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Actin-ADF/cofilin rod formation in *Caenorhabditis elegans* muscle requires a putative F-actin binding site of ADF/cofilin at the C-terminus

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Abstract

Under a number of stress or pathological conditions, actin and actin depolymerizing factor (ADF)/cofilin form rod-like structures that contain abnormal bundles of actin filaments that are heavily decorated with ADF/cofilin. However, the mechanism of actin rod formation and the physiological role of actin rods are not clearly understood. Here, we report that overexpression of green fluorescent protein-fused UNC-60B, a muscle-specific ADF/cofilin isoform, in *Caenorhabditis elegans* body wall muscle induces formation of rod-like structures. The rods contained GFP-UNC-60B, actin-interacting protein 1 (AIP1), and actin, but not other major actin-associated proteins, thus resembling actin-ADF/cofilin rods found in other organisms. However, depletion or overexpression of AIP1 did not affect formation of the actin-GFP-UNC-60B rods, suggesting that AIP1 does not play a significant role in the rod assembly. Truncation of the C-terminal tail, a putative F-actin binding site, of UNC-60B abolished induction of the rod formation, strongly suggesting that stable association of UNC-60B with F-actin, which is mediated by its C-terminus, is required for inducing actin-ADF/cofilin rods. This study suggests that *C. elegans* can be a new model to study functions of actin-ADF/cofilin rods.

Keywords

Actin filaments; nematode; myofibrils; stress; overexpression

Introduction

Abnormal aggregates or rigid rod-like bundles of actin filaments are often found in cells and tissues under a variety of pathological conditions (Clarkson et al. 2004; Fechheimer et al. 2002; Hirano 1994; Laing 2007). In some cases, mutations of actin are associated with formation of actin aggregates or rods (Bathe et al. 2007; Costa et al. 2004; Domazetovska et al. 2007; Procaccio et al. 2006), suggesting that alterations in the biochemical properties and/or structure of actin itself cause these abnormalities. However, in the absence of actin mutations, mutations in other genes or environmental changes can also cause formation of actin aggregates or rods perhaps by different mechanisms. Thus, actin aggregates and rods represent one aspect of cellular dysfunctions, but their physiological significance is not clearly understood.

A number of stress conditions, including heat shock, oxidative stress, and ATP depletion, induce formation of intranuclear or cytoplasmic actin rods that are heavily decorated by actin depolymerizing factor (ADF)/cofilin (Ashworth et al. 2003; Bamburg and Wiggan

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2002; Minamide et al. 2000; Nishida et al. 1987; Ono et al. 1993). Actin-ADF/cofilin rods are also formed under neurodegenerative conditions (Fulga et al. 2007; Maloney et al. 2005; Minamide et al. 2000). These rods disturb functions of neurites and synapses in neurons (Jang et al. 2005; Minamide et al. 2000). On the other hand, stabilization of actin in the rods preserves consumption of ATP for actin turnover and may serve as a transient defense mechanism against stress (Bernstein et al. 2006). ADF/cofilin not only severs and depolymerizes actin filaments but also stays bound to the side of actin filaments at high concentrations (Ono 2007). Microinjection or overexpression of ADF/cofilin is sufficient to induce formation of rods (Jang et al. 2005; Nagaoka et al. 1995; Ono et al. 1996). Mammalian ADF/cofilin can be inactivated by phosphorylation of Ser-3, and its dephosphorylation under stress is one mechanism to activate ADF/cofilin and induce rod formation (Huang et al. 2008; Ohta et al. 1989). However, dephosphorylation of ADF/cofilin is not necessarily associated with rod formation in some cell types (Abe et al. 1993; Saito et al. 1994), and regulation of ADF/cofilin during rod formation by other mechanism, possibly by direct binding by phosphoinositides, has been proposed (Hosoda et al. 2007).

The body wall muscle of the nematode *Caenorhabditis elegans* is striated muscle and has been used as a model to study structure and function of sarcomeric proteins (Moerman and Fire 1997; Waterston 1988). Mutations in UNC-60B, a muscle-specific ADF/cofilin isoform, cause disorganization of sarcomeric actin filaments and formation of actin aggregates (Ono et al. 2003; Ono et al. 1999). This phenotype is similar to actin accumulations found in human nemaline myopathy that is caused by a mutation of muscle-type cofilin (cofilin-2) (Agrawal et al. 2007). However, these actin aggregates are morphologically different from stress-induced actin rods. In this study, we found that overexpression of GFP-tagged UNC-60B in the *C. elegans* body wall muscle induces formation of actin rods that resemble stress-induced actin-ADF/cofilin rods in mammalian cells. These rods contained GFP-UNC-60B, UNC-78 (actin-interacting protein 1/AIP1), and actin but not other major actin-associated proteins. Using this system, we demonstrated that AIP1 is not required for rod formation and that a putative F-actin binding site at the C-terminus of UNC-60B is required for rod formation, indicating that F-actin binding by ADF/cofilin plays a primary role in assembly of actin rods. Thus, the results suggest that *C. elegans* has a conserved mechanism to assemble actin-ADF/cofilin rods and could be used as a new model to study mechanisms of pathological cytoskeletal reorganization.

Materials and methods

C. elegans Strains and Culture

Wild-type *C. elegans* strain N2 was obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). *unc-60B(su158)* (an *unc-60B*-null mutant) was described previously (Ono et al. 2003; Zengel and Epstein 1980). Transgenic expression of GFP-tagged UNC-60B in the body wall muscle was driven by the *myo-3* promoter (Okkema et al. 1993). Plasmid vectors for expressing GFP-tagged UNC-60B(WT), UNC-60B(Δ 150), and UNC-60B(Δ 152) under the control of the *myo-3* promoter were described previously (Ono et al. 2008). The plasmid vectors at 20 μ g/ml were mixed with 80 μ g/ml pET-32a (Novagen) as carrier DNA and injected into the distal arm of the hermaphroditic gonad as described (Mello and Fire 1995). Transgenic expression of GFP-UNC-78 was driven by the *unc-78* promoter as described (Mohri et al. 2006), and the vector plasmid was co-injected with pRF6 that contains a dominant *rol-6* marker into wild-type background. Transformants were selected by expression of GFP as observed by epifluorescence, and the transgenes maintained as extrachromosomal arrays (Mello et al. 1991). Nematodes were maintained under standard conditions at 20°C (Brenner 1974).

Fluorescence Microscopy

To observe GFP signals in live worms, they were anesthetized in 0.1% tricaine, 0.01% tetramisolein M9 buffer for 30 min and mounted on 2% agarose pads. Staining of whole animals with tetramethylrhodamine-phalloidin (Sigma-Aldrich, St. Louis, MO) was performed as described previously (Ono 2001). For immunofluorescent staining of adult body wall muscle, adult worms were cut in halves near the vulva by needles on poly-lysine-coated slides, permeabilized by a freeze-crack method (Epstein et al. 1993), and fixed with methanol at -20°C for 5 min. Additional fixation was applied for staining vinculin or α -actinin by acetone at -20°C for 5 min, or for staining tropomyosin by 4% paraformaldehyde in cytoskeleton buffer (10 mM MES-KOH, pH 6.1, 138 mM KCl, 3 mM MgCl_2 , 2 mM EGTA) containing 0.32 M sucrose for 20 min at room temperature. The samples were washed with phosphate-buffered saline (PBS) and incubated with a primary antibody, which is then visualized by Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories), Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories), or Alexa555-conjugated goat anti-guinea pig IgG (Invitrogen). Primary antibodies used are mouse anti-actin monoclonal antibody C4 (MP Biomedicals), rabbit anti-UNC-78 antibody (Mohri and Ono 2003), mouse anti-MYO-3 monoclonal antibody 5–6 (Miller et al. 1983), mouse anti- α -actinin monoclonal antibody MH40 (Francis and Waterston 1985), mouse anti-vinculin monoclonal antibody MH24 (Francis and Waterston 1985), guinea pig anti-*C. elegans* tropomyosin antibody (Ono and Ono 2002), mouse anti-*C. elegans* kettin monoclonal antibody MH44 (Francis and Waterston 1985; Ono et al. 2006), rabbit anti-TMD-1 antibody (Stevenson et al. 2007), guinea pig anti-UNC-87 antibody (Yamashiro et al. 2007).

Samples were viewed by epifluorescence using a Nikon Eclipse TE2000 inverted microscope with a 40 or 60x CFI Plan Fluor objective. Images were captured by a SPOT RT monochrome charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI) and processed by the IPLab imaging software (Scanalytics, Rockville, MD) and Adobe Photoshop CS2 (Adobe Systems, Mountain View, CA).

RNA Interference Experiments

RNAi against *unc-78* was performed by feeding with *E. coli* expressing double stranded RNA (MRC GeneService clone X-2A18) as described previously (Ono and Ono 2002). L4 larvae were treated with RNAi, and phenotypes were characterized in the F1 generation.

Western Blot

Thirty adult worms were picked and lysed in 20 μl of SDS-lysis buffer (2% SDS, 80 mM Tris-HCl, 5% β -mercaptoethanol, 15% glycerol, and 0.05% bromophenol blue, pH 6.8), heated at 97°C for 2 min, homogenized by brief sonication, and heated again at 97°C for 2 min. The samples were resolved by SDS-PAGE by using a 12% acrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). The membrane was blocked in 5% nonfat milk in phosphate-buffered saline containing 0.1% Tween 20 for 30 min and incubated with anti-UNC-60B antibody (Ono et al. 1999) or anti-UNC-78 antibody (Mohri and Ono 2003) for 1 h followed by treatment with peroxidase-labeled goat anti-rabbit IgG (Pierce Chemical, Rockford, IL). The reactivity was detected with a SuperSignal West Pico chemiluminescence reagent (Pierce Chemical). The membrane was treated with a buffer containing 2% SDS, 100 mM β -mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8, at 50°C for 30 min to remove bound probes and reprobed with mouse monoclonal anti-actin antibody (C4; MP Biomedicals, Irvine, CA) as a loading control. To determine relative amounts of GFP-UNC-60B and endogenous UNC-60B, the films were scanned by a UMAX Powerlook III scanner at 300 dots per inch, and band intensity was densitometrically quantified by Image J.

Results

Overexpression of UNC-60B ADF/cofilin Induces Formation of Actin-UNC-60B Rods in *C. elegans* Muscle

To determine effects of overexpression of ADF/cofilin in the *C. elegans* body wall muscle, UNC-60B, a muscle-specific *C. elegans* ADF/cofilin isoform (McKim et al. 1994), was fused with green fluorescent protein (GFP) and expressed under the control of the *myo-3* promoter that strongly drives expression of a transgene in the body wall muscle (Okkema et al. 1993). Western blot analysis showed that GFP-UNC-60B (40 kDa) was expressed in the transgenic animals in addition to the endogenously expressed UNC-60B (18 kDa) and that the level of endogenous UNC-60B is not significantly different from control animals (Fig. 1A). Densitometric analysis indicated that the transgenic animals had endogenous UNC-60B and GFP-UNC-60B at a 1:1.7 ratio.

Overexpression of GFP-UNC-60B in wild-type background caused formation of rod-like structures that contain GFP-UNC-60B (Fig. 1C–J). In living worms without any fixation, GFP-UNC-60B predominantly localized to rod-like structures in the cytoplasm (Fig. 1C and D) and weakly localized in a striated manner (Fig. 1C, arrowheads). By immunofluorescent staining of fixed worms by anti-actin antibody, actin was co-localized with GFP-UNC-60B to the rod-like structures (Fig. 1E–G, arrowheads) and also incorporated in striated myofibrils (Fig. 1F). However, tetramethylrhodamine-phalloidin, a probe for F-actin, only stained striated myofibrils but not GFP-UNC-60B-containing rods (Fig. 1H–J). These are very similar to the characteristics of actin-ADF/cofilin rods that are formed in various cell types in other organisms. ADF/cofilin and phalloidin binds to actin in a mutually exclusive manner (Nishida et al. 1987; Yonezawa et al. 1988). As actin filaments in the rods are heavily decorated by ADF/cofilin, actin in the rods is poorly stained by fluorescently labeled phalloidin (Nishida et al. 1987; Ono et al. 1996). Therefore, in *C. elegans* muscle cells, overexpression of UNC-60B (ADF/cofilin) is sufficient to induce formation of actin-ADF/cofilin rods without any stress. It should be noted that the striated actin organization was not disrupted by overexpression of GFP-UNC-60B (Fig. 1F and I). This is similar to previous reports that induction of actin-ADF/cofilin rods in vertebrate muscle cells did not cause major disorganization of sarcomeric actin filaments (Abe et al. 1993; Nagaoka et al. 1995). This is perhaps due to a stable nature of sarcomeric actin filaments, which are associated with F-actin stabilizers including tropomyosin (Bernstein and Bamberg 1982; Ono and Ono 2002; Yu and Ono 2006).

Actin-interacting protein 1 (AIP1) cooperates with ADF/cofilin and promotes actin filament disassembly (Ono 2003) and is the only other known component of actin-ADF/cofilin rods. However, the presence of AIP1 in actin-ADF/cofilin rods has been demonstrated only in *Dictyostelium* (Aizawa et al. 1999). We found that UNC-78 (an AIP1 protein in *C. elegans* body wall muscle) localized to the actin-GFP-UNC-60B rods in *C. elegans* muscle (Fig. 2A–C). In the body wall muscle of wild-type worms, UNC-78 is present in the diffuse cytoplasm and also localizes to actin filaments in the striated myofibrils (Mohri et al. 2006; Mohri and Ono 2003). When GFP-UNC-60B was overexpressed (Fig. 2A), UNC-78 was concentrated in the rods (Fig. 2B) and co-localized with GFP-UNC-60B (Fig. 2C). These results strongly suggest that AIP1 is a conserved component of actin-ADF/cofilin rods.

We further examined localization of other actin-associated proteins, but none of them localized to the actin-UNC-60B rods (Fig. 2D–X). The following proteins did not localize to the rods: myosin heavy chain A (MYO-3) (Fig. 2D–F), α -actinin (ATN-1) (Fig. 2G–I), vinculin (DEB-1) (Fig. 2J–L), tropomyosin (LEV-11) (Fig. 2M–O), kettin (KETN-1) (Fig. 2P–R), tropomodulin (UNC-94/TMD-1) (Fig. 2S–U), and a calponin-like protein (UNC-87)

(Fig. 2V–X). It should also be noted that localization of these myofibrillar proteins was not affected by overexpression of GFP-UNC-60B (Fig. 2).

AIP1 Does Not Play A Significant Role in Actin-ADF/cofilin Rod Formation

Although AIP1 localizes to the actin-ADF/cofilin rods, role of AIP1 in the actin-ADF/cofilin rod formation is not understood. To determine if UNC-78 (AIP1 in *C. elegans* muscle) is required for the rod formation, we initially attempted to examine rod formation by expressing GFP-UNC-60B in an *unc-78* null mutant background (Ono 2001). However, the actin filaments were severely disorganized in the absence of UNC-78 and accumulated in aggregates where GFP-UNC-60B was also accumulated (our unpublished data). Although the rod formation was significantly reduced in the *unc-78* mutant (our unpublished data), we were not able to determine if UNC-78 has a critical role in rod formation, or this reduction is caused by a secondary effect of disorganized actin filaments.

We then tested whether depletion of UNC-78 by RNA interference affects formation of the actin-GFP-UNC-60B rods. *unc-78(RNAi)* resulted in 80 % reduction in the UNC-78 protein level (Fig. 3A), and thus the conditions were less severe than the complete loss of UNC-78 in the null mutant. UNC-78 was significantly reduced in the body wall muscle as observed by immunofluorescence microscopy (Fig. 3, compare C and D). Nonetheless, actin-GFP-UNC-60B rods were formed similarly in control (100 %: 300/300 positive for rods) and *unc-78(RNAi)* (99.3 %: 298/300 positive for rods) worms (Fig. 3, E and F). These results suggest that UNC-78 (AIP1) is not required for formation of the actin-ADF/cofilin rods.

Furthermore, we found that overexpression of GFP-UNC-78 in wild-type background did not induce actin-UNC-60B rod formation (Fig. 4). Western blot analysis showed that GFP-UNC-78 (90 kDa) was expressed in the transgenic worms in addition to the endogenously expressed UNC-78 (65 kDa) (Fig. 4A). Densitometric analysis indicated that the transgenic animals had endogenous UNC-78 and GFP-UNC-78 at a 1:1.9 ratio. GFP-UNC-78 localized to the actin filaments and the diffuse cytoplasm and did not form rod-like structures or aggregates (Fig. 4C). Immunofluorescent staining of UNC-60B and actin did not show rod-like structures (0/300 positive for rods) (Fig. 4D and E). Therefore, although AIP1 is a conserved component of actin-ADF/cofilin rods, it is not likely to be a critical factor for rod formation.

The C-terminal Tail of UNC-60B Is Required for Actin-UNC-60B Rod Formation

ADF/cofilin exhibits complex actin-regulating activities (Ono 2007), and the mechanism of actin-ADF/cofilin rod formation is not clearly understood. One of the critical determinants of the ADF/cofilin activity is the putative F-actin-binding site at the C-terminus. Truncation of one to three C-terminal amino acids (C-terminal tail) of UNC-60B eliminates its stable binding to F-actin and significantly weakens actin severing activity, but does not affect its G-actin binding activity (Ono et al. 2008; Ono et al. 2001). These amino acids are also required for its cooperation with UNC-78 (AIP1) (Mohri and Ono 2003). Therefore, we tested whether the C-terminal tail of UNC-60B is involved in rod formation. UNC-60B(Δ 150) lacks three C-terminal amino acids and show no detectable actin severing activity, while UNC-60B(Δ 152) lacks one C-terminal amino acid and has weaker actin severing activity than UNC-60B(WT) (Ono et al. 2008; Ono et al. 2001).

We generated transgenic worm strains that overexpress GFP-UNC-60B(Δ 150) or GFP-UNC-60B(Δ 152) (Fig. 5A and B). Western blot analysis and quantification of band intensity indicated that ratios of endogenous UNC-60B to GFP-UNC-60B(Δ 150) (1:1.4) and GFP-UNC-60B(Δ 152) (1:1.6) were comparable to that of endogenous UNC-60B to GFP-UNC-60B(WT) (1:1.7) (see Fig. 1A and B).

The C-terminal tail of UNC-60B was indeed required for rod formation in *C. elegans* muscle (Fig. 5C–E). When GFP-UNC-60B(Δ 150) was overexpressed, it did not induce rod formation (0/100 positive for rods) (Fig. 5D) while GFP-UNC-60B(WT) induced rods in 100 % of examined worms (100/100 positive for rods) (Fig. 5A). Instead, GFP-UNC-60B(Δ 150) localized to the diffuse cytoplasm in a somewhat ambiguous striated pattern and the nucleus except for a small spot which is most likely the nucleolus (Fig. 5D, asterisk). GFP-UNC-60B(Δ 152) also showed a similar localization pattern to GFP-UNC-60B(Δ 150) and did not induce rod formation (0/100 positive for rods) (Fig. 5E). These results strongly suggest that stable association of UNC-60B with F-actin, which is mediated by the C-terminal tail of UNC-60B, is required for actin-UNC-60B rod formation.

Discussion

In this study, we demonstrated that overexpression of GFP-UNC-60B in the nematode *C. elegans* muscle induces formation of actin-UNC-60B rods. These rods were similar to actin-ADF/cofilin rods that are found in other organisms based on the following observations: (1) the rods contained UNC-60B (ADF/cofilin), UNC-78 (AIP1), and actin but not other major actin-associated proteins, and (2) the rods were stained by anti-actin antibody but not by fluorescently labeled phalloidin. The actin-UNC-60B rods are clearly different from the actin aggregates that are formed when UNC-60B is defective, as these aggregates contain tropomyosin, tropomodulin, and a calponin-like protein (Ono and Ono 2002; Yamashiro et al. 2008; Yamashiro et al. 2007). Thus, these results strongly suggest that *C. elegans* has a conserved mechanism to assemble actin-ADF/cofilin rods and is a good model to investigate functions of actin-ADF/cofilin rods.

We found that UNC-78 (AIP1) does not play a critical role in actin-ADF/cofilin rod formation. This is a rare example in which ADF/cofilin and AIP1 do not cooperate. AIP1 is conserved among eukaryotes and promotes disassembly of actin filaments in the presence of ADF/cofilin (Ono 2003). Thus, ADF/cofilin and AIP1 appear to be intimate partners in the regulation of actin dynamics and are involved in common events such as cell migration, cytokinesis, and myofibril assembly (Ono 2003). *In vitro* studies showed that ADF/cofilin binds to F-actin independent of AIP1, but that AIP1 requires ADF/cofilin to bind to F-actin (Aizawa et al. 1999; Mohri and Ono 2003; Okada et al. 1999; Rodal et al. 1999). Mutational studies identified specific surface residues that are required for the interaction among ADF/cofilin, AIP1, and actin (Clark and Amberg 2007; Clark et al. 2006; Mohri et al. 2004; Okada et al. 2006). These studies suggest that AIP1 can efficiently interact with F-actin only after ADF/cofilin binds to F-actin. Therefore, heavy decoration of F-actin by ADF/cofilin might be primarily important for rod formation, but association of AIP1 with the rods might be simply a secondary result of the dense concentration of ADF/cofilin-decorated actin filaments.

We showed that the C-terminal tail of UNC-60B is required for inducing actin-UNC-60B rods. The C-terminal tail of UNC-60B is critical for its stable association with F-actin but not with G-actin *in vitro* (Ono et al. 2001), suggesting that this region mediates rigid association of UNC-60B with actin filaments in the rods *in vivo*. Although the C-terminally truncated UNC-60B variants as GFP-fusion proteins did not induce formation of actin rods, the same recombinant proteins are biologically active in regulating actin organization in the somatic gonad where weaker actin-severing activity of ADF/cofilin is favored (Ono et al. 2008). This indicates that their inability to induce rods is attributed to the specific role of the C-terminal tail of UNC-60B in F-actin binding but not due to total loss of actin-binding or interference by the GFP moiety. The F-actin binding site (F-site) of ADF/cofilin also includes the F-loop that is spatially close to the C-terminus (Lappalainen et al. 1997; Pope et al. 2000). Structural analysis of human cofilin suggest that a change in pH alters the

conformation of the F-site (Frantz et al. 2008; Pope et al. 2004) and modulates F-actin binding and severing activities (Bernstein et al. 2000; Hawkins et al. 1993; Hayden et al. 1993; Yonezawa et al. 1985). Therefore, it should be interesting to examine if intracellular pH affects the F-site and influences actin-ADF/cofilin rod formation.

This work is the first demonstration that actin-ADF/cofilin rods can be induced in *C. elegans*. However, it still needs to be investigated whether any stress or disease conditions can induce actin-ADF/cofilin rods with an endogenous level of ADF/cofilin in *C. elegans*. We used GFP-actin and preliminarily tested whether heat shock (30 – 37 °C for 1 hr) or DMSO (10 – 20 % for 1 hr) can induce actin rods, but found no evidence of rod formation. Therefore, the activity of UNC-60B might be negatively regulated in *C. elegans* cells, and activation of UNC-60B might be required for rod formation. In mammalian cells, activation of ADF/cofilin by dephosphorylation induces rod formation upon ATP depletion (Huang et al. 2008). However, *C. elegans* does not have a homolog of mammalian LIM-kinases or testicular protein kinases, and the presence of a phospho-regulatory mechanism for ADF/cofilin in *C. elegans* is currently unknown. Phosphorylation-independent regulation of ADF/cofilin involving direct binding by PIP2 might also be involved in activation of ADF/cofilin during rod formation (Hosoda et al. 2007; van Rheenen et al. 2007; Yonezawa et al. 1990). Further analyses of actin-ADF/cofilin rod formation should help to understand the mechanism of pathological actin reorganization and physiological roles of actin-ADF/cofilin rods.

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Fig. 1.

Formation of actin rods in the *C. elegans* body wall muscle by overexpression of GFP-UNC-60B. (A and B) Transgenic overexpression of GFP-UNC-60B in *C. elegans*. Worm lysates from wild-type, an *unc-60B*-null mutant (as a negative control), and a GFP-UNC-60B(WT)-expressing strains in wild-type background were subjected to Western blot using anti-UNC-60B (A) or anti-actin antibody (B). Positions of molecular weight markers are shown on the left of A. (A) Bands at ~18 kDa are endogenously expressed UNC-60B as this band is absent in the *unc-60B*-null mutant. In the transgenic strain, an additional 40 kDa band of GFP-UNC-60B was detected. (C and D) Localization of GFP-UNC-60B in the body wall muscle of live worms without any fixation. GFP-UNC-60B predominantly localized to needle-like rods. Faint striated localization was sometimes observed (C, arrowheads). (E–J) Fixed worms were stained with anti-actin antibody (F) or tetramethylrhodamine-phalloidin (I). Co-localization of GFP-UNC-60B and actin in rods was observed by staining with anti-actin antibody (E and F, arrowheads). However, phalloidin did not detect rods (I). Merged images of GFP-UNC-60B (green) and anti-actin or phalloidin (red) are shown in G and J. Bar, 10 μm.

Fig. 2.

Investigation of other components of actin-GFP-UNC-60B rods. GFP-UNC-60B- expressing worms were stained for UNC-78 (AIP1) (B), myosin heavy chain A (MYO-3) (E), α -actinin (ATN-1) (H), vinculin (DEB-1) (K), tropomyosin (LEV-11) (N), Ce-kettin (Q), tropomodulin (UNC-94/TMD-1) (T), or UNC-87 (W). Localization of GFP-UNC-60B is shown in the left column (A, D, G, J, M, P, S, and V). Merged images of GFP-UNC-60B (green), actin associated proteins (red) are shown in the right column (C, F, I, L, O, R, U, and X). Only UNC-78 co-localized with GFP-UNC-60B to the rods (B). Bar, 10 μ m.

Fig. 3. Knockdown of UNC-78 does not affect actin-GFP-UNC-60B rod formation. Worm lysates from control RNAi-treated and *unc-78(RNAi)*-treated GFP-UNC-60B-expressing strains were subjected to Western blot using anti-UNC-78 (A) or anti-actin (B) antibody. (C–H) Localization of UNC-78 (C and D) and GFP-UNC-60B (E and F) in the control RNAi-treated (C, E, and G) or *unc-78(RNAi)*-treated (D, F, and H) GFP-UNC-60B-expressing strain. Merged images of UNC-78 (red) and GFP-UNC-60B (green) are shown in G and H. Bar, 10 μm.

Fig. 4.

Overexpression of GFP-UNC-78 does not induce rod formation. Worm lysates from wild-type and a GFP-UNC-78-expressing strains in wild-type background were subjected to Western blot using anti-UNC-78 (A) or anti-actin antibody (B). Positions of molecular weight markers are shown on the left of A. (A) Bands at ~65 kDa are endogenously expressed UNC-78. In the transgenic strain, an additional 90 kDa band of GFP-UNC-78 was detected. (C–E) Localization of GFP-UNC-78 (C), UNC-60B (D), and actin (E) in the body wall muscle of the GFP-UNC-78- expressing worms. None of these proteins form rod-like structures. Bar, 10 μ m.

Fig. 5.

Essential role of the C-terminal tail of UNC-60B for rod formation. (A and B) Transgenic overexpression of C-terminally truncated GFP-UNC-60B variants in *C. elegans*. Worm lysates from wild-type, an *unc-60B*-null mutant (as a negative control), and a GFP-UNC-60B(Δ150)- or GFP-UNC-60B(Δ152)-expressing strains in wild-type background were subjected to Western blot using anti-UNC-60B (A) or anti-actin antibody (B). Positions of molecular weight markers are shown on the left of A. In the transgenic strains, GFP-UNC-60B(Δ152) and GFP-UNC-60B(Δ152) were overexpressed at similar levels to GFP-UNC-60B(WT) in Fig. 1. (C–E) Localization of GFP-UNC-60B(WT) (C), GFP-UNC-60B(Δ150) (D) or GFP-UNC-60B(Δ152) (E) were observed in live worms without fixation. GFP-UNC-60B(WT) induced rod formation (C), but GFP-UNC-60B(Δ150) (D) and GFP-UNC-60B(Δ152) (E) localized to the diffuse cytoplasm with vague striation and the nucleus (asterisks) and did not induce rods. Bar, 10 μm.