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ATP-dependent regulation of actin monomer-filament equilibrium by cyclase-associated protein and ADF/cofilin

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SYNOPSIS

Cyclase-associated protein (CAP) is a conserved regulator of actin filament dynamics. In the nematode *Caenorhabditis elegans*, CAS-1 is an isoform of CAP that is expressed in striated muscle and regulates sarcomeric actin assembly. Here, we report that CAS-2, a second CAP isoform in *C. elegans*, attenuates the actin-monomer-sequestering effect of actin depolymerizing factor (ADF)/cofilin to increase steady-state levels of actin filaments in an ATP-dependent manner. CAS-2 binds to actin monomers without a strong preference to either ATP- or ADP-actin. CAS-2 strongly enhances exchange of actin-bound nucleotides even in the presence of UNC-60A, which is a *C. elegans* ADF/cofilin that inhibits nucleotide exchange. UNC-60A induces depolymerization of actin filaments and sequesters actin monomers, whereas CAS-2 reverses the monomer-sequestering effect of UNC-60A in the presence of ATP but not in the presence of only ADP or absence of ATP or ADP. A 1:100 molar ratio of CAS-2 to UNC-60A is sufficient to increase actin filaments. CAS-2 has two independent actin-binding sites in the amino- and carboxyl-terminal halves, and the carboxyl-terminal half is necessary and sufficient for the observed activities of the full-length CAS-2. These results suggest that CAS-2 (CAP) and UNC-60A (ADF/cofilin) are important in ATP-dependent regulation of actin monomer-filament equilibrium.

Keywords

Actin dynamics; polymerization; depolymerization; nucleotide exchange; ADF/cofilin; cyclase-associated protein

INTRODUCTION

Modulation of actin filament turnover is critical for many cell biological events. Actin by itself can undergo turnover or treadmilling. However, the rate of spontaneous actin turnover is very slow and it needs to be accelerated to account for most of dynamic cellular processes involving rapid dynamics of the actin cytoskeleton. Severing and depolymerization of actin

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AUTHOR CONTRIBUTION

Kazumi Nomura designed and performed the experiments and analyzed the data. Shoichiro Ono developed the study, designed the experiments, analyzed the data, and wrote the paper.

filaments are important mechanisms to enhance actin filament dynamics [1], as slow dissociation of actin monomers from filaments is a rate-limiting step of actin turnover. Actin depolymerizing factor (ADF)/cofilin is a major class of conserved actin-regulatory proteins that enhance actin filament turnover by severing and depolymerizing actin filaments. ADF/cofilin is sufficient to promote the rate of actin turnover *in vitro*. However, when ADF/cofilin is present at excessive amounts, it is not effective in promoting actin turnover. ADF/cofilin preferentially binds to ADP-actin and inhibits exchange of actin-bound ADP/ATP [2, 3], and some ADF/cofilin isoforms sequester actin monomers and prevent polymerization [4, 5]. Therefore, under certain conditions, ADF/cofilin can cause accumulation of ADP-actin that is much less competent for polymerization than ATP-actin and does not support treadmilling of actin filaments.

Profilin is a factor that can overcome some of the inhibitory effects of ADF/cofilin on actin dynamics and promote recycling of actin monomers for new rounds of polymerization at the barbed ends. Profilin competes with ADF/cofilin for G-actin binding and enhances exchange of actin-bound nucleotides [6, 7], and profilin-bound actin monomers can contribute to elongation of actin filaments from the barbed ends [8]. As a result, profilin and ADF/cofilin can synergistically promote actin turnover [6, 7]. However, profilin binds to ADP-actin with relatively low affinity [8], and yeast profilin fails to promote nucleotide exchange when ADF/cofilin is tightly bound to ADP-actin [9]. Therefore, profilin may not be an efficient nucleotide exchange factor immediately after ADF/cofilin depolymerizes actin filaments. In addition, plant profilins do not enhance nucleotide exchange [10, 11], indicating that this is not a conserved function.

Cyclase-associated protein (CAP) also enhances exchange of actin-bound ADP for ATP and promotes recycling of ADF/cofilin-bound actin monomers for polymerization [12–15]. CAP has multiple domains with distinct functions. Studies on human CAP1 and yeast CAP (Srv2/CAP) show that the N-terminal helical-folded domain binds to ADF/cofilin-actin complex, and that the central Wiscott Aldrich syndrome protein homology 2 (WH2) domain and the C-terminal CAPs and X-linked retinitis pigmentosa 2 protein (CARP) domain [16] mediate nucleotide exchange [9, 12, 14, 15]. However, the mechanism by which CAP promotes actin turnover remains elusive. Yeast Srv2/CAP preferentially binds to ADP-actin over ATP-actin [17], which is one of the bases of the current model in which CAP as an initial processor of ADF/cofilin-bound ADP-actin monomers before handing recharged ATP-actin monomers to profilin [14]. However, *Arabidopsis* CAP and *C. elegans* CAP (CAS-1) do not have a strong preference to either ADP-actin or ATP-actin but still cooperate with ADF/cofilin to promote actin turnover [13, 18], suggesting that strong binding of CAP to ADP-actin is not critical. In addition, how the two functional domains in the N- and C-terminal halves coordinate to induce dissociation of ADF/cofilin from actin and exchange of actin-bound nucleotides remains unknown. Recent studies have shown that CAP promotes severing of actin filaments [19–21], but how this activity contributes to cytoskeletal regulation is not clearly understood.

CAP was originally identified in yeast as an adenylyl cyclase-associated protein that is involved in the Ras-cAMP signaling [22, 23]. Subsequent studies have shown that CAP is also a regulator of actin filament dynamics in a wide variety of cell types in different

organisms. Vertebrates have two CAP isoforms, CAP1 and CAP2 [24]. CAP1 is widely expressed in non-muscle tissues, while CAP2 is predominantly expressed in heart and skeletal muscle [25]. Nevertheless, whether CAP1 and CAP2 have different functions is currently unknown. The nematode *Caenorhabditis elegans* also has two CAP isoforms, CAS-1 and CAS-2. CAS-1 is specifically expressed in striated muscle and several other tissues and required for sarcomeric actin organization [18]. In contrast, mRNA of CAS-2 is enriched in non-muscle tissues as reported in the Nematode Expression Pattern Database [26], but its function remains uncharacterized. In this study, we characterized biochemical properties of CAS-2 and found that, unlike CAS-1, the C-terminal half of CAS-2 containing WH2 and C-terminal CARP domain has equivalent activities to the full-length protein in G-actin binding and nucleotide exchange. Moreover, we found that CAS-2 antagonizes ADF/cofilin and shifts actin monomer-filament equilibrium to increase filamentous actin in an ATP-dependent manner. These observations suggest a new function of CAP and ADF/cofilin in the ATP-dependent regulation of actin filament dynamics.

EXPERIMENTAL

Proteins and materials

Rabbit muscle actin was purified from acetone powder (Pel-Freeze Biologicals) as described [27]. G-actin was further purified by gel filtration by Sephacryl S-300 in G-buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM CaCl₂, 0.2 mM dithiothreitol, pH 8.0). Pyrene-labeled rabbit muscle actin was prepared as described [28]. ADP-G-actin was prepared using hexokinase (Worthington Biochemical Corp.) as described previously [18]. UNC-60A was expressed in *Escherichia coli* and purified as described [29]. The bacterial expression vector for chicken capping protein (CapZ) was kindly provided by Dr. Takashi Obinata (Chiba University, Japan), and it was expressed in *E. coli* and purified as described [30]. Latrunculin A was purchased from Enzo Life Sciences, Inc.

Expression and purification of recombinant CAS-2 proteins

The full-length protein coding sequence of CAS-2 was amplified from a cDNA clone yk1478g02 (kindly provided by Yuji Kohara, Mishima, Shizuoka, Japan), and cloned into the pGEX-2T vector. Originally, we attempted bacterial expression of CAS-2 using a glutathione S-transferase-fusion system, but solubility of recombinant fusion proteins was very poor (our unpublished observations). Then, full-length CAS-2, CAS-2N (residues 1 – 207), CAS-2C (residues 208 – 457), or CAS-2C WH2 (residues 275 – 457) sequences were re-cloned into the pDEST-HisMBP, a vector for bacterial expression as fusion proteins with maltose-binding protein (MBP) with an N-terminal histidine-tag as described previously using the Gateway® technology with Clonase™ II (Invitrogen) [31]. pDEST-HisMBP was developed by the group of Dr. David Waugh [31] and obtained through Addgene. The protein coding sequences were verified by DNA sequencing. *E. coli* BL21(DE3) was transformed with the expression vectors, and protein expression was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside for 3 hr at room temperature. The cells were harvested by centrifugation at 5000 × g for 10 min and disrupted by a French pressure cell at 360 – 580 kg/cm² in phosphate-buffered saline. The homogenates were cleared at 20000 × g for 15 min and applied to a His60 Ni Superflow™ column (Clontech) and washed with 150

mM NaCl, 3 mM imidazole, 50 mM Na-PO₄, pH 7.9 to remove unbound proteins. Bound proteins were eluted with 150 mM NaCl, 250 mM imidazole, 50 mM Na-PO₄, pH 7.9. MBP-CAS-2 (full-length) was further purified with a HiPrep 26/60 Sephacryl S-300 column (GE Healthcare), while MBP-CAS-2N, MBP-CAS-2C, and MBP-CAS-2C WH2 were further purified with a HiTrapQ FF column (GE Healthcare). MBP was used without additional chromatographic purification. Finally, purified proteins were dialyzed against F-buffer (0.1 M KCl, 20 mM Hepes-NaOH, pH 7.5, 2 mM MgCl₂ and 0.2 mM DTT) containing 50 % glycerol and stored at -20 °C.

Monitoring kinetics of actin polymerization

Kinetics of actin polymerization was monitored by measuring fluorescence of pyrene-labeled actin. Pyrene-labeled G-actin (final 5 μ M, 9 % labeled) was mixed with MBP-CAS-2, MBP-CAS-2N, MBP-CAS-2C, MBP-CAS-2C WH2, or MBP in G buffer, and polymerization was initiated by adding salts to final concentrations of 0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, 20 mM Hepes-NaOH, pH 7.5. Fluorescence of pyrene (excitation at 365 nm and emission at 407 nm) was monitored for 10 min with an F-4500 fluorescence spectrophotometer (Hitachi High-Technologies).

Determination of apparent critical concentration of actin

Varying concentrations of pyrene-labeled ATP- or ADP-G-actin (20 % labeled) were polymerized in 0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, 20 mM Hepes-NaOH, 0.2 mM DTT, 0.2 mM ATP or ADP, pH 7.5 for 18 hr (2 h for ADP-G-actin) at room temperature. Fluorescence of pyrene (excitation at 366 nm and emission at 384 nm) was measured with an F-4500 fluorescence spectrophotometer (Hitachi High-Technologies). Equilibrium dissociation constant for CAS-2 binding to G-actin was calculated from the change in the apparent critical concentration as described [32].

Assays for exchange of actin-bound nucleotides

Effects of CAS-2 variants and UNC-60A on the exchange rate of actin-bound nucleotides were examined by monitoring increase in the fluorescence of etheno-ATP that is associated with G-actin binding. ADP-G-actin (1.3 μ M) was prepared in 150 μ l of G-buffer without ATP, and then 50 μ l of etheno-ATP (160 μ M) with or without CAS-2 variants and UNC-60A was mixed. Final concentrations of G-actin and UNC-60A were both 1 μ M. Then, fluorescence of etheno-ATP (excitation at 360 nm and emission at 410 nm) was monitored for 10 min with an F-4500 fluorescence spectrophotometer (Hitachi High-Technologies). The exponential rates (k_{obs}) were calculated by curve fitting using SigmaPlot 10 (Systat Software).

F-actin sedimentation assays

F-actin (10 μ M) in F-buffer (0.1 M KCl, 2 mM MgCl₂, 0.2 mM dithiothreitol, 20 mM Hepes-NaOH, pH 7.5) with or without ATP or ADP (concentrations are indicated in Figure legends) was pre-incubated with 20 μ M UNC-60A for 30 min at room temperature. Then, varying concentrations of MBP-CAS-2, MBP-CAS-2N, MBP-CAS2C, MBP-CAS-2C WH2 or MBP were added to the mixtures and incubated for 30 min at room

temperature. The mixtures were ultracentrifuged at $436,000 \times g$ for 15 min at 20 °C. Supernatant and pellet fractions were adjusted to the same volumes and subjected to SDS-PAGE and staining with Coomassie Brilliant Blue R-250 (National Diagnostics). Gels were scanned by an Epson Perfection V700 photo scanner at 300 dots per inch, and band intensity was quantified using Image J. When the effects of latrunculin A (20 μ M) or capping protein (0.1 μ M) were examined, these were included at the time of assembling the preincubation mixtures.

Light scattering assays

5.2 μ M F-actin in F-buffer with or without 0.5 mM ATP or ADP was mixed with 10.3 μ M UNC-60A at time 0 to initiate depolymerization, and light scattering at an angle of 90° and a wavelength of 500 nm was measured over time with an F-4500 fluorescence spectrophotometer (Hitachi High-Technologies). After 30 min (1800 sec), varying concentrations of MBP-CAS-2, MBP-CAS2N, MBP-CAS2C, MBP-CAS-2C WH2, or MBP were added (final 5 μ M F-actin and 10 μ M UNC-60A), and light scattering was measured for another 30 min. Slit width was set at 5 nm each for excitation and emission.

RESULTS

CAS-2 is a second isoform of cyclase-associated protein in *C. elegans*

The *C. elegans* *C18E3.6* gene (GenBank accession number CCD65141) on chromosome I encodes a previously uncharacterized protein of 457 amino acids. Its sequence is homologous to cyclase-associated protein (CAP) and 41 % identical to CAS-1, a muscle isoform of CAP in *C. elegans* [18]. Therefore, we designated this gene as *cas-2* (*cyclase-associated protein-2*) as a second CAP isoform in *C. elegans*. Predicted domain structure of CAS-2 is similar to that of CAS-1 with an N-terminal helical folded domain (HFD), a proline-rich region (P), a Wiscott Aldrich syndrome protein-homology 2 (WH2) domain, and a C-terminal CAPs and the X-linked retinitis pigmentosa 2 protein (CARP) domain (Fig. 1A). Vertebrates also have two CAP isoforms: CAP1 (a non-muscle isoform) and CAP2 (a muscle isoform) [24]. However, our previous phylogenetic analysis suggested that the two *C. elegans* CAP isoforms have evolved separately from the vertebrate isoforms [18].

CAS-2 binds to actin monomers

To determine whether CAS-2 has actin-regulatory functions, we produced recombinant CAS-2 proteins and characterized their properties *in vitro*. We have shown that CAS-1 has two independent actin-binding sites in the N- and C-terminal halves [18]. Therefore, in addition to the full-length CAS-2 protein (residues 1–457), we made the N-terminal half (CAS-2N) (residues 1–207), the C-terminal half (CAS-2C) (residues 208–457), and the C-terminal half lacking WH2 (CAS-2C WH2) (residues 275–457) (Fig. 1A). These proteins were expressed as fusion proteins with maltose binding protein (MBP). Since cleavage of MBP by TEV protease was very inefficient, we used MBP-fusion proteins (Fig. 1B, lanes 2–5) in the following biochemical experiments and included purified MBP (Fig. 1B, lane 1) in control experiments.

Binding of CAS-2 to actin monomers was examined by two methods. First, we examined effects of CAS-2 on the initial phase of spontaneous actin polymerization from G-actin (Fig. 2). MBP-CAS-2 inhibited actin polymerization in a concentration-dependent manner (Fig. 2B), while a high concentration of MBP (15 μ M) had no effect on actin polymerization (Fig. 2A). These results suggest that CAS-2 bound to G-actin and inhibited initial nucleation and elongation, as previously reported for yeast Srv2/CAP [9, 33]. While MBP-CAS-2N showed no detectable effects on actin polymerization (Fig. 2C), MBP-CAS-2C had nearly as strong effect as MBP-CAS-2 (Fig. 2D). MBP-CAS-2C WH2 also inhibited the initial phase of actin polymerization (Fig. 2E) but required higher concentrations than MBP-CAS-2C to cause comparable effects (compare with Fig. 2D). These results show that the C-terminal CARP domain of CAS-2 is necessary and sufficient for binding to G-actin, and that WH2 enhances G-actin binding.

Next, we examined effects of CAS-2 on the apparent critical concentration (C_c) of ATP- and ADP-actin (Fig. 3). Various concentrations of pyrene-labeled G-actin were polymerized in the absence or presence of 2 or 5 μ M MBP, MBP-CAS-2, MBP-CAS-2N, MBP-CAS-2C, or MBP-CAS-2C WH2 and steady-state levels of polymerized actin were quantified by the pyrene fluorescence. Above the C_c , amounts of polymerized actin were linearly correlated with the concentrations of total actin (Fig. 3). The C_c values of actin alone were 0.15 μ M for ATP-actin (Fig. 3A, white circles) and 2.5 μ M for ADP-actin (Fig. 3B, white circles). MBP did not affect these values (Fig. 3A and B, black circles). MBP-CAS-2 shifted C_c to higher values for both ATP-actin (Fig. 3A, Table 1) and ADP-actin (Fig. 3B, Table 1), indicating that CAS-2 bound to G-actin and sequestered it from polymerization. From the shifts in C_c , dissociation constant for binding of MBP-CAS-2 to G-actin was estimated to be 0.60 – 0.88 μ M for ATP-actin and 0.83 – 2.3 μ M for ADP-actin (Table 1), suggesting that their binding is not strongly influenced by the actin-bound nucleotides. MBP-CAS-2N only altered C_c of ADP-actin with K_d of 0.83 – 2.9 μ M (Fig. 3D) without affecting C_c of ATP-actin (Fig. 3C). MBP-CAS-2C shifted C_c to higher values (Fig. 3E and F) and did not exhibit a strong preference for binding to ATP-actin (K_d = 0.95 – 1.0 μ M) or ADP-actin (K_d = 1.7 – 2.9 μ M) (Table 1) in a similar manner to MBP-CAS-2. However, MBP-CAS-2C WH2 only slightly shifted C_c of ATP-actin (K_d = 2.9 – 3.6 μ M) (Fig. 3G), but shifted C_c of ADP-actin to similar extents to MBP-CAS-2 (K_d = 1.2 – 1.7 μ M) (Fig. 3H). Thus, CAS-2 also has at least two separate G-actin binding sites; the N-terminal site that preferentially binds to ADP-actin and the C-terminal site that binds to both ATP- and ADP-actin. Furthermore, WH2 of CAS-2 appears to enhance binding to ATP-actin, which is similar to WH2 in yeast Srv2/CAP and human CAP1 [9, 15]

CAS-2 antagonizes ADF/cofilin (UNC-60A) to promote exchange of actin-bound nucleotides

As previously demonstrated for *C. elegans* CAS-1 [18], CAS-2 also accelerated the rate of exchange of actin-bound nucleotides (Fig. 4). ADP-bound G-actin (1 μ M) was incubated with various concentrations of MBP-CAS-1 in the presence of etheno-ATP whose fluorescence is increased upon binding to actin. Changes in its fluorescence were monitored over time, and rates of exchange [k_{obs} (s^{-1})] were determined (Fig. 4). UNC-60A strongly inhibited nucleotide exchange on ADP-G-actin (Fig. 4, compare first and second bars), and

MBP had no effect on this inhibition (Fig. 4, third bar). However, MBP-CAS-2 relieved this inhibition and accelerated nucleotide exchange in a concentration-dependent manner (Fig. 4, fourth and fifth bars). In the presence of 0.5 μ M MBP-CAS-2, rate of nucleotide exchange was faster than that of ADP-G-actin alone. MBP-CAS-2 also enhanced nucleotide exchange on ADP-G-actin or ATP-G-actin in the absence of UNC-60A indicating that CAS-2 is capable of enhancing nucleotide exchange on G-actin (K. Nomura and S. Ono, unpublished observations).

Among the truncated CAS-2 fragments tested, MBP-CAS-2N did not enhance nucleotide exchange (Fig. 4, sixth bar), while MBP-CAS-2C enhanced nucleotide exchange nearly as strongly as MBP-CAS-2 (Fig. 4, seventh and eighth bars). However, MBP-CAS-2C WH2 failed to promote nucleotide exchange in the presence of UNC-60A (Fig. 4, ninth bar) as well as in the absence of UNC-60A (K. Nomura and S. Ono, unpublished observations). Therefore, the C-terminal half of CAS-2 is necessary and sufficient for enhancement of exchange of actin-bound nucleotides, and WH2 plays an essential role in this function. This is in contrast to *C. elegans* CAS-1 in which the C-terminal half is necessary but not sufficient for enhancement of nucleotide exchange [18]. These results indicate that CAS-2 is a strong nucleotide exchange factor for G-actin that functions antagonistically to ADF/cofilin.

CAS-2 antagonizes UNC-60A to reduce actin monomer sequestration in an ATP-dependent manner

Next, we examined how CAS-2 influences UNC-60A-mediated actin filament dynamics *in vitro*. UNC-60A is a somewhat unusual member of the ADF/cofilin family, since it has very weak actin-filament severing activity and strong actin-monomer sequestering activity [5, 29]. Under physiological conditions including 0.5 mM ATP, less than 5 % of control actin (10 μ M) remained in the supernatant after ultracentrifugation (Fig. 5A, lanes 1 and 2). UNC-60A (20 μ M) promoted actin depolymerization and increased actin in the supernatant to ~ 60 % of total actin (Fig. 5A, lanes 3 and 4). To determine the effect of MBP-CAS-2, F-actin was pre-incubated with UNC-60A for 30 min, and then MBP-CAS-2 was added and incubated for another 30 min, which was followed by ultracentrifugation. Although MBP did not affect UNC-60A-induced actin depolymerization (Fig. 5A, lanes 5 and 6), addition of 0.2 μ M MBP-CAS-2 significantly decreased actin in the supernatant to ~30 % of total actin (Fig. 5A, lanes 7 and 8). MBP-CAS-2N did not alter the amount of actin in the supernatants (Fig. 5A, lanes 9 and 10), whereas MBP-CAS-2C had nearly equal activity as MBP-CAS-2 (Fig. 5A lanes 11 and 12). MBP-CAS-2C WH2 at 0.2 μ M did not alter the distribution of actin (Fig. 5A, lanes 13 and 14). By testing various concentrations of MBP-CAS-2, 0.1 – 1 μ M of MBP-CAS-2 were sufficient to cause a decrease in actin in the supernatants in the presence of 10 μ M actin and 20 μ M UNC-60A (Fig. 5B, white circles), and MBP-CAS-2C exhibited a similar optimal concentration range (Fig. 5B, white squares). At a high concentration (5 μ M), the effects of MBP-CAS-2 and MBP-CAS-2C were diminished (Fig. 5B), most likely due to their own actin-monomer sequestering activities as shown in Fig. 3 thereby increasing actin in the supernatants. MBP-CAS-2C WH2 showed weak activity to decrease actin in the supernatants at high concentrations (2–5 μ M) (Fig. 5B,

white diamonds), indicating that removal of WH2 does not completely abolish but significantly weakens this function.

The effect of CAS-2 to reduce UNC-60A-sequestered actin was dependent on ATP (Fig. 5). Under ATP/ADP-free conditions, neither MBP-CAS-2 nor MBP-CAS-2C decreased actin in the supernatants (Fig. 5C and D). MBP, MBP-CAS-2N, and MBP-CAS-2C WH2 also did not have significant effects (Fig. 5C and D). Similarly, in the presence of 0.5 mM ADP, MBP or none of the MBP-CAS-2 variants reduced actin in the supernatants in the presence of UNC-60A (Fig. 5E and F). Pelleting assays in the presence of various concentrations of ATP showed that both MBP-CAS-2 and MBP-CAS-2C required >0.1 mM ATP to relieve actin-monomer sequestration by 20 μ M UNC-60A (Fig. 5G, white circles and squares). The weak activity by a high concentration (2 μ M) of MBP-CAS-2C WH2 was not further enhanced by increasing ATP concentrations (Fig. 5, white diamonds).

The effect of CAS-2 to antagonize UNC-60A was prevented by latrunculin A, an actin-monomer sequestering drug (Fig. 6A, lanes 5 – 8, and Fig. 6B), indicating that MBP-CAS-2 did not inhibit UNC-60A-induced actin depolymerization. Similarly, the effect of MBP-CAS-2C was inhibited by latrunculin A (Fig. 6A, lanes 13 – 16, and Fig. 6B). MBP and MBP-CAS-2N did not affect UNC-60A-mediated actin depolymerization in the absence or presence of latrunculin A (Fig. 6A and B). These results indicate that MBP-CAS-2 and MBP-CAS-2C contribute to increasing F-actin after UNC-60A depolymerizes actin filaments, rather than by inhibition of UNC-60A-induced depolymerization. Furthermore, the effects of MBP-CAS-2 and MBP-CAS-2C were significantly, but not completely, inhibited by capping protein (CapZ) (Fig. 6C and D), suggesting that CAS-2-induced increase in pelletable actin depends on association of actin monomers to the barbed ends of pre-existing actin filaments.

To better understand how CAS-2 antagonizes UNC-60A in actin depolymerization and polymerization, UNC-60A and MBP-CAS-2 were sequentially incubated with actin filaments, and time course of depolymerization and polymerization was monitored by the light scattering assay (Fig. 7). UNC-60A (10 μ M) was mixed with F-actin (5 μ M) at time 0, and actin depolymerization was detected by the decrease in light scattering (Fig. 7A–G). After 30 min (1800 sec), MBP, MBP-CAS-2, MBP-CAS-2N, MBP-CAS-2C, or MBP-CAS-2C WH2 was added, and light scattering was continuously measured. In the presence of ATP, 0.5 μ M MBP did not affect the light scattering (Fig. 7A), while MBP-CAS-2 (at 0.2 and 0.5 μ M) enhanced light scattering immediately after its addition indicating that actin polymerization was induced after depolymerization by UNC-60A (Fig. 7B). MBP-CAS-2N did not have an effect (Fig. 7C), while MBP-CAS-2C (> 0.1 μ M) triggered polymerization (Fig. 7D). A high concentration (3.7 μ M) of MBP-CAS-2C WH2 did not strongly induce polymerization (Fig. 7E), which is consistent with its weak effect in the sedimentation assays (Fig. 5B). These results indicate that, in the presence of ATP, CAS-2 can release actin monomers for polymerization after depolymerization by UNC-60A. However, in the absence of ATP or ADP (Fig. 7F) or in the presence of only ADP (Fig. 7G), MBP and all MBP-CAS-2 variants did not affect polymerization or depolymerization, confirming that the effect of CAS-2 is ATP-dependent and that the C-terminal half of CAS-2 containing WH2 and CARP is necessary and sufficient for this function.

DISCUSSION

We report that the *C. elegans cas-2* gene encodes a second isoform of cyclase-associated protein that has actin regulatory activities *in vitro*. CAS-2 binds to both ATP-actin and ADP-actin and strongly promotes exchange of actin-bound nucleotides. The nucleotide exchange activity of CAS-2 is antagonistic to UNC-60A (ADF/cofilin). As a result, CAS-2 can shift actin monomer-filament equilibrium from UNC-60A-sequestered G-actin to increased F-actin in the presence of ATP. Importantly, CAS-2-mediated increase in F-actin is not induced in the absence of ATP or in the presence of only ADP, suggesting that the role of CAS-2 is to recharge UNC-60A-depolymerized actin monomers with ATP and release UNC-60A from actin monomers for subsequent actin polymerization.

Based on these results, we hypothesized a model of regulation of actin dynamics and monomer-filament equilibrium by CAS-2 and UNC-60A (Fig. 8). In the presence of ADP-G-actin, UNC-60A, and CAS-2 (Fig. 8A), ADP-F-actin can be formed but will not be stable, as UNC-60A depolymerizes and sequesters ADP-G-actin. When a CAS-2 concentration is low, the CAS-2-ADP-G-actin complex should be negligible without contributing to monomer sequestration. ADP-G-actin will be rapidly converted to ATP-G-actin by CAS-2 in a catalytic manner, if free ATP is available. UNC-60A binds to ATP-G-actin with low affinity, which allows ATP-G-actin to polymerize into ATP-F-actin. A recent report shows that actin mutations that enhance nucleotide exchange are sufficient to repel ADF/cofilin from G-actin [34]. ATP hydrolysis by F-actin increases ADP-F-actin and promotes subsequent depolymerization and monomer sequestration by UNC-60A. Thus, we predict that CAS-2 and UNC-60A cooperate to promote ATP-dependent actin filament dynamics (Fig. 8A). However, if CAS-2 is absent, UNC-60A sequesters ADP-G-actin and inhibits nucleotide exchange on ADP-G-actin (Fig. 8B). Therefore, the cycle of actin dynamics will not be promoted.

These results suggest a new aspect of the role of CAP in actin filament turnover. Previous studies on mammalian CAP1 and yeast Srv2/CAP propose models in which the N-terminus of CAP binds and dissociates the G-actin-ADF/cofilin complex and the C-terminus of CAP enhances nucleotide exchange on G-actin [12, 14]. Our results are inconsistent with these models in that CAS-2 does not require the N-terminal helical-folded domain for enhancing actin turnover in the presence of UNC-60A. In addition, we were not able to detect binding of MBP-CAS-2N to the G-actin-UNC-60A complex in a pull-down assay (our unpublished data) in similar experiments that we reported previously for CAS-1 [18]. Therefore, a role of the N-terminal domain of CAS-2 is currently unclear. Recent studies show that CAP promotes actin filament severing [19, 20], which is mediated by its N-terminal domain [21]. Unlike human CAP1 that directly binds to F-actin [19], MBP-CAS-2 did not co-sediment with F-actin in a range of pH 6 – 8 (our unpublished results). Whether *C. elegans* CAS-1 and CAS-2 have similar actin-severing activity is currently unknown and will be investigated in the future.

The antagonistic role of CAS-2 against UNC-60A-mediated actin depolymerization is likely to be a key mechanism to regulate dynamic reorganization of the actin cytoskeleton. In *C. elegans*, UNC-60A is widely expressed in non-muscle tissues and essential for embryonic

cytokinesis [35] and assembly of contractile actin networks in the somatic gonad [36]. However, UNC-60A is an unusual member of the ADF/cofilin family as it primarily binds to actin monomers and exhibits strong actin-monomer sequestering activity *in vitro* [5, 29]. UNC-60A can enhance actin depolymerization and initiate actin reorganization. However, in order to assemble new cytoskeletal structures, actin polymerization needs to be promoted from the UNC-60A-sequestered monomer pool. Our observations strongly suggest that CAS-2 can play a role to release actin monomers for assembly of new actin filaments and/or elongation of pre-existing filaments after old filaments are disassembled by UNC-60A. We have not been successful in precisely determining expression patterns of CAS-2 in *C. elegans* due to lack of antibody and functional transgenic expression constructs for CAS-2. However, a previous study suggested that CAS-2 is expressed in non-muscle tissues. Messenger RNA of CAS-2 is enriched in the distal part of the hermaphroditic gonad as reported in the Nematode Expression Pattern Database (<http://nematode.lab.nig.ac.jp>) [26]. The *cas-2* gene is in an operon that also contains *ppw-1*, a gene encoding a protein of the Argonaute family essential for RNA interference in the germline [37]. Genes in the same operon are often co-expressed, suggesting that both *ppw-1* and *cas-2* are expressed in the germline. We are currently investigating *in vivo* functions of CAS-2 and its cooperation with UNC-60A.

Biochemical characterization of the two *C. elegans* CAP isoforms, CAS-1 and CAS-2, revealed a functional difference in their C-terminal halves. We compared the sequences of CAS-1 and CAS-2 and designed C-terminal fragments such that each contains the proline-rich domain, the WH2 domain, and the putative actin-binding domain. The C-terminal half of CAS-1 (CAS-1C) binds to G-actin but is not sufficient for enhancing nucleotide exchange on G-actin [18], while CAS-2C is sufficient for both G-actin binding and nucleotide exchange (this study). An additional C-terminal fragment of CAS-2 lacking WH2 revealed that WH2 is critical for its activity to promote nucleotide exchange (this study). Similarly, in yeast Srv2/CAP, the WH2 domain plays an important role in nucleotide exchange [9]. The WH2 domains of CAS-1 and CAS-2 are very similar and essential residues “LKKV” are conserved, but a minor difference in the surrounding sequences may contribute to the biochemical difference. Alternatively, CAS-1 may require additional parts in the N-terminal half for the nucleotide exchange activity. The N-terminal halves of CAS-1 and CAS-2 binds to G-actin independently of the C-terminal halves [18]. However, additional studies such as chimeric analysis of CAS-1 and CAS-2 are required to determine differential mechanisms utilized by the two CAP isoforms to promote nucleotide exchange.

Profilin has a similar function to CAP to promote exchange of actin-bound nucleotides [2, 10, 38, 39]. Profilin also competes with ADF/cofilin for G-actin binding and promotes actin turnover synergistically with ADF/cofilin [6, 7]. However, profilin preferentially binds to ATP-G-actin, and yeast profilin fails to promote nucleotide exchange on ADF/cofilin-bound ADP-G-actin [9]. Therefore, in yeast, a model of sequential processing of G-actin by ADF/cofilin, Srv2/CAP, and profilin was proposed: Srv2/CAP dissociates ADF/cofilin from ADP-G-actin and promotes ATP/ADP exchange before profilin can bind to ATP-G-actin [17]. Functional relationship between profilin and CAP in animal cells is currently unknown. Mammalian profilins have much higher activity to promote nucleotide exchange

on G-actin than yeast profilin [38]. Therefore, profilin and CAP could play independent roles to recharge G-actin with ATP in mammalian cells. *C. elegans* expresses three profilin isoforms [40], but their activities to catalyze nucleotide exchange have not been characterized. In *C. elegans* striated muscle, two profilin isoforms, PFN-2 and PFN-3, are expressed, but a double null mutation for these profilins causes only minor actin disorganization and does not enhance ADF/cofilin-mutant phenotypes [40, 41]. In contrast, CAS-1 knockout is embryonic lethal with severe muscle defects [18], suggesting that CAS-1 functions independently from profilin in *C. elegans* muscle. PFN-1 is expressed in non-muscle cells [40] and likely to be co-expressed with CAS-2. Therefore, further biochemical and genetic investigations of PFN-1 and CAS-2 should reveal their functional relationship in actin dynamics.

This study on CAS-2 suggests a new role of CAP in promoting actin polymerization when ADF/cofilin maintains high levels of actin monomers. Actin depolymerization is a rate-limiting step in spontaneous actin treadmilling, and enhancement of actin depolymerization by ADF/cofilin is sufficient to enhance actin turnover *in vitro* [1]. However, under certain conditions, ADF/cofilin sequesters actin monomers and prevents them from polymerization. Vertebrate ADF (or destrin) and cofilin exhibit higher actin depolymerizing activity at alkaline pHs, and ADF has higher actin-monomer sequestering activity than cofilin [4, 42]. Indeed, ADF/cofilin plays a major role in increasing concentrations of actin monomers during lamellipodial extension [43]. High concentrations of actin monomers can support a subsequent burst of actin assembly. To release the sequestered actin monomers for polymerization, phosphorylation of ADF/cofilin by LIM-kinase or TESK kinase is one mechanism to dissociate the actin-ADF/cofilin complex [44]. However, dissociation of the actin-ADF/cofilin complex by phosphorylation does not enhance exchange of actin-bound ADP for ATP. In contrast, if other CAPs have similar properties to *C. elegans* CAS-2, CAP can promote both recharging of actin monomers with ATP and dissociation of ADF/cofilin, such that free ATP-G-actin can be utilized for actin nucleation and elongation.

Several ADF/cofilin proteins from protozoan parasites primarily function as actin-monomer sequestering proteins *in vitro* [45–47]. They are required for cellular events involving dynamic reorganization of the actin cytoskeleton including gliding motility in *Toxoplasma* [48] and cell division in *Leishmania* [49]. These parasites express small CAP orthologs (C-CAP) with homology to the C-terminal G-actin binding (CARP) domain of CAP but lacking the N-terminal helical-folded, proline-rich, and WH2 domains [50]. C-CAP binds to actin monomers and sequesters them from polymerization [50] and enhances exchange of actin-bound nucleotides [15], suggesting that the C-terminal domain is a minimal requirement for G-actin binding. Thus, conservation of ADF/cofilin and CAP-related proteins across eukaryotic species suggests that they are essential core regulators of actin filament dynamics.

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Abbreviations

ADF	actin depolymerizing factor
CAP	cyclase-associated protein
CARP	CAPs and the X-linked retinitis pigmentosa 2 protein
CAS-1	cyclase-associated protein-1
CAS-2	cyclase-associated protein-2
Cc	apparent critical concentration
MBP	maltose-binding protein
WH2	Wiscott Aldrich syndrome protein homology-2

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Figure 1. Domain structure of *C. elegans* CAS-2

A, schematic representation of domain structures of CAS-1, CAS-2, and CAS-2 fragments used in this study. Highlighted domains are helical-folded domain (HFD), proline-rich region (P), WASP-homology 2 domain (WH2), and CAPs and X-linked retinitis pigmentosa 2 protein (CARP) domain. **B**, Purified MBP (lane 1), MBP-CAS-2 (lane 2), MBP-CAS-2N (lane 3), MBP-CAS-1C (lane 4), and MBP-CAS-1C WH2 (lane 5) were analyzed by SDS-PAGE (0.5 μ g protein/lane) and Coomassie blue staining. Molecular weight markers in kDa (lane M) are shown on the left of the gel.

Figure 2. Effects of CAS-2 on the initial phase of spontaneous actin polymerization

G-actin (5 μM, 9 % pyrene-labeled) was polymerized by addition of salt at time 0 in the presence of various concentrations of MBP (A), MBP-CAS-2 (B), MBP-CAS-2N (C), MBP-CAS-2C (D), or MBP-CAS-2C ΔWH2 (E) and intensity of the pyrene fluorescence (arbitrary units; AU) was monitored over time. Note that different lots of pyrene-labeled actin were used in A-C and D-E, resulting in different fluorescence intensity and rates of polymerization.

Figure 3. Effects of CAS-2 on apparent critical concentration of ATP-actin and ADP-actin
 Various concentrations of pyrene-labeled (20 %) ATP-actin (A-C) or ADP-actin (D-F) were polymerized in the presence of 0–5 μ M MBP (A and B, black circles), MBP-CAS-2 (A and B, white symbols), MBP-CAS-2N (C and D, white symbols), MBP-CAS-2C (E and F, white symbols), or MBP-CAS-2C Δ WH2 (G and H, white symbols) for 18 hr (ATP-actin) or 2 hr (ADP-actin), and the intensity of the pyrene fluorescence (arbitrary units; AU) was measured. The apparent critical concentrations were determined as the inflection points of actin concentrations at which a linear increase of the fluorescence was initiated and summarized in Table 1.

Figure 4. Effects of CAS-2 on exchange of actin-bound nucleotides in the presence of UNC-60A
 One μM ADP-G-actin was incubated with etheno-ATP in the absence or presence of 1 μM UNC-60A without or with 0.2 – 1 μM MBP, MBP-CAS-2, MBP-CAS-2N, MBP-CAS-2C, or MBP-CAS-2C ΔWH2 as indicated on the figure and the fluorescence of etheno-ATP was monitored over time. Rates of exchange of nucleotides [k_{obs} (s^{-1})] were determined from the data and shown in the graph. Data are means $\pm$ s.d. of three independent experiments.

Figure 5. ATP-dependent effects of CAS-2 on the amounts of F-actin in the presence of UNC-60A

10 μ M F-actin was pre-incubated without or with 20 μ M UNC-60A for 30 min with 0.5 mM ATP (A, B), no ATP or ADP (C, D), or 0.5 mM ADP (E, F), and then mixed and incubated with buffer only or buffer with MBP, MBP-CAS-2, MBP-CAS-2N, MBP-CAS-2C, or MBP-CAS-2C Δ WH2 for 30 min. The mixtures were ultracentrifuged, and the supernatants and pellets were analyzed by SDS-PAGE. Experiments were performed at various concentrations of MBP and MBP-CAS-2 variants. Representative results of gels with 0.2 μ M of MBP or MBP-CAS-2 variants are shown in A, C, and E. Molecular weight markers in kDa (lane M) are shown on the left of the gels. A and U on the right of the gels indicate positions of actin and UNC-60A, respectively. Percentages of actin in the supernatants were quantified by densitometry and plotted as a function of concentrations of the CAS-2 variants (in a logarithmic scale) in B, D, and F. Data are means $\pm$ s.d. of three independent experiments. (G) Similar experiments were performed at various concentrations of ATP with pre-incubation of 10 μ M F-actin and 20 μ M UNC-60A, which were followed by addition of 0.2 μ M MBP (black circles), 0.2 μ M MBP-CAS-2 (white circles), 0.2 μ M MBP-CAS-2N (white triangles), 0.2 μ M MBP-CAS-2C (white squares), or 2 μ M MBP-CAS-2C Δ WH2 (white diamonds). Percentages of actin in the supernatants were quantified

by densitometry and plotted as a function of ATP concentrations. Data are means $\pm$ s.d. of three independent experiments.

Figure 6. Inhibitory effects of latrunculin A and capping protein on CAS-2-induced actin polymerization in the presence of UNC-60A

10 μ M F-actin and 20 μ M UNC-60A were pre-incubated in the absence or presence of 20 μ M latrunculin A (A, B) or 0.1 μ M capping protein (C, D) for 30 min, which were followed by addition and incubation of 0.2 μ M MBP (lanes 1–4), MBP-CAS-2 (lanes 5–8), MBP-CAS-2N (lanes 9–12), or MBP-CAS-2C (lanes 13–16) for 30 min. The mixtures were ultracentrifuged, and the supernatants and pellets were analyzed by SDS-PAGE. Representative gels are shown in A and C. Molecular weight markers in kDa (lanes M) are shown on the left of the gels. Positions of actin and UNC-60A are indicated on the right of the gels. Percentages of actin in the supernatants were quantified by densitometry (B and D). Data are means $\pm$ s.d. of three independent experiments.

Figure 7. Time-course monitoring of sequential UNC-60A-induced depolymerization and CAS-2-induced polymerization by light scattering measurement

5.2 μM F-actin was mixed with 10.3 μM UNC-60A at time 0 in a buffer containing 0.5 mM ATP (A-E), no ATP or ADP (F), or 0.5 mM ADP (G), and light scattering at a wavelength of 500 nm was monitored over time. After 30 min (1800 sec; indicated by arrows), the same reaction buffer without or with MBP or MBP-CAS-2 variants was mixed (final concentrations are indicated on the figure), and light scattering was continuously monitored. Final concentrations of actin and UNC-60A were 5 μM and 10 μM, respectively. In all graphs, control experiments with buffer only are shown in gray curves with black circles. In F and G, added proteins are shown in the box on the right. Note that not all symbols are visible due to overlaps.

Figure 8. Model of the regulation of actin filament dynamics by UNC-60A (ADF/cofilin) and CAS-2 (CAP)

(A) G-actin, UNC-60A, and CAS-2 are in equilibrium with UNC-60A-G-actin, CAS-2-G-actin, and F-actin, since binding of UNC-60A or CAS-2 to F-actin is negligible. UNC-60A binds to ADP-G-actin with high affinity and sequesters it. CAS-2 shifts the equilibrium by converting ADP-G-actin to ATP-G-actin, which weakens G-actin sequestration by UNC-60A and allows polymerization of ATP-actin. (B) If CAS-2 is absent, UNC-60A inhibits ATP/ADP exchange on ADP-G-actin, and ADP-G-actin remains sequestered by UNC-60A.

Table 1

Effects of CAS-2 on apparent critical concentration of actin.

Actin	MBP/MBP-CAS-2	Cc (μM)	Dissociation constant (μM)
ATP-actin	None	0.15	-
ATP-actin	5 μM MBP	0.14	ND (not determined)
ATP-actin	2 μM MBP-CAS-2	0.55	0.60
ATP-actin	5 μM MBP-CAS-2	0.88	0.88
ATP-actin	2 μM MBP-CAS-2N	0.15	ND
ATP-actin	5 μM MBP-CAS-2N	0.15	ND
ATP-actin	2 μM MBP-CAS-2C	0.41	1.0
ATP-actin	5 μM MBP-CAS-2C	0.83	0.95
ATP-actin	2 μM MBP-CAS-2C WH2	0.23	3.6
ATP-actin	5 μM MBP-CAS-2C WH2	0.40	2.9
ADP-actin	None	2.5	-
ADP-actin	5 μM MBP	2.5	ND
ADP-actin	2 μM MBP-CAS-2	4.0	0.83
ADP-actin	5 μM MBP-CAS-2	5.1	2.3
ADP-actin	2 μM MBP-CAS-2N	4.0	0.83
ADP-actin	5 μM MBP-CAS-2N	4.8	2.9
ADP-actin	2 μM MBP-CAS-2C	3.7	1.7
ADP-actin	5 μM MBP-CAS-2C	4.8	2.9
ADP-actin	2 μM MBP-CAS-2C WH2	3.7	1.7
ADP-actin	5 μM MBP-CAS-2C WH2	5.9	1.2