Specific requirement for two ADF/cofilin isoforms in distinct actin-dependent processes in Caenorhabditis elegans

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Summary
Actin depolymerizing factor (ADF)/cofilin is an essential enhancer of actin turnover. Multicellular organisms express multiple ADF/cofilin isoforms in different patterns of tissue distribution. However, the functional significance of different ADF/cofilin isoforms is not understood. The Caenorhabditis elegans unc-60 gene generates two ADF/cofilins, UNC-60A and UNC-60B, by alternative splicing. These two ADF/cofilin proteins have different effects on actin dynamics in vitro, but their functional difference in vivo remains unclear. Here, we demonstrate that the two isoforms are expressed in different tissues and are required for distinct morphogenetic processes. UNC-60A was ubiquitously expressed in most embryonic cells and enriched in adult gonads, intestine and oocytes. In contrast, UNC-60B was specifically expressed in the body wall muscle, vulva and spermatheca. RNA interference of UNC-60A caused embryonic lethality with variable defects in cytokinesis and developmental patterning. In severely affected embryos, a cleavage furrow was formed and progressed but reversed before completion of the cleavage. Also, in some affected embryos, positioning of the blastomeres became abnormal, which resulted in embryonic arrest. In contrast, an unc-60B-null mutant was homozygous viable, underwent normal early embryogenesis and caused disorganization of actin filaments specifically in body wall muscle. These results suggest that the ADF/cofilin isoforms play distinct roles in specific aspects of actin reorganization in vivo.

Movies available online

Key words: Myofibrils, Thin filaments, Actin dynamics, Embryogenesis, Cytokinesis

Introduction
The actin cytoskeleton is organized into many different structures and adapted for diverse cellular functions. A number of actin-binding proteins regulate dynamic reorganization and stabilization of the actin filaments and determine appropriate architecture depending on the circumstances (Cooper and Schafer, 2000; Pollard et al., 2000). In multicellular organisms, many actin-binding proteins are present as multiple isoforms that are expressed from multigene families and/or generated by alternative splicing of a single gene. These isoforms are often expressed in a cell-type-specific manner and involved in different aspects of cellular activities. Therefore, having multiple isoforms of actin-binding proteins could increase complexity in the structure and function of the actin cytoskeleton.

Actin depolymerizing factor (ADF)/cofilins are a family of actin-binding proteins that promote rapid turnover of the actin cytoskeleton (Bamburg, 1999; Bamburg et al., 1999; Carlier et al., 1999; Maciver and Hussey, 2002). ADF/cofilins preferentially bind to ADP-actin (Carlier et al., 1997; Maciver and Weeds, 1994) and enhance the turnover of actin filaments by increasing the rate of depolymerization from their pointed ends (Carlier et al., 1997; Maciver et al., 1998) and by severing filaments (Du and Frieden, 1998; Hawkins et al., 1993; Hayden et al., 1993; Ichetovkin et al., 2000; Maciver et al., 1991; Nishida et al., 1984; Nishida et al., 1985). Filament binding by ADF/cofilin changes the twist of the actin filaments (McGough et al., 1997) and weakens lateral contacts in the filaments (McGough and Chiu, 1999). ADF/cofilin binds to F-actin in a cooperative manner and generates an unstable population of filaments (McGough et al., 1997; Ressad et al., 1998).

Multicellular organisms have multiple ADF/cofilin genes that are often expressed in tissue-specific patterns, whereas simple organisms, such as yeast, have a single essential gene for cofilin (Iida et al., 1993; Lappalainen and Drubin, 1997; Lappalainen et al., 1997; Moon et al., 1993). Vertebrates have two or three ADF/cofilin genes that are designated as ADF (also known as destrin) (Abe et al., 1990; Adams et al., 1990; Moriyama et al., 1990), non-muscle-type cofilin/cofilin-1 (Matsuzaki et al., 1988) and muscle-type cofilin/cofilin-2 (Gillett et al., 1996; Ono et al., 1994; Thirion et al., 2001). In mice, cofilin-1 is expressed in most cell types, whereas cofilin-2 is predominant in muscle cells and ADF is specific for epithelia and endothelia (Mohri et al., 2000; Ono et al., 1994; Vartiainen et al., 2002). These ADF/cofilin isoforms exhibit different actin-regulatory activities. ADF is more potent in depolymerization than cofilin (Abe and Obinata, 1989; Giuliano et al., 1988; Nishida et al., 1985), whereas cofilin apparently remains bound to filaments (Abe et al., 1989; Nishida et al., 1984). Detailed comparisons of ADF and cofilin revealed that they have nearly identical activities to accelerate...
depolymerization and filament severing, but are different in their nucleating activities to initiate polymerization when they make complexes with actin (Yeoh et al., 2002). The cofilin-actin complex initiates spontaneous polymerization more efficiently than the ADF-actin complex. The difference is significant at a high pH and confers their pH-sensitive activities (Yeoh et al., 2002; Yonezawa et al., 1985). Cofilin-2 is more effective in enhancing polymerization than ADF and cofilin-1 (Vartiainen et al., 2002), suggesting that the critical concentration of the cofilin-2-actin complex is very low.

In cells where multiple ADF/cofilin isoforms are expressed, the isoforms behave very similarly in localizing to stress-induced intranuclear actin rods (Ono et al., 1993) and in translocating to the lamellipodia after growth factor stimulation (Meberg et al., 1998). Also, many extracellular stimuli regulate phosphorylation levels of both ADF and cofilin in common pathways (Kanamori et al., 1995; Meberg et al., 1998; Saito et al., 1994). However, ADF and cofilin respond differently to changes in some of the cellular states. Colocalization of ADF with monomeric actin is enhanced upon an increase in intracellular pH, whereas that of cofilin is less sensitive to pH changes (Bernstein et al., 2000), which is consistent with their isoforms from studies using model organisms in which cofilin might have both redundant and non-redundant roles. Colocalization of ADF with actin is more significant at a high pH and confers their pH-sensitive activities (Yeoh et al., 2002; Yonezawa et al., 1985). Cofilin-2 is more efficient than ADF in enhancing polymerization than ADF and cofilin-1 (Vartiainen et al., 2002), suggesting that the critical concentration of the cofilin-2-actin complex is very low.

There are some functional differences among ADF/cofilin isoforms from studies using model organisms in which mutations in ADF/cofilin genes have been isolated. The slime mold Dictyostelium discoideum has two cofilin genes, cofilin-1 and cofilin-2 (Aizawa et al., 2001). A null mutant of cofilin-1 has not been isolated in previous attempts of gene knockout (Yeoh et al., 2002). Expression of ADF, but not cofilin, is downregulated by an increase in the actin monomer pool (Minamide et al., 1997). In contrast, expression of cofilin, but not ADF, is upregulated in dystrophic muscles (Hayakawa et al., 1993; Nagaoka et al., 1996). Thus, ADF and cofilin might have both redundant and non-redundant functions.

Materials and Methods

Nematode strains

Nematodes were grown at 20°C as described previously (Brenner, 1974). Wild-type strain N2 was obtained from the Caenorhabditis Genetics Center (Minneapolis, MN). unc-60 (e677) (Waterston et al., 1980) and unc-60 (s1586); sup-12 (st203) (McKim et al., 1994) were provided by David Baillie (Simon Fraser University, Burnaby, Canada). unc-60 (su158) (Zengel and Epstein, 1980) was provided by Henry Epstein (Baylor College of Medicine, Houston, Texas) and outcrossed five times with N2 before phenotypic characterization. All strains used in this study were homozygous for each allele.

Northern blot

A northern blot containing total RNA from different developmental stages was kindly provided by Edward Kipreos (University of Georgia, Athens, Georgia). This blot was first hybridized with a probe for 18S rRNA as described previously (Kipreos et al., 1996), then stripped and re-hybridized with a probe specific for unc-60A, stripped and re-hybridized with a probe specific for unc-60B. unc-60 probes were created by PCR from a cDNA library (kindly provided by Robert Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK), and 50 ng was labeled with 32P by random primer method. Hybridization was conducted overnight at 42°C in 150 μg/ml single-stranded salmon sperm DNA, 5×SSC (0.75 M NaCl, 0.075 M trisodium citrate, pH 7.0), 1×Blocking Quencher (Molecular Research Center, Inc.), 0.2% SDS and 50% formamide. Final washes were at 65°C in 0.5× SSC, 1% SDS and 2×Denhardt solutions (0.04% Ficoll 400, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin).

Immunofluorescence microscopy

Worm embryos were obtained by cutting gravid adults on poly-lysine-coated slides, freeze-cracked as described previously (Epstein et al., 1993) and fixed with methanol at −20°C for 5 minutes. They were washed with phosphate-buffered saline (PBS) for 10 minutes and stained with antibodies diluted in 1% bovine serum albumin in PBS. Gonads were dissected by cutting adult worms at the level of the pharynx on poly-lysine-coated slides, freeze-cracked, fixed and stained in the same manner as embryo staining. Antibody staining of adult worms was performed as described elsewhere (Finney and Ruvkun, 1990).

Rabbit polyclonal anti-UNC-60A and UNC-60B antibodies were described previously (Ono et al., 1999). An anti-UNC-60B antibody was further absorbed with acetone-fixed powder of the unc-60 (su158) (unc-60B-null) mutant worms to remove non-specific reactivity (Miller and Shakes, 1995). Mouse monoclonal anti-myoA antibody (clone 5.6) (Miller et al., 1983) was provided by Henry Epstein (Baylor College of Medicine, Houston, Texas). Mouse monoclonal anti-vinculin antibody (MH24) and mouse monoclonal anti-α-actinin antibody (MH40) (Francis and Waterston, 1985) were provided by Michelle Hresko (Washington University School of Medicine, St. Louis, MO). Mouse monoclonal anti-actin antibody (C9) was purchased from ICN Biomedicals. Rabbit polyclonal anti-actin antibody was purchased from Cytoskeleton Inc. Mouse monoclonal anti-α-tubulin antibody was purchased from Amersham Biosciences. Secondary antibodies used were Alexa488-labeled goat anti-mouse IgG (Molecular Probes) and Cy3-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). To stain DNA, 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Sigma-Aldrich) was included in the solution of secondary antibodies at 0.1 μg/ml.

Samples were viewed by epifluorescence using a Nikon Eclipse TE2000 inverted microscope with a 40× CFI Plan Fluor objective. Images were captured by a SPOT RT Monochrome CCD camera (Diagnostic Instruments) and processed by the IPLab imaging software (Scanalytics, Inc.) and Adobe Photoshop 6.0.

RNA interference experiments

The cDNAs containing entire coding regions of UNC-60A or UNC-60B were cloned into the pBlueScript vector (Stratagene) and transcribed into RNA by T7 RNA polymerase. Adult worms were fed E. coli expressing the UNC-60 RNA interference constructs as described (Gordon and Waterston, 1993). Each isoform was required for distinct actin-dependent processes. The results suggest that multicellular organisms have functionally different ADF/cofilin isoforms to support the complexity of tissue organization.
Functions of ADF/cofilin isoforms

![Fig. 1. Expression of mRNAs for UNC-60A and UNC-60B during C. elegans development. 25 μg of total RNA from the indicated stages was loaded on each lane and probed with radiolabeled cDNAs for unc-60A, unc-60B or 18S rRNA. Samples are embryos (lane 1), L1 larvae (lane 2), L2 larvae (lane 3), L3 larvae (lane 4), L4 larvae (lane 5), young adults (lane 6) and gravid adults (lane 7).](image)

**Time-lapse DIC microscopy**

Embryos were mounted on a 2% agarose pad with egg salts and covered with a glass coverslip. They were set on a Nikon Eclipse TE2000 inverted microscope and observed with a 40x CFI Plan Fluor objective with DIC optics. Images were captured by a SPOT RT Monochrome CCD camera (Diagnostic Instruments) and recorded every 15 seconds using the IPLab imaging software (Scanalytics, Inc.).

**Sequencing of genomic DNA**

Total DNA was prepared from unc-60 homozygotes as described previously (Sulston and Brenner, 1974) and used as a template for PCR reactions. Several primer sets were used to amplify 1 to 2 kb genomic DNA fragments from different regions of the unc-60 gene by REDTiag DNA polymerase (Sigma-Aldrich) to determine the location of deletions in the unc-60 alleles. DNA fragments with deletions were sequenced with an ABI PRIZM dye terminator cycle sequencing kit and an ABI310 genetic analyzer (Applied Biosystems) to determine precise deletion sites.

**Western blot**

Total worm lysates were prepared as described previously (Ono and Ono, 2002). Protein concentrations of the lysates were determined by a filter paper dye-binding assay (Minamide and Bamburg, 1990). 10 μg of proteins from each lysate was separated on a 15% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P: Millipore) using a Genie Blotter (Idea Scientific). The membranes were blocked in 5% non-fat milk in PBS containing 0.1% Tween 20 and incubated for 1 hour with anti-UNC-60A, anti-UNC-60B or anti-actin (C4; ICN Biomedicals) antibodies followed by treatment with peroxidase-labeled goat anti-rabbit IgG or goat anti-mouse IgG (Pierce Chemical Co.). The reactivities were detected with a SuperSignal chemiluminescence reagent (Pierce Chemical Co.).

**Motility assay**

A motility assay was performed as described previously (Epstein and Thomson, 1974). Briefly, adult worms were placed in M9 buffer (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 85.5 mM NaCl, 1 mM MgSO₄). Then, one beat was counted when a worm swung its head to either left or right. The total number of beats in 30 seconds was recorded.
UNC-60A is essential for early embryonic development

To characterize the in vivo roles of UNC-60A and UNC-60B, we suppressed expression of each ADF/cofilin isoform by RNAi and characterized the resultant phenotypes. When worms were fed with bacteria expressing dsRNA for unc-60A, they produced many dead embryos and a greatly reduced number of developed progeny (Table 1). However, treatment with dsRNA for unc-60B caused did not alter brood size (Table 1), morphology or motility (data not shown). Immunostaining of the unc-60A (RNAi) embryos showed remarkable reduction of the UNC-60A protein to somewhat variable extents (Fig. 4). In control embryos (Fig. 4a-c), UNC-60A was distributed in the same patterns as in wild-type embryos under standard culture conditions (Fig. 2). The unc-60A (RNAi) treatment significantly reduced the cytoplasmic staining of UNC-60A (Fig. 4d-f), whereas staining of some structures that resembled cell-cell boundaries or premature cleavage furrows was observed (Fig. 4d, arrowheads), suggesting that UNC-60A was not completely depleted. Western blot analysis indicated that the UNC-60B protein was not decreased by feeding with the unc-60B dsRNA (data not shown). Therefore, we concluded that RNAi was successful in demonstrating the requirement of UNC-60A but not UNC-60B in embryonic development.

Table 1. Effects of RNAi on F1 progeny

| RNA    | Dead embryos* | Abnormal larvae* | Total larvae* |
|--------|---------------|------------------|---------------|
| Control | 2.0±2.6†      | 0.29±0.61        | 184±36        |
| unc-60A (RNAi) | 25±15†      | 5.4±4.0          | 204±44        |
| unc-60B (RNAi) | 1.2±3.0†     | 0.14±0.36        | 157±41        |

*The phenotypes of the F1 progeny were categorized into dead embryos, abnormal larva and normal larva. Abnormal larvae were transparent, deformed or uncoordinated. Total larvae are abnormal larvae plus normal larvae.

†All values represent numbers of embryos or larvae (mean±s.d., n=14).
was relatively rapid (Movie 2). Embryos still underwent multiple rounds of nuclear division and attempts at cytokinesis, which resulted in multinucleated cells or irregularly compartmentalized embryos (Fig. 5n). In a representative positioning-defective embryo (Fig. 5c,f,i,l,o), first and second rounds of cytokinesis were apparently normal except that the cleavage of the P1 blastomere occurred slightly earlier than the control (Fig. 5l). However, subsequent positioning of the blastomeres at the four-cell stage became abnormal (compare Fig. 5m with o). In control embryos, the cleavage plane of the AB blastomere occurs parallel to the long axis of the embryo, whereas that of the P1 blastomere is set perpendicular to the long axis (Fig. 5j, Movie 1). The resultant ABp and EMS blastomeres slide to the sides, and ABa and P2 are located at anterior and posterior poles, respectively (Fig. 5m). However, in some of the unc-60A (RNAi) embryos, although the orientation of the spindles and cleavage planes was normal (Fig. 5l), both ABa and ABp remained at the anterior side and EMS was squeezed into the center of the embryos (Fig. 5o, Movie 3). These embryos continued to divide but were not successful at completing the following morphogenesis.

In addition, we observed a defect in extrusion of the polar body and abnormal cortical activity in the unc-60A (RNAi) embryos (Fig. 6a,b). In 71% (n=17) of the unc-60A (RNAi) embryos, the polar body was not extruded after meiosis, which resulted in the appearance of the third pronucleus during pronuclear migration (Fig. 6a, arrow). This is likely to occur.
be due to a cytokinesis defect in meiosis. Also, 41% of the *unc-60A* (RNAi) embryos showed abnormally active membrane protrusion and retraction after the two-cell stage (Fig. 6b, arrowheads, Movie 3, see the lower side of cell-cell boundary at the two-cell stage), suggesting that the cell cortex became unstable, as observed with RNAi of the Arp2/3 complex (Severson et al., 2002).

Actin filaments are important for establishment of the anterior-posterior (A-P) polarity at the one-cell stage (Hill and Strome, 1988; Hill and Strome, 1990). However, the A-P polarity is not severely disturbed by the *unc-60A* (RNAi) treatment (Fig. 6c-e). We measured positions of pronuclear meeting (Fig. 6c) and the first mitotic spindle (Fig. 6d), which are normally posteriorly localized (Golden, 2000). The position of pronuclear meeting was more variable in *unc-60A* (RNAi) embryos than in control embryos (Fig. 6e). In some *unc-60A* (RNAi) embryos, the pronuclei met near the center of the embryos (Fig. 5c), whereas in other embryos, it occurred at a posterior region (Fig. 5b). However, the average value of the meeting position in *unc-60A* (RNAi) embryos (65±8.9% egg length from the anterior pole) was not significantly different from that of control (67±4.1%) (P=0.54 by a t-test). Similarly, positions of the anterior and posterior spindle poles were slightly more variable in *unc-60A* (RNAi) embryos, but the differences were not statistically significant (Fig. 6e). These results suggest that the A-P polarity was not significantly affected by the *unc-60A* (RNAi) treatment.
might be slightly unstable but not severely disturbed when UNC-60A was suppressed.

In control embryos, actin was uniformly localized to the cortex (Fig. 7a). However, in the unc-60A (RNAi) embryos with the multinucleated phenotype, cortical actin was unevenly distributed and enriched at one end of the cell cortex (Fig. 7d,g) and sometimes accumulated at a cleavage-furrow-like structure (Fig. 7g; indicated by an arrow). These phenotypes suggest that UNC-60A is required for even distribution of cortical actin and proper progression of a cleavage furrow. Mitotic spindles were often found around nuclei (Fig. 7e), indicating that separation of the spindle poles and spindle assembly were not significantly affected by depletion of UNC-60A.

A null mutation of unc-60B causes specific defects in actin assembly in body wall muscle

Previously characterized mutations of unc-60B were all point mutations that resulted in expression of mutant UNC-60B proteins (Ono et al., 1999). Therefore, although these mutants were homozygous viable and showed disorganization of actin filaments in body wall muscle, we were not able to exclude the possibility that the mutant UNC-60B proteins function sufficiently in some cellular activities other than myofilibr assembly. To clearly determine the requirement of unc-60B in vivo, we need to characterize a null phenotype of unc-60B.

We collected unc-60 mutant strains from other researchers, determined their sequence alterations and found that two unc-60 alleles contain deletions in the unc-60B region (Fig. 8A). unc-60 (s1586) is homozygous lethal at a late larval stage (McKim et al., 1994). We found that this allele had a deletion that completely removes exon 2B (Fig. 8A). However, the deletion also extends to the unc-60A region and removes approximately 250 bp of the 3'-untranslated region in exon 5A that contains a putative polyadenylation signal for unc-60A. Therefore, this deletion is likely to affect expression of both ADF/cofilin isoforms, which may be a cause of the lethal phenotype. In contrast, we found that unc-60 (su158) (Zengel and Epstein, 1980) had a deletion of 600 bp that completely removed exons 3B and 4B and did not disturb the unc-60A region (Fig. 8A). Even if exons 2B and 5B are connected by an aberrant splicing event, exons 1 and 2B encode only 30

(e677) has a greatly reduced amount of UNC-60B (d, lane 3), whereas the unc-60 (su158) mutant has no detectable UNC-60B (d, lane 2). Molecular mass markers in kDa (lane M) are indicated on the left of a.
amino acids from the N-terminus of UNC-60B and the coding region in exon 5B will be out of frame. The unc-60 (su158) mutant is homozygous viable and shows a more severe motility defect than a strong loss-of-function mutant unc-60 (e677) (Fig. 8B). In the unc-60 (su158) homozygotes, the UNC-60B protein was not detected by western blotting, whereas UNC-60A was present at an equivalent amount to that found in wild-type worms (Fig. 8C). Thus, unc-60 (su158) is a null allele of unc-60B that does not interfere with unc-60A.

The unc-60B null mutants were homozygous viable and showed a muscle-specific defect in actin organization. During embryonic development, morphological observation by DIC microscopy revealed no abnormalities (data not shown). However, staining with an anti-actin antibody revealed that, although early embryogenesis from the one-cell to 1.75-fold stages appeared to be normal (Fig. 9Ad), actin became discontinuous in the body wall muscle after the two-fold stage (Fig. 9Bd,Cd, indicated by arrows). The defect in actin organization was specifically detected in the body wall muscle cells. In addition, other myofibrillar components, myoA myosin heavy chain (Fig. 9A-Cb,e) and vinculin (Fig. 9A-Cc,f), appeared undisturbed, suggesting that initial assembly of the myosin thick filaments and adhesion structures are independent of UNC-60B-mediated actin dynamics. Nonetheless, as a result of the muscle defects, movement of the mutant embryos in the egg shell was significantly slower than wild-type embryos (data not shown). In addition, RNAi of unc-60A on the unc-60B-null mutant resulted in similar cytokinesis and patterning defects (40% and 27%, respectively, n=15) to wildtype, and no enhancement of the phenotype was observed, suggesting that UNC-60B does not function in early embryogenesis.

When the unc-60B-null mutants grew into adult worms, they were nearly paralyzed and large actin aggregates were formed in the body wall muscle (Fig. 10). The extent of actin disorganization was slightly more severe than a strong loss-of-function mutant unc-60 (e677) (Ono et al., 1999). However, unlike the embryos, the organization of the myosin filaments and dense bodies in the adults was disturbed (Fig. 10). In wildtype, myosin was clearly arranged in a striated pattern (Fig. 10Aa). In the unc-60B-null mutant, myosin was assembled into wide and uneven bands with very obscure striation (Fig. 10Ad). Dense bodies are adhesion structures that appear as discrete spots in the center of the I-bands, as shown by staining for α-actinin and vinculin (Fig. 10B,C). In the unc-60B null mutant, both α-actinin and vinculin were aligned in striation, but the staining patterns were often continuous as if several dense bodies were fused together (Fig. 10Bd,Cd). In addition, vinculin was somewhat diffuse in some regions (Fig. 10Cd). Double staining of the dense body components with actin showed that α-actinin and vinculin were not the components of actin aggregates in the mutant (Fig. 10Bf,Cf). These results suggest that UNC-60B-dependent actin dynamics are required for proper alignment of thick filaments and dense bodies during post-embryonic development, and that the actin-bundling activity by myosin, α-actinin or vinculin does not contribute to formation of the actin aggregates in the unc-60B mutants. In addition, the expression pattern of UNC-60A in the unc-60 (su158) mutant
was not significantly different from wild-type (data not shown), suggesting that there is no compensatory mechanism to upregulate UNC-60A in the absence of UNC-60B.

**Discussion**

Our study demonstrated that the two ADF/cofilin isoforms in *C. elegans* are required for distinct actin-dependent processes. The two ADF/cofilins, UNC-60A and UNC-60B, have different activities to enhance actin dynamics (Ono and Benian, 1998) and are expressed from the unc-60 gene by alternative splicing in developmentally regulated and tissue-specific manners. RNAi suppression of UNC-60A causes embryonic lethality with cytokinesis or patterning defects during early embryogenesis. In contrast, a null mutation of unc-60B specifically impairs myofibril assembly in body wall muscle without affecting early embryogenesis. In contrast, a null mutation of unc-60B specifically impairs myofibril assembly in body wall muscle without affecting early embryogenesis. The biochemical difference between the two ADF/cofilin isoforms are consistent with the cell biological observations that UNC-60A functions in a highly dynamic actin rearrangement during embryogenesis, whereas UNC-60B is involved in a less dynamic event in muscle cells.

**Role of UNC-60A in embryogenesis**

We found that UNC-60A was required for cytokinesis in early *C. elegans* embryos. Similarly, ADF/cofilin has been reported to be required for cytokinesis in *Drosophila* (Gunsalus et al., 1995; Somma et al., 2002) and *Xenopus* eggs (Abe et al., 1996), indicating that ADF/cofilin is a conserved regulator of cell division. Our immunostaining indicated that UNC-60A was not concentrated in the early accumulation of actin at the cleavage site. Rather, it was localized to progressing cleavage furrows in the late phase of cytokinesis and remained associated with cortical actin at cell-cell contacts. This is also consistent with the observations that cofilin is localized to the cleavage furrow in cultured cells (Nagaoka et al., 1995) and *Xenopus* eggs (Abe et al., 1996). This localization pattern suggests that the role of UNC-60A is in late cytokinesis and explains the cytokinesis defect by RNAi of unc-60A. In the unc-60A (RNAi) embryos, a cleavage furrow was often formed and progressed, but prematurely disassembled, suggesting that UNC-60A-mediated actin dynamics are required for efficient completion of cytokinesis. Also intriguingly, progression of a cleavage furrow in cytokinesis-defective embryos was much slower than that in control embryos, suggesting that UNC-60A-dependent actin dynamics might be a driving force for furrow progression. The contractile ring in dividing cells has been shown to be an active site of actin polymerization (Noguchi and Mabuchi, 2001; Pelham and Chang, 2002). Therefore, actin depolymerization and severing by ADF/cofilin would be required to maintain dynamic actin turnover during cytokinesis.

Similar phenotypes in late cytokinesis have been described for mutants or RNAi suppression of the formin-like protein CYK-1 (Swan et al., 1998), the kinesin-like protein ZEN-4/CeMKLP1 (Powers et al., 1998; Raich et al., 1998; Severson et al., 2000), CYK-4 Rho-GAP (Jantsch-Plunger et al., 2000), the aurora-related kinase AIR-2 (Kaitna et al., 2000; Schumacher et al., 1998; Severson et al., 2000) and the syntaxin SYN-4 (Jantsch-Plunger and Glotzer, 1999). Formin has been shown to nucleate actin polymerization (Pruyne et al., 2002; Sagot et al., 2002), but it is not known how ADF/cofilin affects this process. ZEN-4 and CYK-4 physically interact and are
required for assembly of the central spindle, which is implicated in completion of cytokinesis (Mishima et al., 2002). Syntaxin mediates vesicle fusion and may be required for membrane addition at the cleavage furrow (Skop et al., 2001). However, the mechanism by which these proteins promote the completion of cytokinesis is poorly understood. How UNC-60A might be involved in these processes remains to be determined.

Defects in early embryonic patterning in the unc-60A (RNAi) embryos are probably weaker phenotypes than the cytokinesis defects, because embryos with patterning defects were successful in multiple rounds of cell division. Nonetheless, the observed phenotype is novel. Embryonic polarity and asymmetric cell division are essential for determining cell fate and embryonic patterning. Actin and myosin are required for polarized localization of the products of polarity genes (Rose and Kemphues, 1998). However, the unc-60A (RNAi) embryos showed an apparently normal pattern of spindle orientation and cleavage pattern at the second cell division, whereas subsequent positioning of the blastomeres was defective. It is still possible that the embryonic polarity is partially disrupted so that the blastomeres were not able to locate at appropriate positions. Alternatively, cortical rigidity or activity might be disturbed by unc-60A (RNAi). UNC-60A is colocalized with cortical actin at the cell-cell contacts, and unc-60A (RNAi) alters cortical distribution of actin and induces irregular membrane activity. Therefore, the cell cortex might have lost its rigidity and, thus, might be unable to push or to be pushed by the neighboring cells.

**Specific requirement of UNC-60B in myofibril assembly and development**

Phenotypic analysis of the unc-60B-null mutant confirms our previous observations that UNC-60B is specifically required for assembly of actin (Ono et al., 1999). In addition, our extended analysis demonstrates that UNC-60B is not required for early development and that it is required for organized assembly of other myofibrillar components during postembryonic development. This indicates that the presence of the UNC-60A isoform is sufficient for viability of worms. UNC-60A is expressed in every embryonic cell but not in adult body wall muscle cells. The expression of UNC-60A in muscle was already low in larval stages, and so we were not able to determine when UNC-60A is downregulated (K.O. and S.O., unpublished). Although the antibody failed to detect UNC-60A in adult muscle, it is possible that a low level of UNC-60A is expressed in adult muscle and supports cell viability.

Phenotypic characterization of the unc-60B-null mutant showed that actin was disorganized from the embryonic stages onwards, whereas myosin thick filaments and dense bodies were disorganized only in adults. This suggests that UNC-60B is primarily required for actin assembly and that the effects on myosin and dense bodies are secondary and are due to disrupted thin filament organization. Therefore, the initial assembly of thick filaments and dense bodies may not require UNC-60B-mediated actin dynamics. This supports early observations that myosin and actin are initially assembled into nascent structures that are distinct from myofibrils (Epstein et al., 1993), suggesting that the assembly processes of myosin and actin are independent. However, the subsequent development and maintenance of these structures may need an organized myofibril structure that can generate contractile forces. In vertebrate striated muscle cells, inhibition of muscle contraction causes disorganization of myofibrils (De Deyne, 2000; Soeno et al., 1999). These observations suggest that a regulated actin-myosin interaction facilitates proper alignment of other myofibrillar components.

**Functional significance of ADF/cofilin isoforms**

We demonstrated that the two *C. elegans* ADF/cofilin isoforms have different functions in vivo. This is consistent with the different actin-regulating activities of UNC-60A and UNC-60B (Ono and Benian, 1998). We also observed that UNC-60A accelerates subunit dissociation from F-actin more rapidly than UNC-60B (S. Yeoh, S.O. and A. Weeds, unpublished). Our results are consistent with biochemical studies using different methods showing that UNC-60A is ADF-like, whereas UNC-60B is cofilin-like (H. Chen and J. Bamburg, personal communication). UNC-60A is a more potent depolymerizing agent than UNC-60B, suggesting that UNC-60A is suitable in cells where actin filaments are dynamic, whereas UNC-60B is adapted to cells, such as muscle, in which actin filaments are relatively stable. In addition, we have recently found that UNC-78/actin-interacting protein 1 (Ono, 2001) disassembles actin filaments more efficiently in the presence of UNC-60B than UNC-60A (K. Mohri, A. G. Weeds and S. Ono, unpublished), indicating that isoform-specific interactions with other cytoskeletal proteins are also important determinants of actin dynamics.

The other mechanism that differentiates these isoforms is their differential expression in different cell types. RNAi suppression or mutations of each isoform causes a specific phenotype in which the expression of the target isoform is predominant. However, it is still possible that the two ADF/cofilin isoforms have a redundant function in some biological aspects when both isoforms co-exist. UNC-60A and UNC-60B are produced by alternative splicing of the unc-60 gene (McKim et al., 1994). Therefore, the tissue-specific splicing machinery seems to be involved in the regulation of expression of the ADF/cofilin isoforms. It would be interesting to examine if misexpression of the ADF/cofilin isoforms causes a dominant phenotype or if forced expression of UNC-60B in early embryos or UNC-60A in muscle suppresses the RNAi or mutant phenotypes. The lethal phenotype of the yeast cofilin-null mutant can be rescued by expression of vertebrate ADF/cofilins (Iida et al., 1993) or *Dictyostelium* cofilin-1, but not cofilin-2 (Aizawa et al., 2001). Thus, further functional characterization of the ADF/cofilin isoforms is important to understand how evolution of actin-regulatory proteins has contributed to increasing the diversity in actin cytoskeletal structure and function.

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