Actin filament disassembling activity of *Caenorhabditis elegans* actin-interacting protein 1 (UNC-78) is dependent on filament binding by a specific ADF/cofilin isoform

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Summary

Actin-interacting protein 1 (AIP1) is a conserved WD-repeat protein that enhances actin filament disassembly only in the presence of actin depolymerizing factor (ADF)/cofilin. In the nematode *Caenorhabditis elegans*, an AIP1 ortholog is encoded by the *unc-78* gene that is required for organized assembly of muscle actin filaments. We produced bacterially expressed UNC-78 protein and found that it enhances actin filament disassembly preferentially in the presence of a specific ADF/cofilin isoform. Extensive and rapid filament disassembly by UNC-78 was observed in the presence of UNC-60B, a muscle-specific *C. elegans* ADF/cofilin isoform. UNC-78 also reduced the rate of spontaneous polymerization and enhanced subunit dissociation from filaments in the presence of UNC-60B. However, in the presence of UNC-60A, a non-muscle *C. elegans* ADF/cofilin isoform, UNC-78 only slightly enhanced filament disassembly. Interestingly, UNC-78 failed to enhance disassembly by mouse muscle-type cofilin. Using mutant forms of UNC-60B, we demonstrated that the F-actin-specific binding site of UNC-60B at the C terminus is required for filament disassembly by UNC-78. UNC-78 was expressed in body wall muscle and co-localized with actin where UNC-60B was also present. Surprisingly, UNC-78 was co-localized with actin in *unc-60B* null mutants, suggesting that the AIP1-actin interaction is not dependent on ADF/cofilin in muscle. These results suggest that UNC-78 closely collaborates with UNC-60B to regulate actin dynamics in muscle cells.

Key words: Actin dynamics, myofibrils, WD-repeat, *Caenorhabditis elegans*

Introduction

Disassembly of actin filaments is a critical process of cytoskeletal reorganization and recycling monomers for formation of new filaments. Actin depolymerizing factor (ADF)/cofilin is an essential factor to enhance actin filament dynamics by depolymerizing and severing actin filaments (reviewed by Bamburg, 1999; Bamburg et al., 1999; Carlier et al., 1999; Maciver and Hussey, 2002). Filament severing by ADF/cofilin also increases the number of exposed filament ends that can nucleate actin polymerization (Hawkins et al., 1993; Hayden et al., 1993; Mabuchi, 1983; Maciver et al., 1991; Nishida et al., 1984; Nishida et al., 1985) and the new filaments provide preferential sites for the Arp2/3 complex to form branched filament network (Ichetovkin et al., 2002). Overexpression of cofilin in *Dictyostelium* cells induces filament formation and bundling (Aizawa et al., 1996): this may be a consequence of the spontaneous assembly of cofilin:ADP-actin complexes (Yeoh et al., 2002). Thus, ADF/cofilin can promote both disassembly and growth of filaments depending on the concentration of actin monomers and other cellular factors (Condeelis, 2001).

The activity of ADF/cofilin is inhibited by phosphorylation of a serine residue near the N terminus (Agnew et al., 1995; Moriyama et al., 1996). LIM kinases (Arber et al., 1998; Sumi et al., 1999; Yang et al., 1998) and testicular protein kinases (TESKs) (Toshima et al., 2001a; Toshima et al., 2001b) in vertebrates and a calmodulin-domain like kinase in plants (Allwood et al., 2001) phosphorylate ADF/cofilin and mediate various signals to effect changes in the actin cytoskeleton. Recently, Slingshot has been identified as an ADF/cofilin phosphatase that can reactivate ADF/cofilin (Niwa et al., 2002). Phosphatidylinositol 4,5-bisphosphate directly binds near the actin-binding site of ADF/cofilin and inhibits actin binding (Ojala et al., 2001; Van Troys et al., 2000; Yonezawa et al., 1991a; Yonezawa et al., 1990). Binding of tropomyosin to actin filaments inhibits depolymerization by ADF/cofilin both in vitro (Bernstein and Bamburg, 1982; Nishida et al., 1985) and in vivo (Ono and Ono, 2002). However, one of the non-muscle tropomyosin isoforms co-localizes with ADF/cofilin to dynamic actin filaments, suggesting that such tropomyosins may have a positive role in actin dynamics that are mediated by ADF/cofilin and its associated proteins (Bryce et al., 2003).

In addition, there are a number of other proteins that promote ADF/cofilin-dependent actin dynamics. Profilin competes with ADF/cofilin for actin binding (Blanchoin and

Pollard, 1998; Maciver et al., 1991); it also promotes exchange of actin-bound ADP with ATP in complexes containing ADF/cofilin, thereby promoting dissociation of the complexes and enhancing barbed end elongation (Didry et al., 1998). Cyclase-associated protein 1 binds to ADF/cofilin and actin; it enhances subunit dissociation from the pointed end and stimulates exchange of actin-bound ADP with ATP thereby promoting dissociation of complexes and providing monomers for barbed end elongation (Moriyama and Yahara, 2002b). Actin-interacting protein 1 (AIP1) enhances filament fragmentation only in the presence of ADF/cofilin (Aizawa et al., 1999; Okada et al., 1999; Rodal et al., 1999). Recently, Okada et al. (Okada et al., 2002) reported that *Xenopus* AIP1 by itself has negligible effects on actin dynamics but it caps barbed ends in the presence of ADF/cofilin to prevent re-annealing of severed filaments.

AIP1 is a conserved WD-repeat protein that was originally identified in yeast as one of actin-interacting proteins from a two-hybrid screen (Amberg et al., 1995). Functional links between AIP1 and ADF/cofilin have been reported in several different organisms. In yeast, AIP1 is co-localized with cofilin to actin patches (Iida and Yahara, 1999; Rodal et al., 1999). Overexpression of AIP1 suppresses a temperature-sensitive lethality of a *COF1* (the yeast cofilin gene) allele (Iida and Yahara, 1999), whereas a deletion of *AIP1* is synthetic lethal with mutant *COF1* alleles (Iida and Yahara, 1999; Rodal et al., 1999). In the nematode *Caenorhabditis elegans*, mutations in the *unc-78* gene, which encodes AIP1, cause disrupted actin organization, defects in muscle motility and altered localization of UNC-60B (a muscle-specific ADF/cofilin isoform in *C. elegans*) (Ono, 2001). UNC-60B co-precipitates with F-actin in vitro, has very weak depolymerizing activity and is required for proper actin assembly in body wall muscle (Ono et al., 1999; Ono and Benian, 1998). In addition, colocalization of AIP1 and ADF/cofilin in actin-rich cellular structures has been reported in *Xenopus* eggs (Okada et al., 1999), *Dictyostelium* (Aizawa et al., 1999; Konzok et al., 1999), pollens in green plants (Allwood et al., 2002) and chicken cochlea (Oh et al., 2002).

As reported above, UNC-78/AIP1 is required for organized assembly of actin filaments in body wall muscle of *C. elegans* (Ono, 2001). However, this is the only example to date in which mutations in an AIP1 gene cause drastic disorganization of the actin cytoskeleton. In yeasts (Iida and Yahara, 1999; Rodal et al., 1999) and *Dictyostelium* (Konzok et al., 1999), AIP1 null mutant cells do not exhibit disorganized actin cytoskeleton, although they have partial defects in several actin-dependent processes. Such difference may reflect different demands on actin regulators in different cell types. This could also reflect different biochemical activities among AIP1s from different species. To understand the biochemical properties of UNC-78/AIP1, we prepared bacterially expressed recombinant UNC-78 protein and determined its effects on actin filament dynamics in vitro. We report that UNC-78 has actin filament disassembling activity in the presence of a specific ADF/coflin isoform and that they are both expressed in muscle cells. Furthermore, we provide evidence that F-actin binding by ADF/cofilin is essential for UNC-78 to disassemble filaments. These results provide novel insights into the mechanism of isoform-specific regulation of actin filament dynamics in multicellular organisms.

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, Minnesota, USA). *unc-60 (su158)* (*unc-60B* null mutant) was provided by Dr Henry Epstein (Baylor College of Medicine, Houston, Texas, USA) and described previously (Ono et al., 2003; Zengel and Epstein, 1980). *unc-78 (gk27)* (*unc-78* null mutant) was provided by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, Vancouver, Canada and has been described previously (Ono, 2001). All strains used in this study were homozygous for each allele.

Proteins

Rabbit skeletal muscle actin was purified as described previously (Pardee and Spudich, 1982). *C. elegans* actin was purified from wild-type N2 strain as described previously (Ono, 1999). Bacterially expressed recombinant UNC-60A, UNC-60B and mutant forms of UNC-60B were purified as described (Ono et al., 1999; Ono and Benian, 1998; Ono et al., 2001). Bacterially expressed recombinant mouse muscle-type cofilin (M-cofilin) was a generous gift from Dr Takashi Obinata (Chiba University, Chiba, Japan).

Expression and purification of recombinant UNC-78 protein

The full-length UNC-78 cDNA clone yk185g6 (provided by Dr Yuji Kohara, National Institute of Genetics, Mishima, Japan) was digested by EcoRI and XhoI and cloned into pET-32a (Novagen) between the EcoRI and XhoI sites. The resultant vector expresses a fusion protein of UNC-78 with thioredoxin and 6× His-tag at the N terminus. The *Escherichia coli* strain BL21(DE3) was transformed with the expression vector and cultured in M9ZB medium (Novagen's instruction) containing 50 μg/ml ampicillin at 37°C until A_{600} reached 0.6 cm^{-1}. Then, the culture was cooled to room temperature and protein expression induced by adding 0.4 mM isopropyl β-D-thiogalactopyranoside for 3 hours at room temperature. The cells were harvested by centrifugation at 5,000 ***g*** for 10 minutes and disrupted by a French Pressure cell at 360-580 kg/cm^2 in a buffer containing 0.3 M NaCl, 50 mM $NaPO_4$, 1 mM phenylmethanesulfonyl fluoride, pH 7.0. The homogenates were centrifuged at 20,000 ***g*** and the supernatants applied to a TALON cobalt affinity column (Clontech). Bound proteins were eluted with 0.3 M NaCl, 50 mM $NaPO_4$, 150 mM imidazole, pH 7.0. Fractions containing UNC-78 were dialyzed against 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5 and the protein concentrations determined by an Advanced Protein Assay Reagent (Cytoskeleton, Inc.). The proteins were digested overnight at room temperature by enterokinase (1/200 by weight) (Roche Applied Sciences) to cleave thioredoxin-His-tag. The reaction was stopped by adding 1 mM phenylmethanesulfonyl fluoride. The digested proteins were passed through a TALON column to which cleaved thioredoxin-His-tag and the uncleaved fusion protein strongly bound. UNC-78 exhibited a weak affinity with a TALON column and was eluted by washing the column with 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5. The fractions containing UNC-78 were dialyzed against 0.2 mM dithiothreitol, 20 mM Tris-HCl, pH 8.0, applied to DEAE-cellulose column (DE-52, Whatman) and eluted with a linear NaCl gradient (0-0.2 M). Fractions containing pure UNC-78 were dialyzed against 0.1 M KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 50% glycerol, 20 mM Hepes-NaOH, pH 7.5 and stored at –20°C. The concentration of purified UNC-78 was spectrophotometrically determined in the presence of 6 M guanidine hydrochloride using a calculated extinction coefficient (Gill and von Hippel, 1989) of 85,220 M^{-1} cm^{-1} at 280 nm.

Assays for F-actin binding and depolymerization by co-pelleting

An F-actin co-pelleting assay was performed as described previously

(Ono et al., 1999) with slight modifications. Briefly, 10 μM F-actin was incubated with various concentrations of UNC-78 and/or ADF/cofilin proteins in F-buffer (0.1 M KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM Hepes-NaOH, pH 7.5), incubated for 30 minutes at room temperature and centrifuged in a Beckman TLA-100 rotor at 80,000 rpm for 20 minutes. The supernatants and pellets were adjusted to the same volumes and analyzed by SDS-PAGE (12% acrylamide gel). Gels were stained with Coomassie brilliant blue R-250 (National Diagnostics) and scanned by a UMAX PowerLook III scanner at 300 dots per inch. The band intensity was quantified by Scion Image Beta 4.02 (Scion Corporation).

Light scattering measurements

F-actin (5 μM) was mixed with various concentrations of UNC-78 and/or UNC-60B in F-buffer and light scattering at an angle of 90° and a wavelength of 500 nm was measured with a fluorescence spectrophotometer (Perkin Elmer LS50B). A decrease in light scattering is indicative of filament severing and/or disassembly, while an increase in scattering occurs when filaments bind other proteins along their length (Cooper and Pollard, 1982).

Assay for actin polymerization

The time course of actin polymerization was monitored as changes in turbidity at a wavelength of 310 nm (Carlier et al., 1997). 5 μM G-actin was mixed with UNC-78 and/or UNC-60B in G-buffer and polymerization was initiated by adding salts to final concentrations of 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM Hepes-NaOH, pH 7.5. Turbidity was monitored by an Ultrospec 3000 spectrophotometer (Amersham Biosciences).

DNase I inhibition assay

Quantification of G-actin by a DNase I inhibition assay was performed essentially as described previously (Ono, 1999; Ono et al., 1999). Briefly, 5 μM F-actin was incubated with various concentrations of UNC-78 and/or UNC-60B in F-buffer for 30 minutes at room temperature. 20 μl of the reactions was mixed with 1 μg of bovine pancreas DNase I (Sigma-Aldrich) and the DNase activity was measured from a linear change in A_{260} using 0.1 mg/ml calf thymus DNA, 125 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 3 mM NaN_3, pH 7.5 at 25°C with an Ultrospec 3000 spectrophotometer (Amersham Biosciences). G-actin from rabbit muscle was used as a standard.

Monitoring actin depolymerization by nucleotide exchange

Depolymerization of actin can be monitored by measuring exchange of actin-bound ADP with free 1,N^6-etheno ATP (εATP), a fluorescent analog of ATP, in which fluorescence is increased upon binding to actin (Wang and Taylor, 1981). This assay is generally performed by pre-loading actin with εATP and measuring the loss of fluorescence after addition of ATP. In this study, we used ADP-bound F-actin and measured the increase in fluorescence after addition of free εATP, because, this way, we were able to obtain reproducible signals to detect the effects of UNC-60B and UNC-78 on depolymerization without the need to prepare εATP (ADP)-bound actin filaments. 50 μM F-actin in F-buffer was incubated with a Dowex 1×8-50 resin for 1 hour at 4°C to remove free ATP and used as a stock. F-actin was diluted to 5 μM in F-buffer containing 40 μM εATP (Sigma-Aldrich) and various concentrations of UNC-78 and/or UNC-60B. After gentle mixing, changes in the fluorescence (excitation at 360 nm and emission at 410 nm) were immediately monitored over time with a fluorescence spectrophotometer (Perkin Elmer LS50B). Data were fitted to exponential curves using SigmaPlot 2000 (SPSS Sciences) to obtain rates of increase in the fluorescence.

Preparation of anti-UNC-78 antibody

A synthetic peptide CAGGSGVDSSKAVAN corresponding to residues 395-408 of UNC-78 plus additional cysteine at the N terminus was synthesized and coupled to keyhole limpet hemocyanin by the Microchemical Facility at Emory University. The conjugate was used to raise rabbit antisera at Spring Valley Laboratories Inc. (Woodbine, Maryland, USA). The immunogen peptide was immobilized to SulfoLink Coupling Gel (Pierce Biotechnology) and used for affinity-purification of the antisera. Specificity of the antibody was tested by western blot as described previously (Ono and Ono, 2002).

Immunofluorescence microscopy

Immunofluorescent staining of embryos was performed as described previously (Ono, 2001) with slight modifications. Briefly, worm embryos were obtained by a hypochlorite treatment of gravid adults (Epstein et al., 1993). They were then fixed with 4% formaldehyde, 1× cytoskeleton buffer (10 mM MES-KOH, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, pH 6.1) containing 0.32 M sucrose for 30 minutes at room temperature, permeabilized with methanol at –20°C for 5 minutes, and stained with antibodies. Immunostaining of adult worms was performed as described previously (Finney and Ruvkun, 1990). Primary antibodies used were rabbit polyclonal anti-UNC-78 (described above), mouse monoclonal anti-myoA (clone 5.6; a generous gift from Dr Henry Epstein, Baylor College of Medicine, Houston, Texas, USA) (Miller et al., 1983) and mouse monoclonal anti-actin (C4; ICN Biomedicals). Secondary antibodies used were Alexa488-labeled goat anti-mouse IgG (Molecular Probes) and Cy3-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). 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.

Results

Bacterial expression and purification of recombinant UNC-78 protein

UNC-78 was expressed and purified as a soluble protein in *E. coli* (Fig. 1A). This is the first successful expression of functional and stable AIP1 in *E. coli* other than *Arabidopsis* AIP1 (Allwood et al., 2002). The recombinant UNC-78 protein, which migrated as a 65 kDa protein on SDS-PAGE (Fig. 1A), had an N-terminal extension of AMADIG which was derived from the vector sequence and it lacked Met-1. The calculated M_r is 65,750. In contrast to *Arabidopsis* AIP1, which is relatively unstable (Allwood et al., 2002), recombinant UNC-78 retained full activity (see below) over 6 months when the protein was stored with 50% glycerol at –20°C (data not shown).

Actin filament disassembly by UNC-78 in the presence of UNC-60B

We first tested if UNC-78 has the AIP1-like activity, that is, actin filament disassembling activity in the presence of ADF/cofilin (Aizawa et al., 1999; Okada et al., 1999; Rodal et al., 1999). In a co-sedimentation assay with rabbit muscle F-actin, UNC-78 alone slightly co-precipitated with F-actin and did not disassemble F-actin (Fig. 1B, lanes 5 and 6), while UNC-60B on its own co-sedimented with F-actin and showed very weak steady-state depolymerizing activity (Ono and

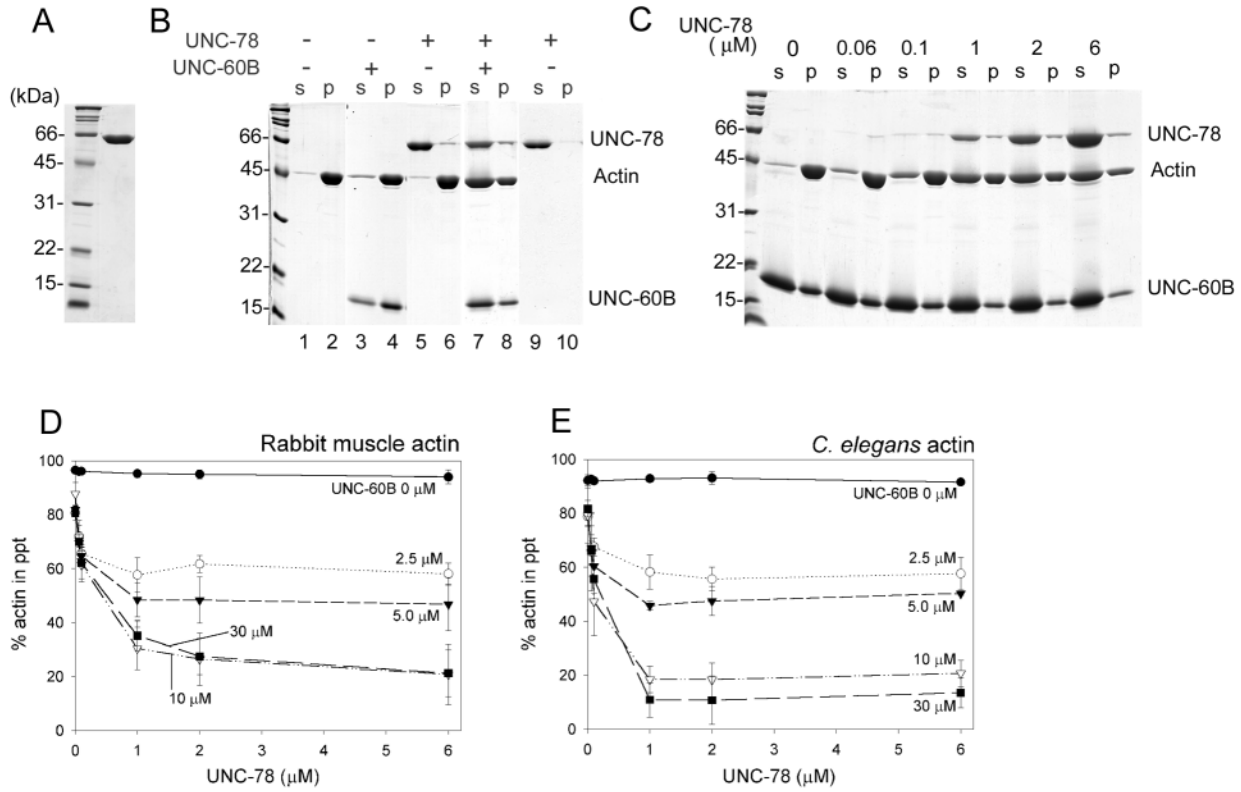

Fig. 1. Actin filament disassembling activity of the recombinant UNC-78 protein. (A) Purified bacterially expressed recombinant UNC-78 protein (3.2 μg). (B) UNC-60B-dependent filament disassembly by UNC-78. Rabbit muscle F-actin (10 μM) was incubated for 30 minutes with buffer (lanes 1 and 2), UNC-60B (10 μM) (lanes 3 and 4), UNC-78 (2 μM) (lanes 5 and 6), or both UNC-60B and UNC-78 (lanes 7 and 8) and separated into supernatants (s) and pellets (p) after ultracentrifugation. UNC-78 was treated in the absence of F-actin as a control (lanes 9 and 10). (C) Dose-dependence of filament disassembly by UNC-78 in the presence of UNC-60B. F-actin (10 μM) and UNC-60B (20 μM) were incubated for 30 minutes with the indicated concentrations of UNC-78 and analyzed by pelleting assays. Molecular mass markers in kDa are indicated on the left of A-C. (D,E) Quantitative analysis of UNC-78-induced filament disassembly. Rabbit muscle actin (D) or *C. elegans* actin (E) was incubated with various concentrations of UNC-60B and UNC-78 and subjected to pelleting assays. Percentages of actin in the pellets were quantified and plotted as a function of the UNC-78 concentration at different UNC-60B concentrations (0-30 μM). Data shown are mean±s.d. of three experiments.

Benian, 1998) (Fig. 1B, lanes 3 and 4). In the presence of both proteins, filaments were disassembled and the unpelletable portion of actin was greatly increased (Fig. 1B, lanes 7 and 8). UNC-78 disassembled F-actin in a concentration-dependent manner in the presence of UNC-60B and showed a maximum activity at 1 μM against 10 μM F-actin and UNC-60B (Fig. 1C,D). The amount of sedimented UNC-78 in the presence of actin (Fig. 1B, lanes 5-8 and Fig. 1C) was slightly greater than that in the absence of actin (Fig. 1B, lanes 9 and 10), but the amounts were small and did not reach saturation within the range of concentrations examined (0-5 μM UNC-78). Small amounts of UNC-78 in the pellets might be from residual supernatant left in the centrifuge tubes or slight aggregation during incubation. In addition, when the pellets were resuspended in F-buffer and centrifuged,

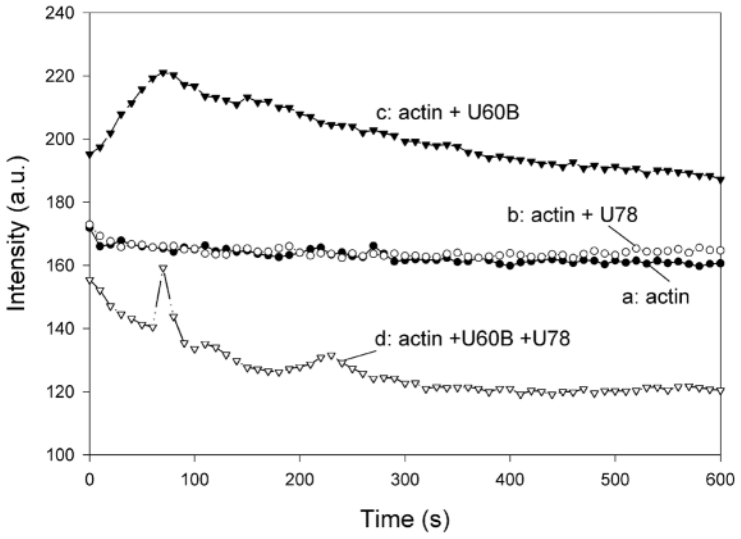

Fig. 2. The effects of UNC-78 and UNC-60B on the kinetics of light scattering of F-actin. F-actin (5 μM) was mixed with buffer (a, black circles), UNC-78 (1 μM) (b, white circles), UNC-60B (5 μM) (c, black triangles), or both UNC-78 and UNC-60B (d, black triangles) and the light scattering intensity (arbitrary units) was monitored over time. Time 0 was when monitoring started, which is after ~20 sec of manual preparation of the samples and setting them in the instrument.

UNC-78 was released into the supernatants (data not shown). Therefore, we were not able to determine whether co-sedimentation was the result of simple trapping in the pellets or weak filament binding. Maximal enhancement was achieved at a 1:1 molar ratio of UNC-60B:actin (Fig. 1D). Note that UNC-60B depolymerizes *C. elegans* actin more strongly than rabbit muscle actin (Ono, 1999). Similarly, UNC-78 disassembled *C. elegans* actin:UNC-60B more efficiently than rabbit muscle actin:UNC-60B, but the difference was not very great (Fig. 1E). These co-sedimentation assays show that bacterially expressed recombinant UNC-78 protein has the AIP1-like activity. Because the difference between rabbit muscle actin and *C. elegans* actin was small, we used rabbit muscle actin in the following experiments unless otherwise specified.

Kinetics of filament disassembly by UNC-78 were characterized by light scattering (Fig. 2). F-actin alone was stable showing little change in the scattering intensity (Fig. 2, black circles) and UNC-78 had no effect on this signal (Fig. 2, white circles). UNC-60B initially increased the scattering intensity, but thereafter the signal declined, consistent with filament binding followed by disassembly (Fig. 2, black triangles). In the presence of both UNC-78 and UNC-60B, the rate and extent of disassembly were greatly enhanced (Fig. 2, white triangles). Note that there were intervals of ~20 seconds for setting the samples in the instrument before time 0 when the measurement started. The differences in the initial intensity indicate that filament binding by UNC-60B (Fig. 2, black triangles) or disassembly by UNC-60B and UNC-78 (Fig. 2, white triangles) progressed during these intervals.

Enhancement of actin depolymerization by UNC-78

The extent of filament disassembly to actin monomers was determined using the DNase I inhibition assay (Fig. 3). UNC-60B alone increased the G-actin concentration from ~0.2 μM to ~0.8 μM (Fig. 3, black circles). Addition of UNC-78 enhanced the G-actin concentration about 2-fold but only in the presence of UNC-60B (Fig. 3, white circles, and white and black triangles). Maximum depolymerization was achieved using 0.2 μM UNC-78 (4% of total actin) and required >1:1 ratio of UNC-60B:actin (>5 μM UNC-60B) (Fig. 3). Interestingly, the maximum amount of G-actin (1.6 μM) determined using this assay was much less than the amount of non-sedimented actin (3.6 μM) in the pelleting assay under the same conditions. This suggests either that the non-sedimented actin contains short oligomers or that the DNase inhibition by complexes of UNC-60B with actin is less than that of G-actin alone.

We also tested the kinetics of actin depolymerization from the exchange rate of actin-bound nucleotides, since rapid exchange of actin-bound nucleotide occurs only in G-actin but

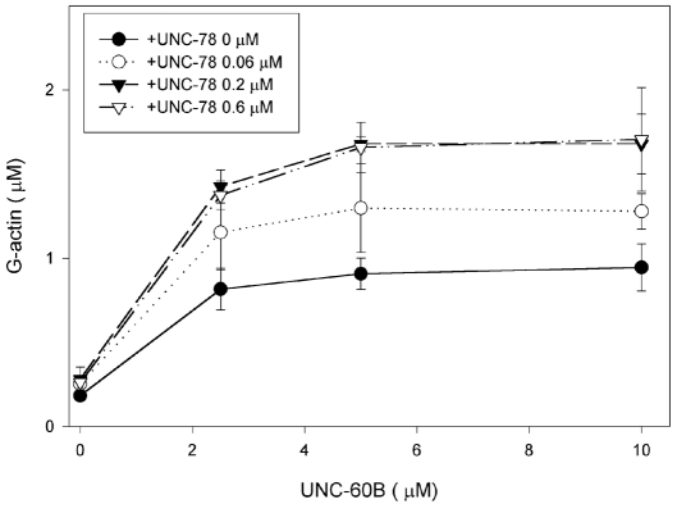

Fig. 3. Effects of UNC-78 and UNC-60B on actin depolymerization as measured by a DNase I inhibition assay. 5 μM F-actin was incubated for 30 minutes with UNC-78 (0-0.6 μM) and UNC-60B (0-10 μM) and the G-actin concentrations were determined by a DNase I inhibition assay. Data shown are mean±s.d. of three experiments.

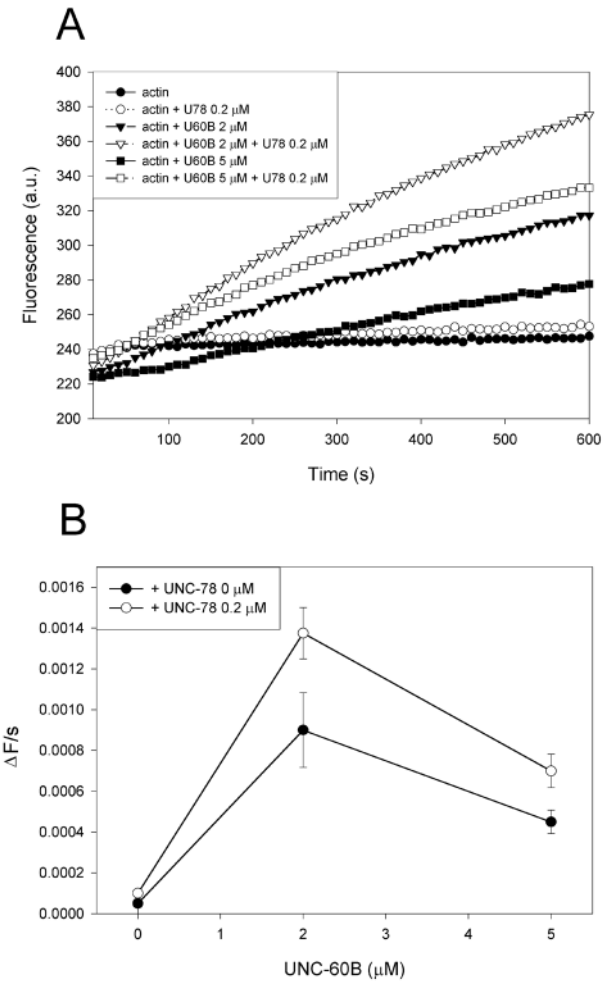

Fig. 4. Effects of UNC-78 and UNC-60B on actin turnover as measured by nucleotide exchange. (A) F-actin (5 μM) was mixed with UNC-78 and/or UNC-60B as indicated in the figure in the presence of 40 μM εATP, and the fluorescence (arbitrary units) of εATP was monitored over time. (B) The data were fitted to exponential curves and the rates of increase in the fluorescence (ΔF/second) were calculated and plotted as a function of concentration of UNC-60B. Data shown are mean±s.d. of four experiments.

not F-actin or complexes of ADF/cofilin with ADP-actin (Fig. 4). F-actin alone showed little nucleotide exchange (Fig. 4, black circles), and addition of UNC-78 had no effect (Fig. 4, white circles). The rate of nucleotide exchange was accelerated by UNC-60B (Fig. 4, black triangles and squares), but addition of 0.2 μM UNC-78 further enhanced this effect by approx. 1.6-fold (Fig. 4A, compare black and white triangles or squares, and 4B for rates of increase in the fluorescence). Optimal acceleration occurred at a ratio of UNC-60B to actin ~0.5:1 (Fig. 4B), as observed previously for human ADF and cofilin (Yeoh et al., 2002).

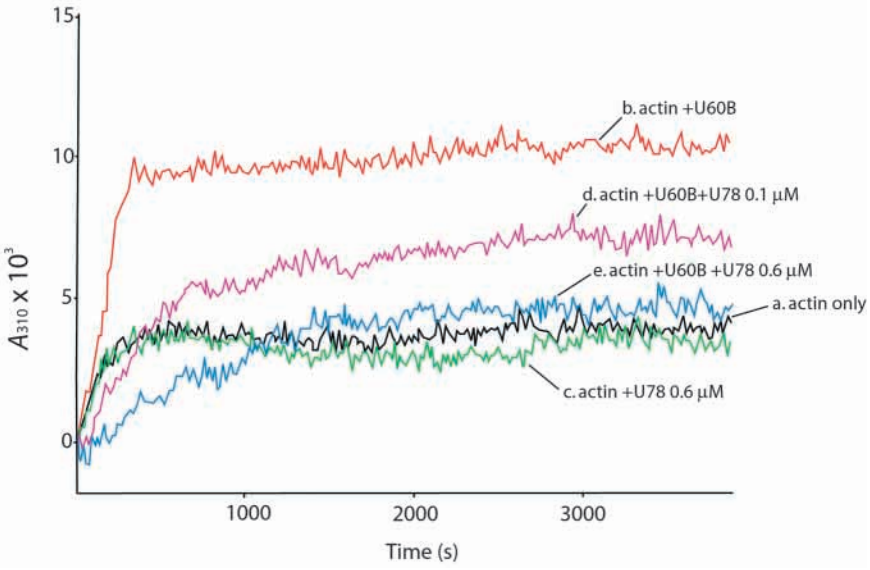

Fig. 5. Effects of UNC-78 and UNC-60B on spontaneous actin polymerization. G-actin (5 μM) was mixed with buffer (a), UNC-60B (5 μM) (b), UNC-78 (0.6 μM) (c), UNC-60B (5 μM) and UNC-78 (0.1 μM) (d), or UNC-60B (5 μM) and UNC-78 (0.6 μM) (e), and the turbidity (absorbance at 310 nm) was monitored over time.

Inhibitory activity of UNC-78 on spontaneous actin polymerization

Turbidimetric methods were used to monitor the spontaneous assembly of G-actin (Fig. 5). Because ADF/cofilins sever actin filaments, UNC-60B accelerates the rate of spontaneous assembly of G-actin (Fig. 5, red) (see also Ono et al., 1999; Ono and Benian, 1998). The marked increase in the final turbidity signal reflects the fact that the filaments are decorated with UNC-60B. UNC-78 reduced this activating effect and also the amplitude of the turbidity change (Fig. 5, pink and blue), suggesting either a lower concentration of polymer or structural differences in the polymer (e.g. less decorated with UNC-60B or shorter lengths). UNC-78 on its own had no effect compared to the control (compare Fig. 5, black and green).

Effects of ADF/cofilin isoforms on UNC-78-induced filament disassembly

We examined how UNC-78 affects the disassembly of F-actin induced by different isoforms of ADF/cofilin (Fig. 6A-C). In comparison to UNC-60B (Fig. 6A), increasing concentrations of UNC-78 had little effect on the depolymerizing activity of UNC-60A (a non-muscle isoform in *C. elegans*) (Fig. 6B), though the slight acceleration may be significant. Interestingly, UNC-78 did not change the behavior of mouse M-cofilin (muscle-type) (Ono et al., 1994), which on its own has little depolymerizing activity (Fig. 6C) (see also Vartiainen et al., 2002). Thus, the most marked increase in depolymerization occurs with the muscle specific isoform of *C. elegans* ADF/cofilin. Similar results were obtained using *C. elegans* actin (data not shown).

Requirement of filament binding by UNC-60B for UNC-78-induced filament disassembly

To understand how UNC-60B supports UNC-78-induced filament disassembly, the activity of UNC-78 was tested in the presence of mutant forms of UNC-60B (Fig. 6D-F). Two actin-binding surfaces on UNC-60B have been predicted: the monomer/filament-binding site containing the putative long helix α3 and the N terminus, and the filament-specific site containing the C terminus (Ono et al., 2001). The A111V mutation at the putative helix α3 reduces the affinity with G- and F-actin (Ono et al., 1999). UNC-78 enhanced disassembly only weakly in the presence of this mutant (Fig. 6D). The S113L mutation (Ono et al., 1999) caused a similar effect to A111V (data not shown). The S112F mutation at the putative helix α3 confers hyper-severing activity (Ono et al., 1999) by inducing different filament conformation than wild-type (A. McGough and S. Ono, unpublished observations). This mutant by itself depolymerized filaments more strongly than wild type, but UNC-78 was able to enhance disassembly only weakly in the presence of this mutant (Fig. 6E). Truncation of the three C-terminal amino acids (Q150*) of UNC-60B abolished filament binding but not monomer binding (Ono et al., 1999; Ono et al., 2001). Although this mutant depolymerized F-actin through monomer binding, UNC-78 did not enhance disassembly (Fig. 6F), suggesting that G-actin binding by UNC-60B is not sufficient to support UNC-78. Likewise, truncation of the two C-terminal amino acids eliminates only F-actin binding (Ono et al., 2001), and UNC-78 had no effect on disassembly in the presence of this mutant (data not shown). These results suggest that filament binding by UNC-60B is essential for UNC-78 to enhance disassembly.

Expression and localization of UNC-78

We prepared a specific antibody against UNC-78 and characterized its expression pattern and intracellular localization. Since the *C. elegans* genome sequencing project predicted the second AIP1 isoform (K08F9.2) that is 68% identical to UNC-78, we used a synthetic peptide corresponding to a unique region of UNC-78 (residues 395-408) to raise rabbit antisera. The affinity-purified antibody specifically reacted with a 65 kDa protein in the total lysates of wild type (Fig. 7B, lane 1) and purified recombinant UNC-

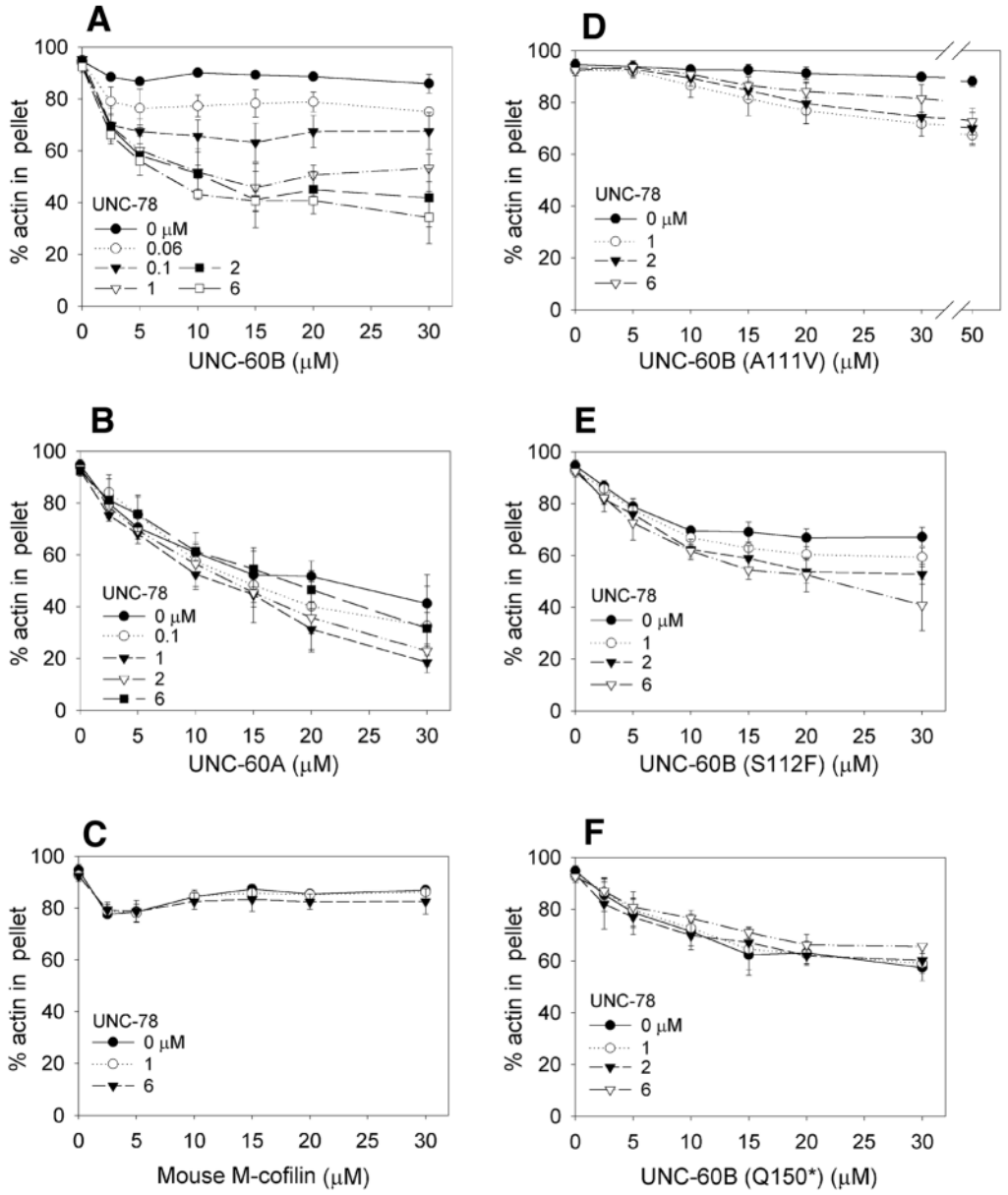

Fig. 6. Effects of ADF/cofilin isoforms and mutants on UNC-78-dependent actin filament assembly. F-actin (10 μM) was incubated for 30 minutes with UNC-78 (0-6 μM) and UNC-60B (A), UNC-60A (B), mouse M-cofilin (C), UNC-60B (A111V) (D), UNC-60B (S112F) (E), or UNC-60B (Q150*) (F), and subjected to pelleting assays. Percentages of actin in the pellets were quantified and plotted as a function of the concentration of ADF/cofilin proteins. Data shown are mean±s.d. of three experiments.

78 protein (Fig. 7B, lane 4). However, the antibody did not show reactivity with the lysates of *unc-78* null mutants (Fig. 7B, lane 2). Therefore, anti-UNC-78 antibody specifically recognizes the UNC-78 protein. The level of the UNC-78 protein was slightly greater in *unc-60B* null mutants than in wild type (Fig. 7B, compare lanes 1 and 3), whereas Coomassie staining of total proteins (Fig. 7B) and western blot of actin (Fig. 7C) did not show a significant difference in the amounts of the loaded proteins. The levels of UNC-60B were not different in wild type and *unc*-78 null mutants (Fig. 7D, compare lanes 1 and 2).

Immunolocalization of UNC-78 revealed that UNC-78 is expressed in a tissue-specific manner. In embryos, faint staining of UNC-78 was first detected in a subset of cells at the 1.5-fold stage (~350 minutes after the first cell division) (Fig. 8A, arrows). The UNC-78-positive regions were adjacent to the nascent myofibrils in body wall muscle (Epstein et al., 1993) where the muscle-specific myosin heavy chain myoA was localized (Fig. 8C,E), suggesting that UNC-78 is localized in the diffuse cytoplasm of the body wall muscle cells. In the later embryonic stages, UNC-78 was expressed in the body wall muscle (Fig. 8B,D,F, arrows) and also more strongly in the pharynx (Fig. 8B, arrowheads). In adults, UNC-78 was expressed in the body wall muscle, pharynx and spermatheca. In the pharynx and spermatheca, subcellular localization of UNC-78 was not clear owing to poor penetration of the antibody into these tissues (data not shown). In the body wall muscle, UNC-78 was localized in a striated pattern (Fig. 9A,B) that was co-localized with actin (Fig. 9C,E) but not with the myosin heavy chain myoA (Fig. 9D,F). However, since the striation of UNC-78 was not as sharp as that of actin, it was difficult to determine precise location of UNC-78 within the thin filaments. Staining of *unc*-78 null mutants with anti-UNC-78 antibody did not yield these patterns (data not shown), indicating that striated staining is specific for reactivity with UNC-78. We were not able to

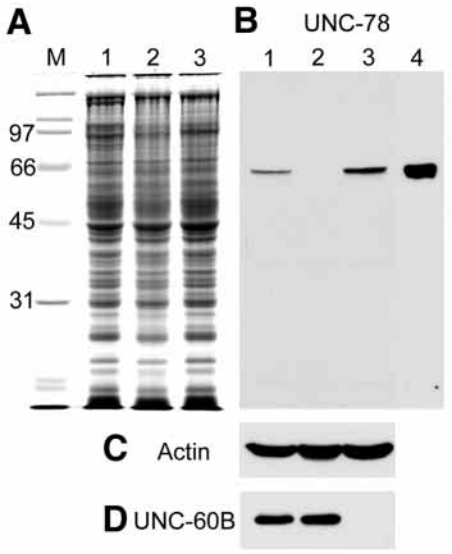

Fig. 7. Specificity of anti-UNC-78 antibody and expression of UNC-78. Total worm lysates (25 μg proteins) of wild type (lane 1), *unc-78 (gk27)* (*unc-78* null) (lane 2), and *unc-60 (su158)* (*unc-60B* null) (lane 3) and purified recombinant UNC-78 protein (0.1 μg) (lane 4 only in B) were resolved by SDS-PAGE (10% acrylamide gel) and visualized by Coomassie Blue (A) or subjected to western blot with anti-UNC-78 (B), anti-actin (C), or anti-UNC-60B (D). Molecular mass markers in kDa (lane M) are indicated on the left of A.

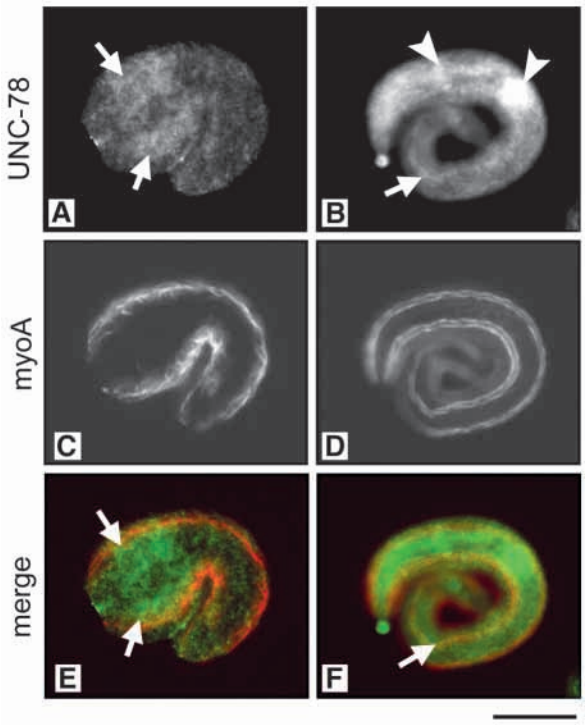

Fig. 8. Localization of UNC-78 in wild-type embryos. Embryos at the 1.5-fold stage (A,C,E) and threefold stage (B,D,F) were stained by anti-UNC-78 (A,B) and anti-myoA (C,D) antibodies. Arrows in A,B,E,F indicate expression of UNC-78 in embryonic body wall muscle. Arrowheads in B indicate the pharynx. Merged images of double-staining of UNC-78 (green) and myoA (red) are shown in E and F. Scale bar: 20 μm.

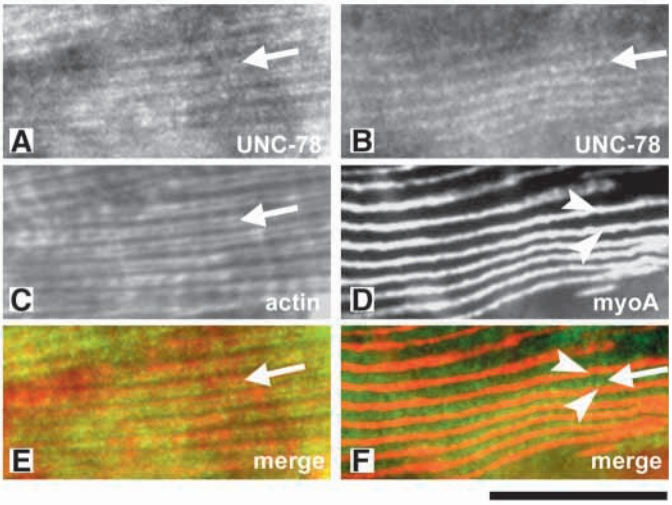

Fig. 9. Localization of UNC-78 in adult wild-type body wall muscle. Adult worms were stained by anti-UNC-78 (A,B) and anti-actin (C) or anti-myoA (D) antibodies. Parts of the body wall muscle cells are shown. Representative locations of UNC-78 are indicated by arrows in A,B,E,F. An arrow in c indicates a line of actin staining that overlaps with UNC-78. Arrowheads in D and F indicate myoA striations that are not co-localized with UNC-78. Merged images of double-staining of UNC-78 (green) and actin or myoA (red) are shown in E and F. Scale bar: 20 μm.

perform double staining of UNC-78 and UNC-60B because the antibody against UNC-60B was also raised in rabbit (Ono et al., 1999). Nonetheless, these results indicate that UNC-78 is expressed in body wall muscle and is associated with myofibrils where UNC-60B, but not UNC-60A, is present (Ono et al., 2003; Ono et al., 1999).

Previously, we demonstrated that, in the absence of UNC-78, UNC-60B is mislocalized to actin aggregates and lost from myofibrils (Ono, 2001). Here, we tested whether myofibrillar localization of UNC-78 is dependent on UNC-60B. Surprisingly, in *unc-60B* null mutants, UNC-78 was still localized to residual myofibrils (Fig. 10, arrowheads), as well as actin aggregates (Fig. 10, arrows). UNC-60A was not detectable in body wall muscle of wild-type or the *unc-60B* null mutants (Ono et al., 2003) (data not shown). These results suggest that UNC-78 is able to associate with myofibrils independent of ADF/cofilin in muscle cells.

Discussion

In this study, we demonstrate that bacterially expressed recombinant UNC-78 protein has AIP1-like activity and provide new insights into the mechanism of actin filament disassembly by AIP1 and ADF/cofilin. Like the other AIP1 proteins, the activities of UNC-78 in disassembling filaments and inhibiting polymerization were dependent on UNC-60B, a muscle-specific ADF/cofilin isoform in *C. elegans*. Using variants of ADF/cofilin proteins, we found that UNC-60B is the preferential ADF/cofilin isoform for efficient filament disassembly by UNC-78, and that filament binding by UNC-60B was required for this interaction. Both UNC-78 and UNC-60B were expressed in body wall muscle cells and are likely

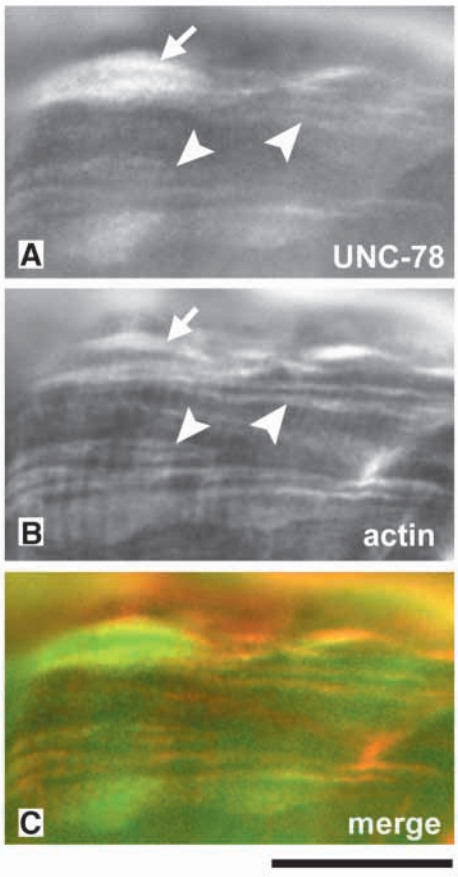

Fig. 10. Localization of UNC-78 in body wall muscle of *unc-60B* null mutants. The *unc-60 (su158)* homozygotes (*unc-60B* null mutants) were stained by anti-UNC-78 (A) and anti-actin (B) antibodies. Arrows indicate aggregates of actin where UNC-78 is also localized. Arrowheads indicate residual striated myofibrils where weak localization of UNC-78 is detected. A merged image of UNC-78 (green) and actin (red) is shown in C. Scale bar: 20 μm.

to regulate actin reorganization during myofibril assembly and maintenance.

The observed effects of UNC-78 on in vitro actin dynamics were generally in agreement with the biochemical data on AIP1s from other organisms and could be explained by the barbed end capping activity of AIP1 (Okada et al., 2002). By the co-sedimentation assay, activity of *Xenopus* AIP1 to increase unsedimented actin in the presence of cofilin (Okada et al., 2002; Okada et al., 1999) is comparable to that of UNC-78 determined in this study. However, there were two experimental results that were not consistent with the previously reported AIP1 activity. First, UNC-78 enhanced depolymerization in both kinetic and steady-state assays (Figs 3 and 4), whereas *Xenopus* AIP1 has no effect on depolymerization as determined by a DNase I inhibition assay (Okada et al., 2002; Okada et al., 1999). This difference may represent a specific activity of UNC-78 and UNC-60B. However, when actin filaments are fragmented and the barbed ends are capped, some depolymerization would be expected to occur because the critical concentration at the pointed end is higher than that of the barbed end (Bonder et al., 1983; Pollard, 1986; Wegner and Isenberg, 1983). Thus, there should be an increase in DNase inhibitory activity of the magnitude observed in Fig. 3. Also, ADF/cofilin accelerates subunit release from the pointed end (Carlier et al., 1997; Maciver et al., 1998; Ressad et al., 1999; Ressad et al., 1998). Barbed end capping by gelsolin has been shown to promote subunit dissociation from the pointed ends by ADF/cofilin (Ressad et al., 1999). Alternatively, depolymerization may be enhanced by increased filament severing in the presence of UNC-78. Some experimental conditions, such as pipetting, dilution and incubation time, could artificially affect depolymerization. Also, our bacterially expressed protein might be slightly different from AIP1 proteins from eukaryotic sources (Aizawa et al., 1999; Okada et al., 1999; Rodal et al., 1999) in its conformation and post-translational modifications, which may affect the activity. Therefore, further comparison of different AIP1 proteins needs to be performed to clarify this discrepancy.

Second, UNC-78 inhibits the elongation phase of actin polymerization (Fig. 5), while *Xenopus* AIP1 shortens the initial lag phase and accelerates the elongation rate (Okada et al., 1999). This property of *Xenopus* AIP1 could be explained by its barbed end capping activity (Okada et al., 2002) that might be accountable for stabilizing actin nuclei as demonstrated for gelsolin (Yin et al., 1981) and capping protein (Isenberg et al., 1980). We preliminarily observed that UNC-78 inhibited actin polymerization from F-actin seeds as observed for *Xenopus* AIP1 (Okada et al., 2002), suggesting that UNC-78 also caps barbed ends. However, we were not able to detect capping by UNC-78 in a nucleation assay using red cell membranes (Pinder et al., 1986) (data not shown). If this is because of relatively low concentration of the barbed ends in this assay, the capping activity of UNC-78 might be weak and not be able to stabilize the actin nuclei efficiently during spontaneous polymerization. Therefore, the apparent difference could be due to small differences in the activities of *Xenopus* AIP1 and UNC-78 and/or ADF/cofilin proteins from different species.

Using mutant forms of UNC-60B, we obtained evidence that filament binding by UNC-60B is important for UNC-78 to enhance filament disassembly. Mutational studies on various ADF/cofilin proteins revealed two actin-binding surfaces: one that is essential for both G- and F-actin binding and a second for F-actin. The G/F-actin binding site (G-site) includes the N terminus (Lappalainen et al., 1997; Pope et al., 2000), a portion of helix α3 (Lappalainen and Drubin, 1997; Moriyama and Yahara, 1999; Moriyama et al., 1992; Yonezawa et al., 1991b), and the turn connecting strand β6 and helix α4 (Lappalainen et al., 1997), while the F-actin binding site (F-site) includes a loop connecting β2 and β3 in mammalian ADF/cofilin and at a similar position in yeast cofilin (Lappalainen et al., 1997; Moriyama and Yahara, 2002a; Pope et al., 2000) and the C-terminal residues (Lappalainen et al., 1997; Ono et al., 2001). Truncation of the three C-terminal residues of UNC-60B abolishes F-actin binding but not G-actin binding (Ono et al., 1999; Ono et al., 2001) and this mutant failed to support UNC-78-enhanced filament disassembly (Fig. 6F). This suggests that the F-site of UNC-60B is required for the UNC-78 activity. Mutations in the G-site of UNC-60B also inhibited its ability to support UNC-78-enhanced disassembly (Fig. 6D,E). However,

mutations in the G-site also affect F-actin binding (Ono et al., 1999; Pope et al., 2000; Ressad et al., 1998). Therefore, the reduced effect of these mutants may reflect overall reduction of affinity with actin and not be a defect only in monomer binding. Interestingly, the S112F mutation causes hyper severing and depolymerizing activity (Ono et al., 1999), but UNC-78 was able to enhance disassembly only weakly in the presence of this mutant. This suggests that the S112F mutant had a different conformation from wild type or induced different F-actin conformation, so that UNC-78 was not able to recognize the altered structure.

We found that UNC-60B is the preferential ADF/cofilin isoform for UNC-78 to disassemble filaments. This is a novel isoform-specific function for ADF/cofilins, suggesting that ADF/cofilin and AIP1 regulate actin dynamics in an isoform-specific manner. We identified that the C terminus of UNC-60B is a critical determinant for the UNC-78 activity. This region is required for filament binding and severing by UNC-60B, but not for monomer binding (Ono et al., 2001). This is also the region where the sequence is quite different between UNC-60B and UNC-60A (McKim et al., 1994) and among other ADF/cofilin proteins (Bamburg et al., 1999; Bowman et al., 2000; Maciver and Hussey, 2002). Indeed, the mapping study of the AIP1-interacting sites on yeast cofilin identified that a cluster of charged residues at the C terminus of yeast cofilin (a part of the F-site) was required for two-hybrid interaction with AIP1 but not with actin (Rodal et al., 1999). UNC-60B changes the twist of actin filament to a similar extent as mammalian ADF/cofilins (McGough et al., 1997; Ono et al., 2001). Therefore, the C terminus of UNC-60B may provide a part of a binding site for UNC-78 or induce a unique filament structure upon filament binding.

The preference of isoforms is consistent with our previous observations that both UNC-60B and UNC-78 are required for actin organization in body wall muscle cells (Ono, 2001; Ono et al., 1999) and current observation that UNC-78 is expressed in body wall muscle (Figs 8, 9). Cytoplasmic concentrations of actin, UNC-60B and UNC-78 in *C. elegans* muscle cells are unknown because of technical difficulty in dissecting tissues from worms. In yeasts, AIP1 is relatively abundant and present at a 1:1 ratio with cofilin and at 10-20% of actin (Rodal et al., 1999). If the *C. elegans* muscle cells express equivalent amounts of UNC-60B and UNC-78, they will be sufficient to induce extensive filament disassembly. Therefore, the function of UNC-78/AIP1 might be to collaborate with UNC-60B/cofilin and enhance actin reorganization during assembly and maintenance of myofibrils. However, since mature myofibrils are relatively stable structures, activity of UNC-78 could be regulated in mature muscle.

We also observed relatively strong expression of UNC-78 in the pharynx. However, pharyngeal morphology and activity in *unc-78* null mutants appeared normal (S. Ono, unpublished observation). In addition, UNC-60B is not detected in the pharynx (Ono et al., 2003; Ono et al., 1999). It is possible that the second AIP1 isoform, K08F9.2, is expressed in the pharynx and has a redundant function. It will also be interesting to determine whether this second isoform has different activity and/or preference for ADF/cofilin isoforms from UNC-78. The function of K08F9.2 is currently unknown, since large-scale RNA interference projects yielded no phenotypes for this gene (Kamath et al., 2003; Piano et al., 2002).

Our observation that UNC-78 is co-localized with actin in *unc-60B* null mutants suggests a novel mechanism of interaction between UNC-78 and actin. In a co-pelleting assay, UNC-78 poorly co-precipitates with F-actin in the absence of UNC-60B. Therefore, an unknown protein(s) may mediate binding of UNC-78 to F-actin in muscle cells. In yeast, AIP1 is localized to cortical actin patches, but this association is disrupted in cofilin mutant (*cof1-19*) cells (Rodal et al., 1999), suggesting that cofilin is required for co-localization of AIP1 with actin. However, the *cof-1-19* mutation (R109A, R110A) does not disrupt two-hybrid interactions of cofilin with actin or AIP1 (Rodal et al., 1999) or cause an apparent phenotype in actin organization (Lappalainen et al., 1997). Therefore, this particular cofilin mutation might indirectly affect localization of AIP1. Localization of AIP1 in cofilin-null yeast cells has not been tested because cofilin is essential for viability. The presence of another protein(s) that mediates the AIP1-actin interaction is also supported by previous reports that partially purified *Physarum* AIP1 (p66) co-precipitates with F-actin but highly purified p66 does not (Matsumoto et al., 1998; Shimada et al., 1992).

Multicellular organisms express multiple ADF/cofilin isoforms with different tissue distribution (Bamburg, 1999). In *C. elegans*, UNC-60A and UNC-60B are expressed in different tissues and involved in distinct morphogenetic processes (Ono et al., 2003). In vertebrates, three isoforms, ADF/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; Vartiainen et al., 2002) are expressed in different patterns of tissue distribution with some overlaps. The *C. elegans* and vertebrate ADF/cofilin isoforms have different activities to depolymerize actin filaments (Ono and Benian, 1998; Vartiainen et al., 2002; Yeoh et al., 2002), which may be important for regulation of actin dynamics to different extents. However, our results suggest AIP1 and, possibly, other proteins can influence ADF/cofilin-mediated actin filament dynamics in an isoform-specific manner in multicellular organisms.

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