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Report

The *Caenorhabditis elegans unc-78* Gene Encodes a Homologue of Actin-interacting Protein 1 Required for Organized Assembly of Muscle Actin Filaments

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Abstract. Assembly and maintenance of myofibrils require dynamic regulation of the actin cytoskeleton. In *Caenorhabditis elegans*, UNC-60B, a muscle-specific actin depolymerizing factor (ADF)/cofilin isoform, is required for proper actin filament assembly in body wall muscle (Ono, S., D.L. Baillie, and G.M. Benian. 1999. *J. Cell Biol*. 145:491–502). Here, I show that UNC-78 is a homologue of actin-interacting protein 1 (AIP1) and functions as a novel regulator of actin organization in myofibrils. In *unc-78* mutants, the striated organization of actin filaments is disrupted, and large actin aggregates are formed in the body wall muscle cells, resulting in defects in their motility. Point mutations in *unc-78* alleles change conserved residues within different WD

repeats of the UNC-78 protein and cause less severe phenotypes than a deletion allele, suggesting that these mutations partially impair the function of UNC-78. UNC-60B is normally localized in the diffuse cytoplasm and to the myofibrils in wild type but mislocalized to the actin aggregates in *unc-78* mutants. Similar Unc-78 phenotypes are observed in both embryonic and adult muscles. Thus, AIP1 is an important regulator of actin filament organization and localization of ADF/cofilin during development of myofibrils.

Key words: myofibrils • AIP1 • ADF/cofilin • WD repeats • actin filament dynamics

Introduction

Myofibrils in striated muscles are highly organized forms of actin cytoskeleton. Actin is the major component of the thin filaments, and their assembly and maintenance require regulation of filament dynamics (Littlefield and Fowler, 1998); but, the mechanisms that controls these processes are not understood. Body wall muscle of the nematode *Caenorhabditis elegans* has obliquely striated myofibrils, which has provided opportunities to study their assembly and function (Waterston, 1988; Moerman and Fire, 1997). In the regulation of thin filament assembly, UNC-60B, a muscle-specific actin depolymerizing factor (ADF)[1]/cofilin isoform, is required for enhancing actin dynamics and proper actin assembly into myofibrils (Ono et al., 1999). This study supports an important function of ADF/cofilin during myofibril assembly in vertebrates,

which has been suggested by the facts that ADF/cofilin is the major G-actin binding protein in embryonic chicken skeletal muscle (Nagaoka et al., 1996) and a muscle-specific cofilin isoform exists in mammals (Ono et al., 1994).

ADF/cofilins are a family of actin regulatory proteins that promote rapid turnover of the actin cytoskeleton (Bamburg, 1999). ADF/cofilin enhances actin filament turnover by increasing the rate of depolymerization from the pointed ends (Carlier et al., 1997) and by severing F-actin, thereby increasing the number of ends (Maciver et al., 1991). Cooperative binding of ADF/cofilin to F-actin changes the twist of the filament (McGough et al., 1997) and weakens lateral contacts in the filament (McGough and Chiu, 1999), which is a likely cause of the severing activity, whereas the structural basis of the depolymerizing activity is not known. These two activities can be uncoupled by several mutations (Moriyama and Yahara, 1999; Pope et al., 2000; Ono et al., 2001). Importantly, genetic studies have shown that mutations that abolish only severing or F-actin binding by ADF/cofilin cause abnormal actin assembly in *C. elegans* (Ono et al., 1999) or defects in actin turnover and viability in yeast (Lappalainen and

Drubin, 1997; Lappalainen et al., 1997), whereas a mutation that only impairs the depolymerizing activity causes no apparent phenotype in yeast (Moriyama and Yahara, 1999). In addition, ADF/cofilin is localized in lamellipodia of motile cells (Bamburg and Bray, 1987; Svitkina and Borisy, 1999) and is involved in increase in the number of free barbed ends at the leading edge (Chan et al., 2000). Thus, the severing activity of ADF/cofilin is crucial for its cellular function. However, the severing activity of purified ADF/cofilin is weak and could be due to a spontaneous breakage of the structurally distorted ADF/cofilin-bound filaments.

Recently, actin-interacting protein 1 (AIP1) has been characterized as a factor that rapidly disassembles ADF/cofilin-bound actin filaments. AIP1 is a conserved WD repeat protein, and was originally identified in yeast as one of several actin-interacting proteins from a two-hybrid screen (Amberg et al., 1995). AIP1 itself is a weak F-actin binding protein, whereas, in the presence of ADF/cofilin, binding of AIP1 to F-actin is enhanced and rapid disassembly of the filaments is induced (Aizawa et al., 1999; Okada et al., 1999; Rodal et al., 1999). The filament disassembly is based on severing rather than depolymerization from the ends (Aizawa et al., 1999; Okada et al., 1999). Genetic studies in yeast agree with the biochemical data. The temperature-sensitive lethality of a *COF1* (the yeast cofilin gene) allele is suppressed by a multicopy plasmid containing *AIP1* (Iida and Yahara, 1999). *AIP1* is not essential for viability, but a deletion of *AIP1* is synthetic lethal in combination with mutant *COF1* alleles (Iida and Yahara, 1999; Rodal et al., 1999). In addition, AIP1 has been implicated in stress response in *Physarum* (Matsumoto et al., 1998), early *Xenopus* development (Okada et al., 1999), response to acoustic damage in the avian auditory epithelium (Adler et al., 1999), and several actin-dependent processes in *Dictyostelium* (Konzok et al., 1999). However, in spite of these observations, the role of AIP1 in the regulation of actin cytoskeleton has not been clear because deletions of the AIP1 genes in yeast and *Dictyostelium* do not cause apparent phenotypes in the actin filament organization of the mutant cells (Iida and Yahara, 1999; Konzok et al., 1999; Rodal et al., 1999).

In this study, I demonstrate that the *C. elegans* *unc-78* gene encodes an AIP1 homologue and is required for actin filament organization in muscle cells. Mutations in *unc-78* cause accumulation of microfilaments in the muscle cells and slow movement of the mutant animals (Waterston et al., 1980; Zengel and Epstein, 1980), which has suggested that *unc-78* is important for myofibril organization. The results presented here provide the first genetic evidence that an AIP1-encoding gene is required for organized actin filament assembly in vivo.

Materials and Methods

Nematode Strains

Nematodes were grown at 20°C as described (Brenner, 1974). The wild-type strain is N2. *unc-78* alleles used are *e1217*, *e1221*, *st43* (provided by Drs. P. Hippe and B. Waterston, Washington University, St. Louis, MO; Waterston et al., 1980), *su135*, *su187* (provided by Dr. H. Epstein, Baylor College of Medicine, Houston, TX; Zengel and Epstein, 1980), and *gk27* (provided by Dr. E. Gilchrist, *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, Vancouver, Canada). The original strain VC34 carrying the *gk27* deletion was outcrossed four times with N2.

unc-60 (s1309) was provided by Dr. D. Baille (Simon Fraser University, Burnaby, Canada). All strains used in this study are homozygous for each allele unless specified.

Sequencing of Genomic DNA

Genomic DNA fragments for C04F6.4 were amplified from the total DNA with *Taq* DNA polymerase as two pieces: a 3.0-kb fragment (exons 1–3) and a 1.1-kb fragment (exons 4–6). All the exons were sequenced with an ABI PRIZM dye terminator cycle sequencing kit and an ABI310 genetic analyzer (Applied Biosystems). Sequencing was performed on at least two independent PCR products to insure that there were no PCR-induced errors.

Phylogenetic Analysis

Sequence alignment and phylogenetic analysis of AIP1 proteins were performed by MEGALIGN (DNASTAR Inc.) with the Clustal method, using the PAM250 matrix. Sequence data are available from GenBank/EMBL/DDBJ under the following accession numbers: human WDR1 (AF020056), mouse WDR1 (AF020055), chicken WDR1 (AF020054), *Xenopus* AIP1 (AF124140), *Drosophila* (AAF49822), *C. elegans* K08F9.2 (CAB03187), *Dictyostelium* DAIP1 (U36936), *Physarum* p66 (U86011), *Arabidopsis* (AAD14533), *Schizosaccharomyces pombe* (CAB11489), *Saccharomyces cerevisiae* AIP1 (U35666).

Motility Assay

A motility assay was performed as described (Epstein and Thomson, 1974) under specific conditions used previously (Ono et al., 1999). A *t* test was performed on the data of wild type and each mutant.

Phalloidin Staining

Nematodes were collected and washed with M9 buffer, fixed with 4% formaldehyde in 1x cytoskeleton buffer (10 mM MES-KOH, pH 6.1, 138 mM KCl, 3 mM MgCl$_2$, 2 mM EGTA) containing 0.32 M sucrose (Cramer and Mitchison, 1993) for 15 min, permeabilized with acetone at −20°C for 5 min, washed with PBS containing 0.5% Triton X-100 and 30 mM glycine (PBS-TG) for 10 min, and stained with 0.2 μg/ml tetramethylrhodamine-phalloidin (Sigma-Aldrich) in PBS-TG for 30 min. After washing with PBS-TG three times for 10 min each, they were mounted with ProLong Antifade (Molecular Probes).

Immunofluorescence Microscopy

Immunofluorescent staining of adult nematodes was performed as described (Finney and Ruvkun, 1990). Embryos were obtained by a hypochlorite treatment of gravid adults (Epstein et al., 1993), fixed with 4% formaldehyde in 1x cytoskeleton buffer containing 0.32 M sucrose for 15 min, permeabilized with methanol at −20°C for 5 min, washed with PBS-TG for 10 min, and stained with antibodies diluted in 1% BSA in PBS-TG. Primary antibodies used are antiactin monoclonal antibody (C4; ICN Biomedicals) and anti–UNC-60B (Ono et al., 1999). They were visualized by labeling with secondary antibodies, Alexa488-labeled goat anti–mouse IgG (Molecular Probes) and Cy3-labeled donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories), respectively.

Results and Discussion

The unc-78 Gene Encodes a Homologue of AIP1

The *unc-78* gene has been mapped to a position between *fax-1* and *sax-3* on the left arm of the X chromosome (Zallen et al., 1998; Much et al., 2000) (Fig. 1 a). Within this interval, a gene encoding a homologue of AIP1 (C04F6.4) has been predicted by the *C. elegans* Sequencing Consortium (Fig. 1 b). By sequencing the genomic DNA from five *unc-78* alleles, I identified sequence alterations in C04F6.4 (Fig. 1 c). Furthermore, *unc-78(gk27)*, a deletion allele of the C04F6.4 gene (Fig. 1 c), caused a similar but stronger Unc-78 phenotype than other *unc-78* alleles (Fig. 2) and failed to complement *unc-78(e1217)*. These results demonstrate that C04F6.4 is the *unc-78* gene.
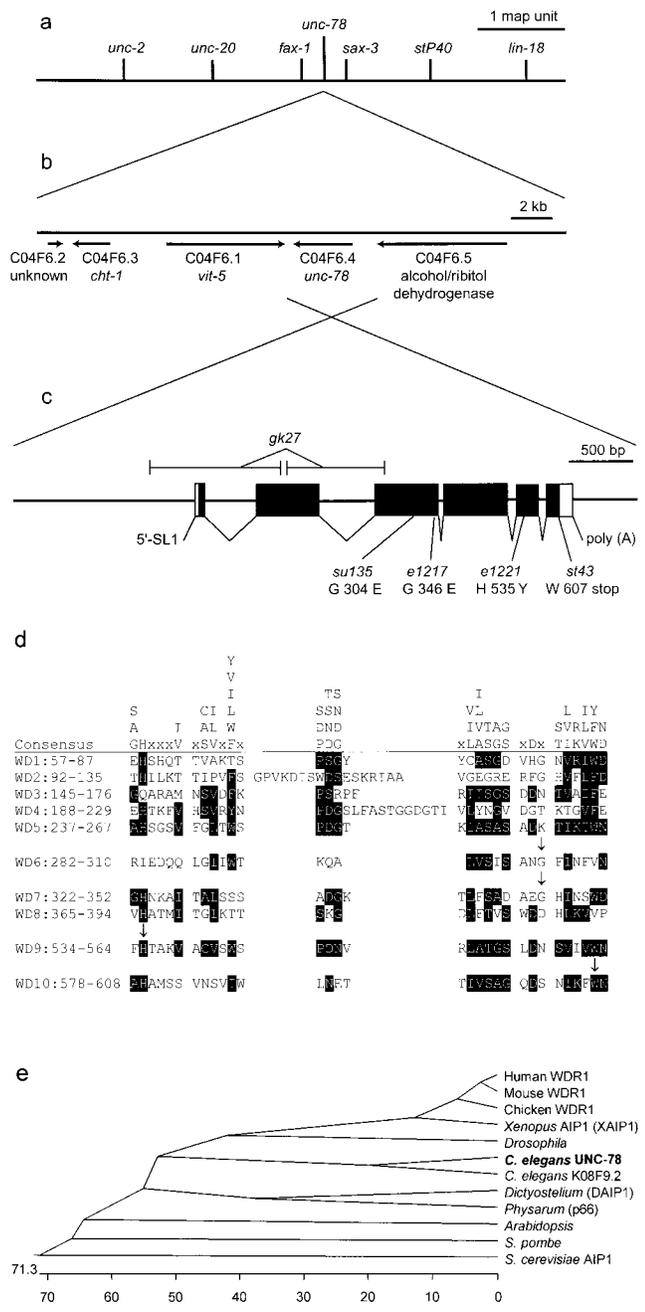
Figure 1. Genetic map and the structure of the *unc-78* gene. (a) A part of the left arm of the X chromosome. (b) Arrangement of genes in the cosmid C04F6 (total 25 kb) predicted by the *C. elegans* Sequencing Consortium. (c) Exon–intron structure of the *unc-78* gene (C06F6.4). Exons are indicated by boxes. The coding regions are represented by black filled regions. SL1 indicates a trans-splicing acceptor site. Locations of deletions and point mutations in *unc-78* alleles are shown. (d) Putative WD repeats in UNC-78. Residues that match the consensus sequence (Smith et al., 1999) are indicated by black boxes. Arrows indicate residues that are altered by *unc-78* mutations. (e) Phylogenetic analysis of AIP1 proteins. The scale shows the distance between sequences; units indicate the number of residue substitutions.

Figure 2. Motility defects by *unc-78* mutations. (a) Motility of adult wild-type and *unc-78* animals. *$p < 0.05$ by a *t* test. (b) Enhancement of the motility defect of *unc-60(s1309)* by the heterozygous *unc-78(gk27)* deletion. *unc-78(gk27)* was balanced by an X chromosome carrying *lon-2* as a recessive marker. As controls, all other animals used in this assay are heterozygous for *lon-2*. Values are the means ± SD; $n = 10$.

The sequence of a full-length cDNA clone yk185g6 (provided by Y. Kohara, National Institute of Genetics, Mishima, Japan) of UNC-78 was determined (sequenc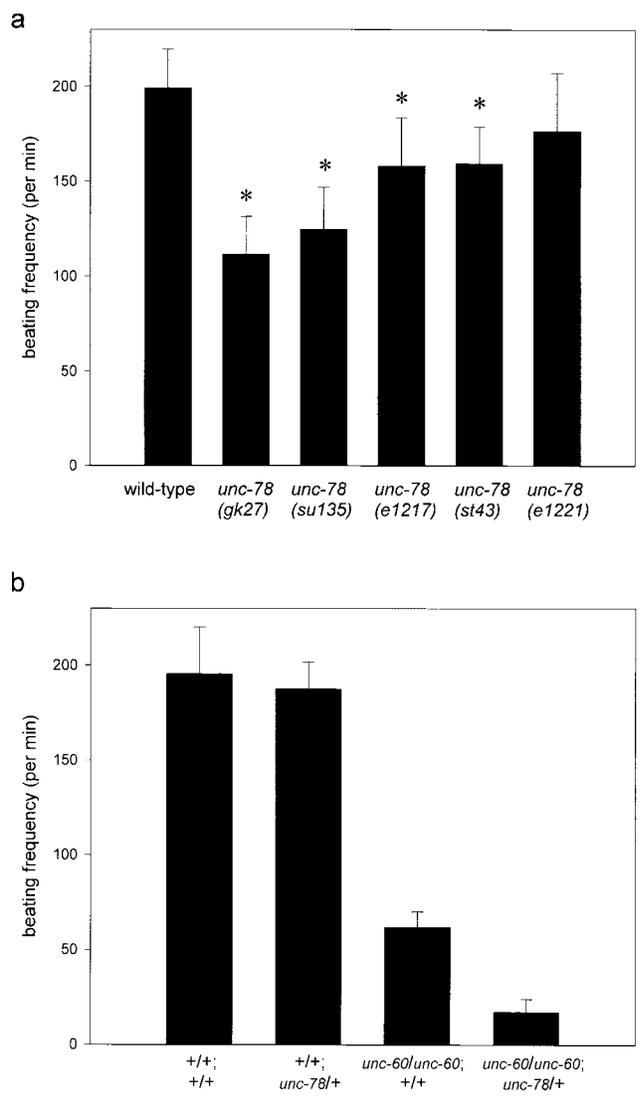e data available from GenBank/EMBL/DDBJ under accession number AF324437) to verify the predicted exon–intron structure by the Sequencing Consortium and characterize the 5′- and 3′-untranslated regions. The UNC-78 protein (611 amino acids, 65 kD) contains 10 putative WD repeats (Fig. 1 d) and is 22–67% identical to those of AIP1 proteins (Fig. 1 e). The point mutations in the *unc-78* alleles alter the sequence of WD repeats (Fig. 1, c and d): they are located in WD6 (G304E; *su135*), WD7 (G346E; *e1217*), WD9 (H535Y; *e1221* and *su187*), and WD10 (W607 to stop; *st43*). These mutations caused variable defects in motility of the homozygous animals (Fig. 2) in the order of *su135*, *e1217*, *st43*, and *e1221* from the strongest to weakest, although the defects were no worse than those of the deletion mutant, *unc-78(gk27)*. These residues are conserved not only in AIP1 proteins but also in WD repeats of other proteins (Smith et al., 1999), suggesting that the WD repeats are functionally important modules of UNC-78.
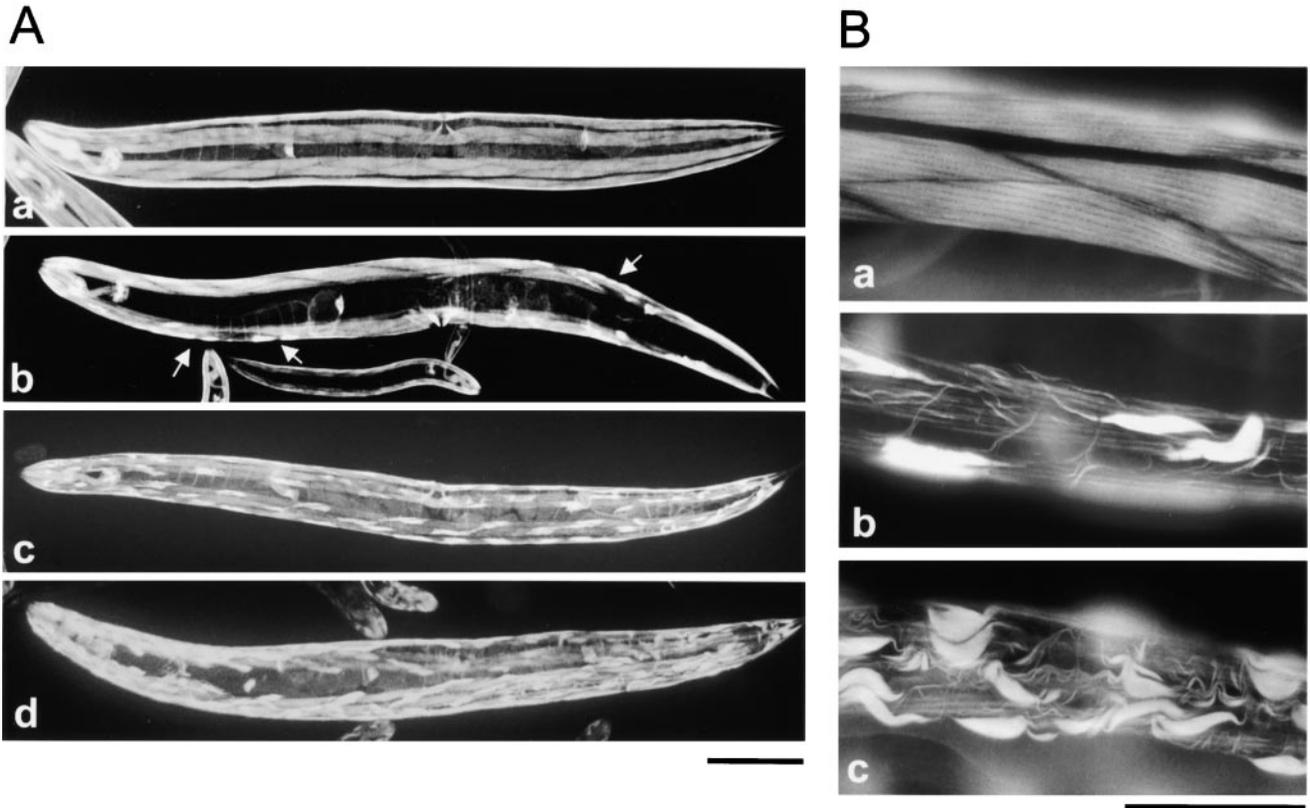
Figure 3. Actin filament organization in wild-type and *unc-78* mutants were visualized by staining adult animals with phalloidin. (A) Micrographs at a low magnification of wild type (a), *unc-78(e1221)* (b), *unc-78(e1217)* (c), and *unc-78(gk27)* (d). Arrows in b indicate cells that contain actin aggregates. The difference in the body size is due to slightly different growth stages. *unc-78* mutants were not significantly different in body size from wild type. (B) High magnification view of body wall muscle of wild type (a), *unc-78(e1217)* (b), and *unc-78(gk27)* (c). These micrographs show that actin filaments are disorganized in *unc-78* mutants and that *unc-78(gk27)* causes a more severe phenotype than *unc-78(e1217)*. Bars: (A) 0.1 mm; (B) 20 μm.

Searching through the *C. elegans* genome sequence, I found a second AIP1 isoform, K08F9.2, on chromosome V. This isoform is 67% identical in the amino acid sequence with UNC-78 and exhibits the highest homology with UNC-78 of all the AIP1 proteins (Fig. 1 e). So far, *C. elegans* is the only organism in which multiple AIP1 genes have been found. *Drosophila*, another multicellular organism for which we have a complete genome sequence, has only a single AIP1 gene (Goldstein and Gunawardena, 2000).

Unc-78 Mutations Disrupt Actin Filament Organization in Body Wall Muscle

Waterston et al. (1980) reported that *unc-78* mutants have large accumulations of thin filaments by electron microscopy. Phalloidin staining revealed that these aggregates contain F-actin (Fig. 3). In *unc-78(e1217)*, striated organization of actin filaments was disorganized and marked aggregates were found in most of the body wall muscle cells (Fig. 3 A, c), whereas, in a weak mutant, *unc-78(e1221)*, the phenotype appeared only in a subset of muscle cells, which randomly occurred throughout the body (Fig. 3 A, b, arrows). The deletion mutant, *unc-78(gk27)*, exhibited a more severe phenotype having more actin aggregates in muscle cells than *unc-78(e1217)* (Fig. 3 A, d). At the light microscopic level, the phenotype was detected only in the body wall muscle. However, the promoter region of the *unc-78* gene was active

not only in body wall muscle but also in the pharynx and the spermatheca (data not shown), suggesting that the second AIP1 gene has a redundant function in some tissues.

Under a higher magnification, the phenotype in *unc-78* mutants was more noticeable (Fig. 3 B). In *unc-78(e1217)*, actin filaments in the striated myofibrils were diminished, and large aggregates and small bundles were detected in the cytoplasm (Fig. 3 B, b), whereas most of the actin filaments were assembled in myofibrils in wild type (Fig. 3 B, a). *unc-78(gk27)* had more severe phenotype than other *unc-78* mutants: actin aggregates and small bundles were more numerous (Fig. 3 B, c).

The extent of the phenotype in actin organization (Fig. 3) correlates well with the severity of motility defects (Fig. 2). This strongly suggests that regulation of actin filament assembly by UNC-78 is an important mechanism in the formation of myofibrils that are able to execute effective contraction. Biochemical analysis of wild-type and mutant UNC-78 proteins should reveal how UNC-78 regulates actin filament dynamics and contributes to the development and maintenance of myofibrils.

UNC-60B (ADF/Cofilin) Is Mislocalized in unc-78 Mutants

Accumulation of actin aggregates in *unc-78* mutants resembles the phenotypes of ADF/cofilin mutants (*unc-60*)
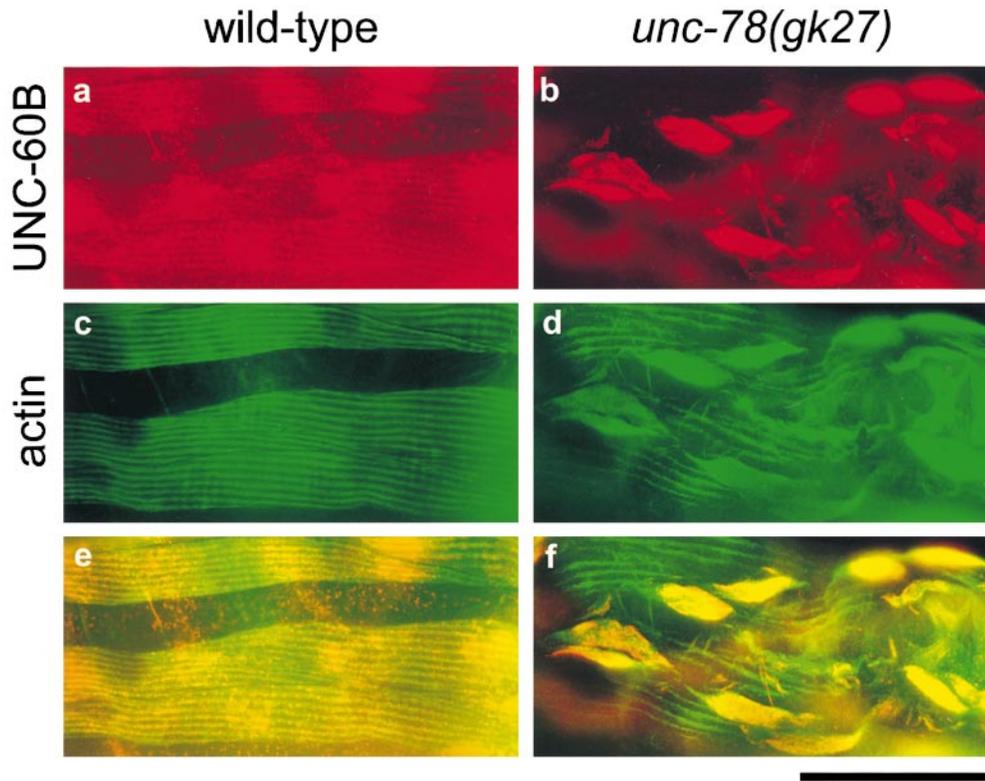
wild-type   *unc-78(gk27)*

UNC-60B

actin

Figure 4. Localization of UNC-60B and actin in adult body wall muscle. Wild type (a, c, and e) or *unc-78(gk27)* (b, d, and f) were double stained with anti–UNC-60B antibody (a and b) and anti-actin antibody (c and d). Merged images of a and c (e) and b and d (f) are shown. Staining of UNC-60B in wild type often results in an uneven cytoplasmic staining pattern (a), which probably reflects insufficient fixation of the protein in the diffuse cytoplasm. Bar, 20 μm.

(Waterston et al., 1980; Ono et al., 1999), suggesting that the two genes are involved in the same genetic pathway. Therefore, I tested for a genetic interaction between *unc-78* and *unc-60*. The *unc-60(s1309)* mutation reduces the actin-depolymerizing activity of UNC-60B, a muscle-specific ADF/cofilin isoform, and is homozygous viable (Ono, et al., 1999). However, the *unc-60(s1309);unc-78(gk27)* homozygotes were lethal at a late larval stage. In addition, heterozygous deletion of *unc-78* strongly enhanced the motility defect of *unc-60(s1309)* (Fig. 2 b). This genetic interaction suggests that ADF/cofilin and AIP1 function together in muscle cells.

The localization of cofilin is altered in AIP1-null yeast cells (Iida and Yahara, 1999; Rodal et al., 1999), but this is not the case in AIP1-null *Dictyostelium* cells (Konzok et al., 1999). In *C. elegans*, the localization of UNC-60B was altered in *unc-78* mutants (Fig. 4). In wild type, UNC-60B was localized in the diffuse cytoplasm and to the myofibrils (Ono et al., 1999) (Fig. 4 a). However, in *unc-78(gk27)*, the majority of UNC-60B was concentrated in the actin aggregates, and its diffuse and myofibrillar localization was reduced (Fig. 4 b). It should be noted that UNC-60B was associated with only part of the regions in the actin aggregates, which were often in the central core of the aggregates (Fig. 4 f). This may be due to the cooperative binding of UNC-60B with F-actin (Ono et al., 2001).

UNC-60B was mislocalized in *unc-78* mutants during embryonic stages (Fig. 5) when myofibril assembly is very active. Accumulation of actin into striated myofibrils becomes evident after the threefold stage (∼520 min after the first cleavage) (Hresko et al., 1994) (Fig. 5 c). UNC-60B was mostly localized in the diffuse cytoplasm (Fig. 5 a). In *unc-78(gk27)* embryos, actin was assembled into myofibrils in a normal pattern, but slightly dense accumula-

tions were formed along the myofibrils (Fig. 5 d, arrows). Remarkably, the localization of UNC-60B in the mutant was predominant in aggregated forms (Fig. 5 b). Some aggregates of UNC-60B were colocalized with the actin accumulations (Fig. 5, b and f). However, many of them were associated with actin in the myofibrils without aggregates of actin, implying that these are the sites where abnormal actin filaments are built up later in development. These mutant phenotypes suggest that the role of UNC-78 is to disassemble UNC-60B–bound actin filaments, prevent formation of the aggregates, and maintain the dynamic state of their interactions.

Conclusion

The results presented here demonstrate that UNC-78 is required for organized assembly of actin filaments into myofibrils and proper localization of UNC-60B in body wall muscle cells. This is the first genetic evidence that AIP1 regulates actin filament organization in vivo. In yeast and slime mold, AIP1-null cells do not show apparent disorganization of the actin cytoskeleton (Iida and Yahara, 1999; Konzok et al., 1999; Rodal et al., 1999), although AIP1-null *Dictyostelium* cells are partially defective in cytokinesis, endocytosis, phagocytosis, and motility, which require actin dynamics (Konzok et al., 1999). Since myofibrils are highly organized forms of actin cytoskeleton, the processes of assembly and maintenance probably require tightly regulated actin filament dynamics, in which UNC-78/AIP1 is a critical factor.

Mislocalization of UNC-60B to actin aggregates in *unc-78* mutants suggests that UNC-60B requires the activity of UNC-78 to enhance actin filament turnover. AIP1 and ADF/cofilin cooperatively disassemble actin filaments by severing in vitro (Introduction). UNC-60B itself is able to
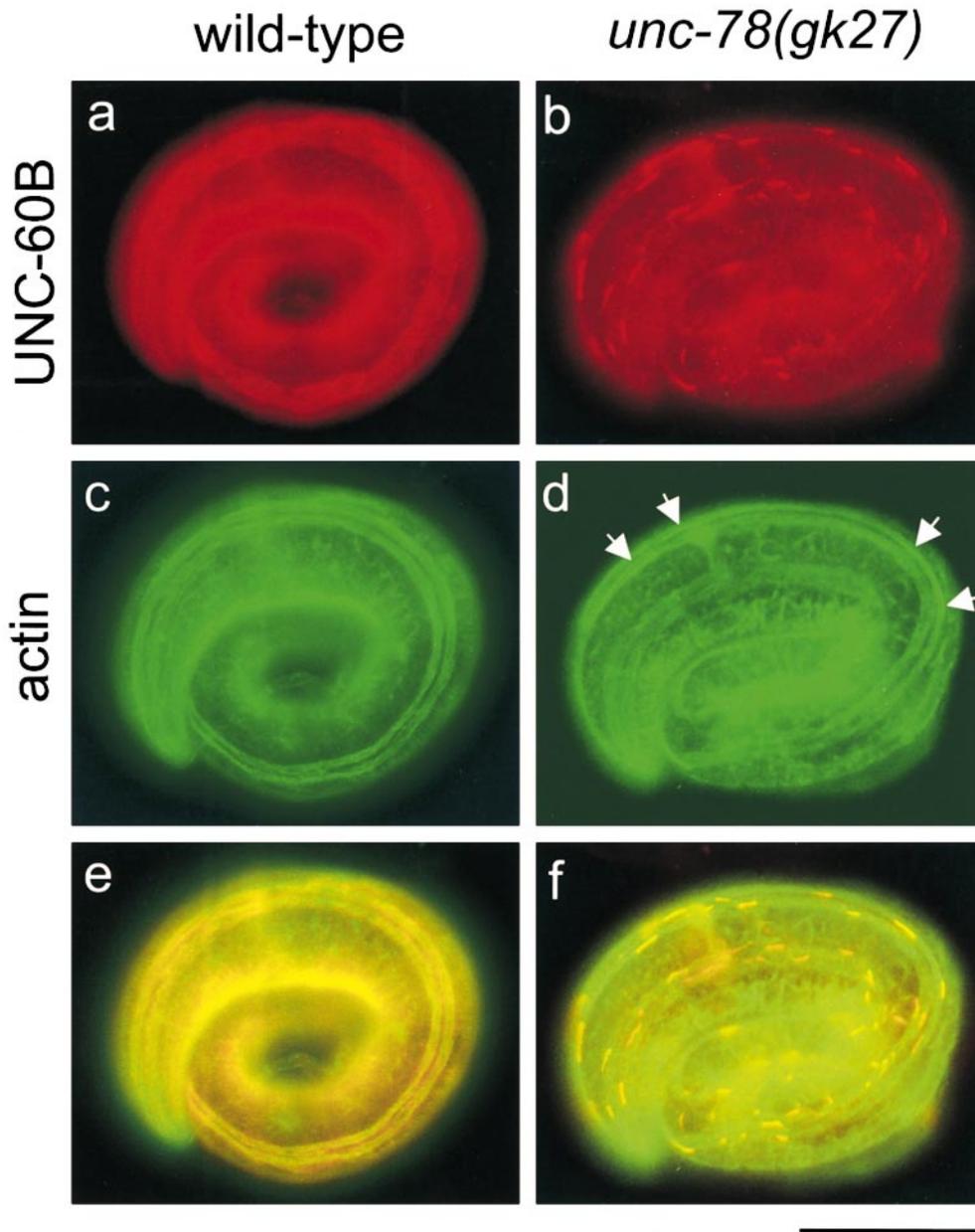
wild-type   *unc-78(gk27)*

UNC-60B

a   b

actin

c   d

e   f

Figure 5. Localization of UNC-60B and actin in embryos. Wild type (a, c, and e) or *unc-78(gk27)* (b, d, and f) were double stained with anti–UNC-60B antibody (a and b) and antiactin antibody (c and d). Merged images of a and c (e) and b and d (f) are shown. Arrows in d indicate slightly dense accumulations of actin. Bar, 10 μm.

depolymerize and sever actin filaments (Ono and Benian, 1998; Ono et al., 1999), but these activities are very weak. Therefore, aggregated forms of actin and UNC-60B in *unc-78* mutants might be a consequence of insufficient actin turnover in the absence of the UNC-78 activity. Biochemical and cell biological analyses of wild-type and mutant UNC-78 will be needed to further characterize specific functions of UNC-78/AIP1 in the regulation of actin filament assembly.

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