaction volume was 50 μl. In addition, we allowed the
in vitro transcription to proceed for 1 to 1.5 hours instead of 30 minutes. For unc-61, both sense and antisense RNA were made using Ambion’s T7 and T3 MEGAscript kits (Ambion, Austin, TX). EST yk109f1 and yk411f2 were used to make unc-61 RNA. Denaturing polyacrylamide gel electrophoresis was used to determine whether the appropriate transcript was made.

We injected 2.4 μg/μl of the antisense RNA or 1.0 μg/μl double stranded RNA (dsRNA) into the ovaries of young N2 hermaphrodites using the same procedures used for DNA transformation (Epstein and Shakes, 1995; Fire et al., 1991). In the double mutant analysis, we co-injected the animals with both unc-59 and unc-61 sense and antisense RNA. In all cases, we used 2.0 μg/μl of each. The injected animals were unmounted in M9 recovery buffer and then transferred to seeded plates (Epstein and Shakes, 1995). After about 24 hours, the injected animals were transferred to new seeded plates. Progeny from the injected animal were mounted on a slide and multifocal plane recordings were made using Nomarski optics to score for defects (Thomas et al., 1996).

RESULTS
Identification and cloning unc-59 and unc-61
Using the Drosophila Peanut amino acid sequence, we performed a sequence match search using the TBLASTN algorithm in NCBI GenBank, which identified a C. elegans expressed sequence tag (EST), cm3a7, of 322 bp that maps to cosmid WO9C5. When translated, this EST shows considerable homology to septin family members at the N terminus of the protein. To determine the 5′ end of the septin message we used reverse transcription-PCR (RT-PCR). We used a specific primer within the EST to reverse transcribe. The purified cDNA was subsequently subjected to PCR using the C. elegans trans-spliced leader sequence SL-1 and another primer within the EST. With the 5′ end fully identified, we screened a C. elegans cDNA library using the EST as our probe (Barstead and Waterston, 1989). We isolated two clones, pBB-TN 5 and pBB-TN 8 that ultimately gave us the full-length cDNA sequence.

Concurrently with our studies, the C. elegans sequencing consortium released the genomic sequence for cosmid WO9C5 that contains the full-length septin gene corresponding to the cm3a7 EST. This cosmid contains only one septin candidate gene. Physically, cosmid W09C5 maps to the region on Chromosome I corresponding to the genetic map of unc-59, which maps to 22.49 centimorgans on the right arm of Chromosome I. When we injected this cosmid into unc-59(e1005), we obtained two transgenic animals. One animal was able to rescue the mutant phenotypes for several generations. Eventually, after the F4, the extra-chromosomal array(s) was lost. The second transgenic animal was able to rescue the F1 generation, but the extra-chromosomal array was lost in subsequent generations. The instability of extra-chromosomal arrays in transgenic animals in C. elegans is a common phenomenon (Swan et al., 1998).

unc-61 was mapped to 6.49 map units to the right arm of chromosome V. Based on the correlation of the genetic and physical maps, a cosmid (C18G4) was found that rescued unc-61 mutants by microinjection. Subsequently, subclones of C18G4 also rescued unc-61 mutants further defining the gene structure. In addition, three over-lapping ESTs: yk109f1, yk411f2 and yk92f8 were identified. Together these three ESTs give rise to a full-length unc-61 cDNA.

Northern blots detected a 1.75 kb band and 2.1 band for unc-59 and unc-61 respectively (data not shown). The full-length cDNA of unc-59 and unc-61 has an open reading frame of 1377 bp and 1383 bp translated into a 459 and 461 amino acid protein, respectively, with a predicted molecular mass of 53 kDa for both.
C. elegans septins have a greater than 34% homology to most of the known septins (Fig. 1). Both predicted proteins contain P-loop regions (predicted to bind to GTP) at their N-termini and a coiled-coil domain at their C-termini (data not shown). Western blot analysis using antibodies raised to the predicted peptide sequences show the apparent molecular masses of the septins to be 58 kDa for UNC-59 and about 53 kDa for UNC-61 (Fig. 2).

To further show that the unc-59 and unc-61 genes encode two C. elegans septins, we amplified and sequenced the two alleles of unc-59 (e261 and e1005) and unc-61 (e228 and n3169). Sequence analysis of the two mutant alleles of unc-59 showed that e261 and e1005 have a base change (G→A) altering a very well conserved Gly at position 29 to Arg. e261 has an additional base change in the second intron at 2040 base pairs from the ATG starting site (T→C). This consistent with our immunoblot results, since the same molecular mass bands are present in both the wild-type and the mutant (Fig. 2A). For unc-61, sequence analyses revealed that both e228 and n3169 have a premature stop codon in their coding message. e228 has a base transition from an Arg (CGA) to a Stop codon (TGA) at position 156 resulting in a truncated protein. Similarly, n3169 has a base transition from a Trp (TGG) to a Stop codon (TGA) at position 316. The absence of mutant UNC-61 bands in both the northern (data not shown) and western data (Fig. 2B) may be a result of nonsense mediated decay (Pulak and Anderson, 1993; reviewed by Hentze and Kulozik, 1999). This mechanism eliminates messages containing inappropriate nonsense codons, which may explain why we were unable to detect either message or protein in the two mutant alleles. The western data also demonstrate that the UNC-61 recognizes a protein of the predicted size. The other bands on the western are cross-reacting bands that are present in both the wild-type and the mutant alleles. These results collectively imply that unc-59 and unc-61 encode two C. elegans septins.

unc-59 and unc-61 are required for post-embryonic cytokinases

C. elegans development can be divided into two major stages: embryogenesis and post-embryonic or larval development. Embryogenesis consists of cell proliferation, organogenesis and morphogenesis giving rise to the first stage larva (L1). During larval development, 10% of the somatic cells in the L1 undergo post-embryonic cell divisions giving rise to additional hypoderms, nervous system, musculature and somatic gonadal structures (Sulston and Horvitz, 1977). Previously, unc-59 has been described (White et al., 1982) as having post-embryonic failure in cytokinesis in the ventral nerve cord precursor cells which normally divide to produce five classes of motor neurons: VA, VB, VC, AS and VD. In unc-59 mutants, these cells variably fail to divide; as a result, not all classes of motor neurons are produced. As a consequence, these animals are uncoordinated. We went on further investigate the mutant phenotypes. Besides being uncoordinated, we found that unc-59 mutants have other defects such as egg-laying defects, gonad extrusion, vulva protrusion and male tail defects (Table 1, Fig. 3B-D and Fig. 4F). In these mutants, cell divisions during embryogenesis occur normally. However, during post-

![Fig. 2. Western analysis of wild type, unc-59 and unc-61 mutants.](image)

**Table 1. Phenotypic characterization of unc-59 alleles**

<table>
<thead>
<tr>
<th>Allele</th>
<th>egglaying defect</th>
<th>gonad extrusion</th>
<th>vulva protrusion</th>
<th>retarded growth</th>
<th>L2/L3 lethality</th>
<th>sterility</th>
<th>wild-type morphology</th>
<th>male tail defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>e261</td>
<td>56.2% (n=73)</td>
<td>31.5% (n=73)</td>
<td>27.4% (n=73)</td>
<td>4.1% (n=73)</td>
<td>4.1% (n=73)</td>
<td>0% (n=73)</td>
<td>4.1% (n=73)</td>
<td>100% (n=44)</td>
</tr>
<tr>
<td>e1005</td>
<td>66.6% (n=78)</td>
<td>14.1% (n=73)</td>
<td>38.5% (n=78)</td>
<td>3.8% (n=78)</td>
<td>2.8% (n=78)</td>
<td>0% (n=78)</td>
<td>0% (n=78)</td>
<td>100% (n=50)</td>
</tr>
<tr>
<td>N2 (control)</td>
<td>0% (n=93)</td>
<td>0% (n=93)</td>
<td>0% (n=93)</td>
<td>0% (n=93)</td>
<td>0% (n=93)</td>
<td>0% (n=93)</td>
<td>0% (n=93)</td>
<td>0% (n=50)</td>
</tr>
</tbody>
</table>

Retarded growth implies that the animals remained at the larva stage for 3-5 days (see Materials and Methods). Each animal was scored independently for the characteristics listed above. An animal can fit into two categories such vulva protrusion and egg laying defect. Wild-type Morphology implies that the animals appear virtually normal under the dissecting microscope, with no obvious defects and are able to lay eggs.
embryonic development, cell divisions fail variably, giving rise to some or all of the defects in the nervous system, vulva and male tail. Similar phenotypes were also observed in unc-61 mutants. (Table 2, Fig. 3E-G and Fig. 4E). Using Nomarski microscopy, we studied the division of the Q/V5 neuroblast precursor in the unc-61(n3169) and unc-59(e1005) mutants at the L1 stage. In wild-type animals, the Q/V5 precursor divides and the Q cell migrates away from its sister, V5 (Fig. 4A). This behavior allowed us to unambiguously score cytokinesis failures in Q/V5 (cell boundaries are difficult to see in the larva). We found that in 30% of unc-61(n3169) mutants, the Q/V5 neuroblast precursor fails to undergo cytokinesis resulting in a binucleate cell (Fig. 4B); similar observations were made with unc-59(e1005) (Fig. 4C).

To further investigate the function of the unc-59 and unc-61 gene, we used RNA-mediated interference (RNAi). RNAi suppression of unc-61 expression gives similar phenotypes to unc-59 and unc-61 mutants. RNAi of unc-59 and the double RNAi suppression of unc-59 and unc-61 expression produced similar results (data not shown). Bars, 20 μm and refer to all panels in a row.

Table 2. Phenotypic characterization of unc-61(e228 and n3169) alleles, unc-59(e261) and the double mutant, unc-59(e261); unc-61(e228)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N2 control</th>
<th>n3169</th>
<th>e228</th>
<th>e261</th>
<th>Double</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg-laying defect:</td>
<td>0% (n=93)</td>
<td>44.9% (n=89)</td>
<td>67.1% (n=79)</td>
<td>56.2% (n=73)</td>
<td>45.5% (n=99)</td>
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<tr>
<td>Gonad extrusion:</td>
<td>0% (n=93)</td>
<td>44.9% (n=89)</td>
<td>24.0% (n=79)</td>
<td>31.5% (n=73)</td>
<td>42.4% (n=99)</td>
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<tr>
<td>Vulva protrusion:</td>
<td>0% (n=93)</td>
<td>10.1% (n=89)</td>
<td>13.9% (n=79)</td>
<td>27.4% (n=73)</td>
<td>17.2% (n=99)</td>
</tr>
<tr>
<td>Retarded growth:</td>
<td>0% (n=93)</td>
<td>1.1% (n=89)</td>
<td>0.0% (n=79)</td>
<td>4.1% (n=73)</td>
<td>6.0% (n=99)</td>
</tr>
<tr>
<td>L2/L3 lethality:</td>
<td>0% (n=93)</td>
<td>3.4% (n=89)</td>
<td>0% (n=79)</td>
<td>4.1% (n=73)</td>
<td>1.0% (n=99)</td>
</tr>
<tr>
<td>Sterility:</td>
<td>0% (n=93)</td>
<td>3.4% (n=89)</td>
<td>3.7% (n=79)</td>
<td>0% (n=73)</td>
<td>3.0% (n=99)</td>
</tr>
<tr>
<td>Wild-type morphology: 1</td>
<td>0% (n=93)</td>
<td>0% (n=89)</td>
<td>5.1% (n=79)</td>
<td>4.1% (n=73)</td>
<td>3.0% (n=99)</td>
</tr>
<tr>
<td>Male tail defects</td>
<td>0% (n=50)</td>
<td>100% (n=52)</td>
<td>100% (n=61)</td>
<td>100% (n=64)</td>
<td></td>
</tr>
</tbody>
</table>

Retarded growth implies that the animals remained at the larva stage for 3-5 days (see Materials and Methods). Each animal was scored independently for the characteristics listed above. An animal can fit into two categories such as vulva protrusion and egg laying defect. Wild-type morphology implies that the animals appeared to be normal under the dissecting microscope with no obvious defects and were able to lay eggs.
method that effectively silences the expression of the endogenous gene product (Fire et al., 1991; Tabara et al., 1998). Antisense and sense unc-59 or/and unc-61 RNA were injected into the adult gonads. The progenies of the injected animals phenocopied the unc-59 and unc-61 mutant phenotypes (Fig. 3H-J). These data further support our identification of the unc-59 and unc-61 coding sequence.

**unc-59 and unc-61 are required for development of somatic gonad and male tail morphogenesis**

To further understand why some mutant animals exhibit sterility, we used a combination of Normarski microscopy and confocal imaging to score various mutant phenotypes (Tables 1 and 2); 2.5% of unc-59(e1005) and 3.4% of the unc-61(n3169) animals are sterile. In the wild type, C. elegans germ cells in the ovaries are syncytial. The individual nuclei reside at the periphery of the syncytial germ cells and are partially enclosed in membrane, but are all joined to a central cytoplasmic core called the rachis (Fig. 3K; White, 1988). This structure is similar to the Drosophila embryo during cellurization of the syncytial blastoderm (Cooper and Keihart, 1996). The germ cells are connected throughout oogenesis. As the oocytes migrate toward the spermatheca, approximately 1300 germ nuclei at the distal ovary export maternal contributed products into the rachis which are transported to the oocytes (White, 1988). We found a small percentage of unc-59(e1005) and unc-61(n3169) germ cells became grossly disorganized (Fig. 4I and H). In these mutants, the rachis did not form properly.

To further characterize these defects, we examined the ovaries of wild-type and mutant animals using indirect immunofluorescence. We found that UNC-61 antibody stained the membranes surrounding the nuclei in the ovaries and testis (Fig. 5A) as did our UNC-59 antibody. However, UNC-59

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**Fig. 4.** (A–C) Post-embryonic failures in cytokinesis in the Q/V5 neuroblast lineage. In the wild type (A), the Q/V5 neuroblast precursor divides; Q migrates to the anterior while V5 migrates to the posterior (Sulston and Horvitz, 1977). However, in unc-61(n3169) mutants, Q/V5 precursor often fails to undergo cytokinesis resulting in one cell with two nuclei (B). (C) An example of a binucleate Q/V5 cell in a later stage larva from an unc-59(e1005) animal. We observed a 100% penetrance of male tail defects in the unc-59 and unc-61 mutants (see Tables 1 and 2). (D) Image of a wild-type male tail. The white arrowheads indicate sensory rays. In homozygous unc-61(n3169) (E) and unc-59(e216) (F) mutants, the male tail fails to develop properly resulting in knob like structures. Both unc-59(e1005) and unc-61(n3169) exhibit defects in the ovary. In the wild type, the germ nuclei are aligned against the periphery of the syncytial germ cell (G) indicated by the white arrows. The center core region (rachis) is devoid of nuclei (white asterisk). However, in the unc-59(e1005) (H) and unc-61(n3169) (I) mutants, the germ nuclei are present in the rachis region as indicated by the black arrows. In addition, the overall structure of the ovary and uterus is deformed. Bars: 10 µm (F); 5 µm (B–C); 25 µm (I), and refer to all panels without bars to the left.
staining of post-embryonic structures are extremely weak compared to UNC-61 (data not shown). There was no detectable staining in the mutants (data not shown). These data suggest that septin expression in the membrane surrounding the rachis contributes to the structural integrity of the syncytial germ cell. If this integrity is perturbed by a lack of septins, then there is small but significant probability that oocytes will not develop and the animals will be sterile.

**Fig. 5.** UNC-61 localizes to the border of the rachis of the syncytial germ cell in the ovary. Wild-type young adult animals were double labeled with UNC-61 antibody (green) and Tro-Pro-3 staining the germ cell nuclei (red). (A) shows a merged image of a whole-mount animal showing the ovary (bottom left). Similar patterns of staining were seen in the syncytial germ cell of the testis (not shown). (B and C) Ventral and lateral views of the transient UNC-61 staining that was observed at the onset of male tail morphogenesis in the early L4. Bar, 10 μm.

**Fig. 6.** The distribution of UNC-59 and UNC-61 in the cleavage furrow and the midbody region. (A-F) illustrate that UNC-59 co-localizes with the actomyosin contractile ring. (A-C) A single embryo with the cleavage furrow about three-quarters ingressed; (D-F) a single embryo with the cleavage furrow fully ingressed. (A and D) Actin; (B and E) UNC-59; (C and F) merged images. (G-L) Embryos were double-labeled with non-muscle myosin II (NMY II)(red) and UNC-61 antibodies (green). The rows show the same embryo taken at the same focal plane. Similar to NMY-II (G), UNC-61 localizes to the leading edge of the cleavage furrow (H). The image (I) is a merged image of the NMY-II and UNC-61. At the terminal phase of cell division, both NMY-II (J) and UNC-61 (K) co-localizes to the midbody region. The image (L) is a merged image of J and K. Bar, 10 μm.
In addition to the germ cell defects, we found that 100% of the unc-59 (e261 and e1005) and unc-61 (e228 and n3169) homozygous males had male tail abnormalities as shown in Fig. 4F and E. When we stained wild-type animals at different stages of development, we consistently observed high expression of UNC-61 during the early L4 stage of development (Fig. 5B and C). This UNC-61 staining was not associated with cleavage furrows. The high level of staining in the tail suggests that UNC-61 may also play a role in male tail morphogenesis that is independent of the role of septins in cytokinases.

**Localization of actin, non-muscle myosin II, UNC-59 and UNC-61 in the cleavage furrow and the midbody**

To determine the localization of UNC-61 relative to other cytoskeletal elements, we labeled UNC-59 and UNC-61 in combination with several proteins that have been shown to localize to the cleavage furrow. In most animal cells, actin filaments and non-muscle myosin II accumulate around the equator during cytokinesis (reviewed by Rapaport, 1986). In *C. elegans*, we observed a similar phenomenon. Actin filaments began to aggregate at the cleavage furrow as cells underwent cytokinesis (Fig. 6A). Eventually, actin filaments concentrated at the boundary of two newly divided cells (Fig. 6D). In double-labeled embryos, *C. elegans* UNC-59 co-localized with actin (Fig. 6C). However, as cytokinesis progressed, and when the cleavage furrow constricted the midbody, UNC-59 became separated from actin. In Fig. 6D, the actin ring had not reached the center of the midbody. However, Fig. 6E shows UNC-59 was already appearing at the center of the midbody at this stage. Similarly, we found that UNC-61 and NMY-II eventually also became localized to the midbody.
Fig. 8. UNC-61 localization in the zyg-9(b244) embryo. (A) Nomarski image of an zyg-9(b244) embryo. There are two orthogonal cleavage furrows in this embryo. The position of the mitotic spindle is represented in a schematic drawing (D). An embryo that resembled the Nomarski image in A was selected and stained to determine the localization of UNC-61. (B and C) The same embryo at different confocal planes. (B) The middle section of the embryo, while C shows the cortex of the same embryo. The furrow to the right is a true furrow (i.e. bisects the mitotic apparatus and usually completes) while the other is a pseudofurrow with no associated mitotic apparatus. Pseudocleavage furrows always regress. Bar, 10 μm.

...region (Fig. 6I-L). UNC-61 also co-localizes with actin in the cleavage furrow (data not shown). In addition, we found that UNC-61 co-localizes with UNC-59 suggesting that these proteins might interact with each other (data not shown).

UNC-59 and UNC-61 depend on each other for proper localization

Budding yeast septins generally depend on each other for proper localization in the mother/bud neck (Haurer and Pringle, 1987; Kim et al., 1991; Ford and Pringle, 1991). Our studies show that this is also the case in developing C. elegans where each septin requires the presence of the other to localize to the cytokinetic furrow, suggesting that septins associate in vivo. UNC-59 and UNC-61 affinity purified antibodies were used in double-labeling experiments with unc-61(n3169) and unc-59(e1005) embryos. An antibody for actin was used as an internal control to indicate that the staining protocols worked well. No UNC-61 product was detected in cleavage furrows of unc-61(n3169) or unc-59(e1005) mutant embryos (Fig. 7A-I). Similarly, no UNC-59 product was detected in cleavage furrows or midbodies of unc-59(e1005) or unc-61(n3169) mutant embryos (Fig. 7J-O). However, the UNC-59 protein was detected in immunoblots of unc59(e1005) (Fig. 2), suggesting that septin encoded by the unc59(e1005) missense allele is synthesized but mislocalized.

UNC-61 localization does not require the mitotic spindle apparatus

In studies of cytokinesis, the question of what component of the mitotic apparatus provides the signals that set up the cleavage furrow is a topic of much debate (see Rappaport, 1996). One of the prime suspects is the spindle midzone which has been shown to be required for cytokinesis in some situations (Wheatley and Wang, 1996). In C. elegans a pseudocleavage furrow forms in fertilized embryos during pronuclear migration (Wood, 1988). Unlike a true furrow, a pseudocleavage furrow does not constrict around a mitotic apparatus spindle. The pseudocleavage furrow ingresses and then regresses; it has an actin ring similar to that of a cytokinetic furrow (Strome, 1986). We double-labeled early embryos with UNC-61 and actin antibodies. We found that UNC-61 localized at the pseudocleavage furrow along with actin (data not shown) suggesting that the mitotic apparatus is not required for septin localization. We also studied the localization of UNC-61 in zyg-9(b244) embryos. zyg-9(b244) is a maternal temperature-sensitive (ts) embryonic lethal mutant that has abnormal furrowing during the first cell division exhibiting both true cleavages and ectopic cleavage furrows (Matthews et al., 1998). We found that UNC-61 localized to both true furrows and ectopic furrows (lacking an associated spindle) in the mutant embryos (Fig. 8A-B). These data suggest that whenever there is a cleavage furrow, septins are likely to be a part of the contractile ring regardless of the presence of a spindle midzone.

DISCUSSION

The unc-59 and unc-61 locus encodes two independent C. elegans septins

We have presented evidence that the unc-59 and unc-61 loci encode two C. elegans septins. First, cosmid containing the full-length septin genes rescued unc-59 and unc-61 mutants independently. Second, we demonstrated that RNAi with the cDNA containing the septin sequences phenocopied the vulva protrusion, gonad extrusion and egg laying defects of unc-59 and unc-61 mutants. Third, the sequence analysis indicated that unc-59 and unc-61 are septins; we found that the two alleles of unc-59 (e261 and e1005) have missense mutations while unc-61 (e228 and n3169) both have a premature stop codon in their transcript. Fourth, western blots detected bands close to the predicted molecular masses of UNC-59 and UNC-61. Finally, UNC-59 and UNC-61 antibodies detected the presence of these septins in the cleavage furrows of wild-type embryos but not in the unc-59(e1005) or unc-61(n3169) mutant embryos. These observations lead us to the conclusion that the C. elegans unc-59 and unc-61 loci are septins.

Both UNC-59 and UNC-61 show a significant homology to...
other known septins; They have a conserved, predicted GTPase domains at their amino termini and a putative coiled-coil domain at their carboxyl termini. The coiled-coil domains may mediate protein-protein interactions between themselves or with other proteins (reviewed by Sander and Field, 1994; Longtine et al., 1996).

**unc-59 and unc-61 are required for reliable post-embryonic cytokinesis**

Our data suggest that *unc-59* and *unc-61* are not required for embryonic development; however, *unc-59* and *unc-61* mutants exhibit consistent defects in their vulva, gonad, and male tails. These structures develop post-embryonically during larval stages (Sulston and Horvitz, 1977; Sulston et al., 1980). Based on what is known about septins and the phenotypes that we observe in *unc-59* and *unc-61* mutants, it is reasonable to suppose that these phenotypes are at least in part due to post-embryonic failure in cytokinesis. Previously, we have described *unc-59(e1005)* as exhibiting failures of cytokinesis during post-embryonic development. Using a combination of light microscope lineage analysis and serial section reconstruction from electron micrographs, we showed that cytokinesis failures occurred in the P-cell derived neuroblasts in the ventral cord (White et al., 1982). Here, we investigated post-embryonic cytokinesis defects by studying the Q neuroblast lineage of the *unc-61(n3169)* and *unc59(e1005)* mutants. We found that the Q/V5 precursor often failed to divide, producing binucleate cells. In wild-type hermaphrodite development, Q and V5 are neuroblast precursor cells; descendants of V5 also generates sensory rays in the tail of wild-type males and posterid sensilla (Hunter et al., 1999). This might explain some of the male tail defects. However, evidence of extensive cell division failures is not apparent in the defective male tails, and the Q/V5 failures only occurred 30% of the time. This together with the high level of UNC-61 expression seen in the early L4 male tails suggest that septins may play a role in male tail morphogenesis that is separate from the role of septins in post-embryonic cytokinesis. Other phenotypes that we observed such as vulva and neuronal defects could be due to post-embryonic failures in cytokinesis since all of these structures develop post-embryonically (Sulston and Horvitz, 1977). However, we once again cannot rule out the possibility of a role for septins that is separate from the requirement for cytokinesis.

**Localization of UNC-59 and UNC-61 in early wild-type and mutant embryos**

Even though we saw no early embryonic defects in septin mutants, we found that UNC-59 and UNC-61 localize to cleavage furrows throughout embryonic development. In *C. elegans* development, F-actin uniformly localizes to the cleavage furrow and at the border of cell-cell contact (Strome, 1986; Waddele et al., 1994). We observed that both UNC-59 and UNC-61 behave in a similar manner to F-actin in the early embryo.

By indirect immunofluorescence of *unc-59* and *unc-61* mutants, we have shown that UNC-59 and UNC-61 depend on each other for proper localization. The absence or mislocalization of one or both of the septins gives similar phenotypes. The *unc-59(e261)* and *unc-61(e228)* double mutant phenotype is virtually identical to the single mutant phenotypes (Table 2). Also, double RNAi using *unc-59* and *unc-61* (data not shown) gave indistinguishable phenotypes to single RNAi experiments (Fig. 3H-J) and to the mutant phenotypes (Fig. 3B-G). In all cases we observed no embryonic phenotype but rather defects in postembryonic development as described (Tables 1 and 2). Although 99% of the *C. elegans* genome has been thus far sequenced, we have not found another septin gene in the database. This suggests that *unc-59* and *unc-61* are likely to be the only two *C. elegans* septins. Therefore, it appears as if septins are not essential for embryonic cytokineses, but they are required for the fidelity of postembryonic cytokineses.

In an attempt to shed some light on the mechanism of septin localization to the furrow, we asked whether the position of the mitotic spindle is essential for recruiting septins to the cleavage furrow. To address this question, we examined the distribution of UNC-61 in pseudocleavage furrows. We found that UNC-61 localized to both normal pseudocleavage furrows and ectopic cleavage furrows as seen in *zyg-9* mutants. These observations suggest that the mitotic apparatus is not required for septin localization to the cleavage furrow.

**Possible functions of septins in *C. elegans***

The observation that UNC-59 and UNC-61 localize to the cleavage furrows in the embryo, yet do not seem to be necessary, begs the question of what the primary function of the septins is in *C. elegans*. Possibly the embryo has some redundant back-up system for ensuring that embryonic cytokineses are executed with maximum fidelity as a failure to produce some of the early blastomeres is likely to have lethal consequences (Sulston et al., 1983) whereas some failure of postembryonic cytokineses can be tolerated as evidenced by the viability of the septin mutants.

Another possible rationalization for our observations is that septins do not have a primary role in cytokinesis but can facilitate that process in certain circumstances. The observation that septins are expressed in post-mitotic cells (Kinoshita et al., 1997; Xie et al., 1999) and our own observations that UNC-61 is expressed at a time when the male tail is undergoing morphogenesis (Fig. 5B and C) suggest that septins may play roles that are independent of cytokinesis. Septins have a well-conserved domain that associates with PIP2 and may provide a membrane anchor that is regulated by GTP (Zhang et al., 1999). The in vitro association experiments (Field et al., 1996; Frazier et al., 1998) suggest that septins could form organized structures in vivo. Perhaps septins could associate whilst anchored to the plasma membrane forming structures, cortically located aggregates. In budding yeast, several proteins have been identified, such as Myo1p and Bni4p, that localize to the mother/bud neck region in a septin dependent manner (DeMarini et al., 1997; Lippincott and Li, 1998). The function of septins may therefore be to define anchor regions on the plasma membrane where other proteins associate.

The mammalian septins Nedd5 and H5 associate with actin (Kinoshita et al., 1997; Xie et al., 1999. This association could be the mechanism by which septins are transported into the cleavage furrow in animal cells (however, actin is not required for localization of septins to the mother/bud neck of *Saccharomyces cerevisiae* (Ayscough et al., 1997)). In this view, the septin accumulation seen in embryonic cytokinetic furrows of *C. elegans* may be a simple consequence of the
accumulation of actin filaments into the contractile ring at the apex of the cleavage furrow and have no functional significance. During postembryonic development, factors that associate with the cortical septin complex at the cleavage furrow may be required for reliable cytokineses. One possibility is syntaxin, which has been shown to be required for cytokinesis in *C. elegans* (Jantsch-Plunger and Glotzer, 1999) and has been also shown to associate with septins in vitro (Beites et al., 1999). This view of septin function suggests that septins have generalized functions of protein targeting and are not an indispensable component of the cytokinetic machinery.

We thank Yuji Kohara for providing us with the three ESTs and the Worm and Genome Research Consortium for providing us with the cosmids. We thank Hiroko Kouke for technical support. We also thank Dr Ken Kemphues for providing us with the non-muscle myosin II antibodies. We thank Michael Myette, Fern Finger, Bill Mohler and particularly Ellen Williams-Masson for critically reading and editing the manuscript, the Kimble lab for the use of the confocal microscope, the Masson lab for the use of their radioactive room, the Anderson lab for providing some lab space and Leanne Olds for graphic advice.

This work was supported by a University of Wisconsin-Madison graduate school AOF fellowship to T.Q.N. and NIH grant (R01 GM-52454) to J.G.W.

REFERENCES


T. Q. Nguyen and others


