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 the dynamic cellular processes involving the rapid dynamics of the actin cytoskeleton. Severing and depolymerization of actin filaments are important mechanisms to enhance actin filament dynamics [1], as slow dissociation of actin monomers from filaments is a rate-limiting step for actin turnover. ADF (actin depolymerizing factor)/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 in excessive amounts, it is not effective in promoting actin turnover. ADF/cofilin preferentially binds to ADP–actin and inhibits the 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 an accumulation of ADP–actin that is much less competent for polymerization than ATP–actin and does not support the treadmilling of actin filaments.

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

CAP (cyclase-associated protein) also enhances the exchange of actin-bound ADP for ATP and promotes the 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 HFD (helical-folded domain) binds to ADF/cofilin–actin complex, and that the central WH2 (Wiscott–Aldrich syndrome protein homology 2) domain and the C-terminal CARP (CAP and X-linked retinitis pigmentosa 2 protein) 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 PFN [14]. However, Arabidopsis CAP and Caenorhabditis elegans CAP (CAS-1) do not have a strong preference for either ADP–actin or ATP–actin, but still co-operate 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 co-ordinate to induce the dissociation of ADF/cofilin from actin and exchange of actin-bound nucleotides remains unknown. Recent studies have shown
that CAP promotes the 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 adenyl cyclase-associated protein that is involved in Ras-cAMP signalling [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, whereas CAP2 is predominantly expressed in the heart and skeletal muscle [25]. Nevertheless, whether CAP1 and CAP2 have different functions is currently unknown. The nematode C. 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, CAS-2 mRNA are enriched in non-muscle tissues as reported in the Nematode Expression Pattern Database [26], but its function remains uncharacterized. In the present study, we characterized the biochemical properties of CAS-2 and found that, unlike CAS-1, the C-terminal half of CAS-2 containing the 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 the actin monomer–filament equilibrium to increase F-actin (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 previously [27]. G-actin was further purified by gel filtration using Sephacryl S-300 in G-buffer [2 mM Tris/HCl, 0.2 mM ATP, 0.2 mM CaCl2 and 0.2 mM dithiothreitol (pH 8.0)]. Pyrene-labelled rabbit muscle actin was prepared as described previously [28]. ADP–G-actin was prepared using hexokinase (Worthington Biochemical) as described previously [18]. UNC-60A was expressed in Escherichia coli cells and purified as described in [29]. The bacterial expression vector for chicken capping protein [CapZ (F-actin-capping protein)] was kindly provided by Dr Takashi Obinata (Chiba University, Chiba, Japan), and it was expressed in E. coli cells and purified as described previously [30]. Latrunculin A was purchased from Enzo Life Sciences.

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 Professor Yuji Kohara, Mishima, Shizuoka, Japan), and cloned into the pGEX-2T vector. Originally, we attempted bacterial expression of CAS-2 using a GST fusion system, but the solubility of the recombinant fusion proteins was very poor (K. Nomura and S. Ono, unpublished results). Therefore full-length CAS-2, CAS-2N (residues 1–207), CAS-2C (residues 208–457) or CAS-2ΔWH2 (residues 275–457) sequences were re-cloned of pDEST-HisMBP, a vector for bacterial expression as fusion proteins with MBP (maltose-binding protein) with an N-terminal histidine tag using the Gateway® technology with ClonaseTM II (Invitrogen) as described previously [31]. pDEST-HisMBP was developed by the group of Dr David Waugh (National Cancer Institute, Frederick, MD, U.S.A.) [31] and obtained through Addgene. The protein coding sequences were verified by DNA sequencing. E. coli BL21(DE3) cells were transformed with the expression vectors, and protein expression was induced by adding 1 mM IPTG for 3 h at room temperature (25°C). The cells were harvested by centrifugation at 5000 g for 10 min at 4°C and disrupted by a French pressure cell at 360–580 kg/cm2 in PBS. The homogenates were cleared at 20000 g for 15 min at 4°C and applied to a His60 Ni SuperflowTM column (Clontech) and washed with 150 mM NaCl, 3 mM imidazole and 50 mM NaPO4 (pH 7.9) to remove the unbound proteins. The bound proteins were eluted with 150 mM NaCl, 250 mM imidazole and 50 mM NaPO4 (pH 7.9). MBP–CAS-2 (full-length) was further purified with a HiPrep 26/60 Sephacryl S-300 column (GE Healthcare), whereas MBP–CAS-2N, MBP–CAS-2C and MBP–CAS-2ΔWH2 were further purified with a HiTrapQ FF column (GE Healthcare). MBP was used without additional chromatographic purification. Finally, the purified proteins were dialysed against F-buffer [0.1 M KCl, 20 mM sodium Hepes (pH 7.5), 2 mM MgCl2 and 0.2 mM DTT] containing 50% glycerol and stored at −20°C.

Monitoring the kinetics of actin polymerization

The kinetics of actin polymerization was monitored by measuring fluorescence of pyrene-labelled actin. Pyrene-labelled G-actin (final 5 μM, 9% labelled) was mixed with MBP–CAS-2, MBP–CAS-2N, MBP–CAS-2C, MBP–CAS-2ΔWH2 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 and 20 mM sodium Hepes (pH 7.5). Fluorescence of pyrene (excitation at 365 nm and emission at 407 nm) was measured for 10 min with an F-4500 fluorescence spectrophotometer (Hitachi High-Technologies).

Determination of the apparent critical concentration of actin

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

Assays for the exchange of actin-bound nucleotides

The effects of CAS-2 variants and UNC-60A on the exchange rate of actin-bound nucleotides were examined by monitoring the increase in the fluorescence of etheno-ATP that is associated with G-actin binding. ADP–G-actin (1.3 μM) was prepared in 150 μl of G-buffer without ATP, and then 50 μl of etheno-ATP (160 μM) with or without CAS-2 variants and UNC-60A was mixed. Final concentrations of G-actin and UNC-60A were both 1 μM. 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. The exponential rates (kobs) were calculated by curve fitting using SigmaPlot 10 (Systat Software).

F-actin sedimentation assays

F-actin (10 μM) in F-buffer [0.1 M KCl, 2 mM MgCl2, 0.2 mM DTT and 20 mM sodium Hepes (pH 7.5)] with or without ATP
or ADP (concentrations are indicated in Figure legends) was pre-incubated with 20 μM UNC-60A for 30 min at room temperature. Then, various concentrations of MBP–CAS-2, MBP–CAS-2N, MBP–CAS2C, MBP–CAS-2CΔWH2 or MBP were added to the mixtures and incubated for 30 min at room temperature. The mixtures were ultracentrifuged at 80,000 rev./min for 15 min at 20°C using a Beckman TLA-100 rotor. Supernatant and pellet fractions were adjusted to the same volumes and subjected to SDS/PAGE (12% gel) 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 ImageJ (http://rsbweb.nih.gov/ij/). When the effects of latrunculin A (20 μM) or capping protein (0.1 μM) were examined these were included at the time of assembling the pre-incubation mixtures.

Light scattering assays
F-actin (5.2 μM) in F-buffer with or without 0.5 mM ATP or ADP was mixed with 10.3 μM UNC-60A at time zero 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. After 30 min, various concentrations of MBP–CAS-2, MBP–CAS2N, MBP–CAS2C, MBP–CAS-2CΔWH2 or MBP were added (final, 5 μM F-actin and 10 μM 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 CAP 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 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. The predicted domain structure of CAS-2 is similar to that of CAS-1 with an N-terminal HFD, a proline-rich region (P), a WH2 domain and a CARP domain (Figure 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-2CΔWH2) (residues 275–457) (Figure 1A). These proteins were expressed as fusion proteins with MBP. Since cleavage of MBP by TEV (tobacco etch virus) protease was very inefficient, we used MBP-fusion proteins (Figure 1B, lanes 2–5) in the following biochemical experiments and included purified MBP (Figure 1B, lane 1) in the control experiments.

Binding of CAS-2 to actin monomers was examined by two methods. First, we examined the effects of CAS-2 on the initial phase of spontaneous actin polymerization from G-actin (Figure 2). MBP–CAS-2 inhibited actin polymerization in a concentration-dependent manner (Figure 2B), whereas a high concentration of MBP (15 μM) had no effect on actin polymerization (Figure 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]. Whereas MBP–CAS-2N showed no detectable effects on actin polymerization (Figure 2C), MBP–CAS-2C had nearly as strong an effect as MBP–CAS-2 (Figure 2D). MBP–CAS-2CΔWH2 also inhibited the initial phase of actin polymerization (Figure 2E), but required higher concentrations than MBP–CAS-2C to cause comparable effects (compare with Figure 2D). These results show that the C-terminal CARP domain of CAS-2 is necessary and sufficient for binding to G-actin and that the WH2 domain enhances G-actin binding.
Next, we examined effects of CAS-2 on the Cc (apparent critical concentration) of ATP– and ADP–actin (Figure 3). Various concentrations of pyrene-labelled G-actin were polymerized in the absence or presence of 2 or 5 μM MBP, MBP–CAS-2, MBP–CAS-2N, MBP–CAS-2C or MBP–CAS-2CΔWH2 and the steady-state levels of polymerized actin were quantified by the pyrene fluorescence. Above the Cc, the amounts of polymerized actin were linearly correlated with the concentrations of total actin (Figure 3). The Cc values of actin alone were 0.15 μM for ATP–actin (Figure 3A, ○) and 2.5 μM for ADP–actin (Figure 3B, ○). MBP did not affect these values (Figures 3A and 3B, ●). MBP–CAS-2 shifted the Cc to higher values for both ATP–actin (Figure 3A and Table 1) and ADP–actin (Figure 3B and Table 1), indicating that CAS-2 bound to G-actin and sequestered it from polymerization. From the shifts in the Cc value, the dissociation constant for binding of MBP–CAS-2 to G-actin was estimated to be 0.60–0.88 μM for ATP–actin and 0.83–2.3 μM for ADP–actin (Table 1), suggesting that their binding is not strongly influenced by the actin-bound nucleotides. MBP–CAS-2N only altered the Cc of ADP–actin with a Kd value of 0.83–2.9 μM (Figure 3D) without affecting the Cc of ATP–actin (Figure 3C). MBP–CAS-2C shifted Cc to higher values (Figures 3E and 3F) and did not exhibit a strong preference for binding to ATP–actin (Kd = 0.95–1.0 μM) or ADP–actin (Kd = 1.7–2.9 μM) (Table 1) in a similar manner to MBP–CAS-2. However, MBP–CAS-2CΔWH2 only slightly shifted the Cc of ATP–actin (Kd = 2.9–3.6 μM) (Figure 3G), but shifted the Cc of ADP–actin to similar extents to MBP–CAS-2 (Kd = 1.2–1.7 μM) (Figure 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, the WH2 domain of CAS-2 appears to enhance binding to ATP–actin, which is similar to the WH2 domain in yeast Srv2/CAP and human CAP1 [9,15]

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

As demonstrated previously for C. elegans CAS-1 [18], CAS-2 also accelerated the rate of exchange of actin-bound nucleotides (Figure 4). ADP-bound G-actin (1 μM) 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 the rates of exchange [kobs (s⁻¹)] were determined (Figure 4). UNC-60A strongly inhibited nucleotide exchange on ADP–G-actin (Figure 4, compare first and second bars), and MBP had no effect on this inhibition (Figure 4, third bar). However, MBP–CAS-2 relieved this inhibition and accelerated nucleotide exchange in a concentration-dependent manner (Figure 4, fourth and fifth bars). In the presence of 0.5 μM MBP–CAS-2 the 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 results).
ATP-dependent actin regulation by CAP and ADF/cofilin

Figure 3 Effects of CAS-2 on the Cc of ATP–actin and ADP–actin

Various concentrations of pyrene-labelled (20%) ATP–actin (A–C) or ADP–actin (D–F) were polymerized in the presence of 0–5 μM MBP (A and B, open symbols), MBP–CAS-2 (A and B, open symbols), MBP–CAS-2N (C and D, open symbols), MBP–CAS-2C (E and F, open symbols), or MBP–CAS-2CΔWH2 (G and H, open symbols) for 18 h (ATP–actin) or 2 h (ADP–actin), and the intensity of the pyrene fluorescence [AU (arbitrary units)] 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.

Among the truncated CAS-2 fragments tested, MBP–CAS-2N did not enhance nucleotide exchange (Figure 4, sixth bar), whereas MBP–CAS-2C enhanced nucleotide exchange nearly as strongly as MBP–CAS-2 (Figure 4, seventh and eighth bars). However, MBP–CAS-2CΔWH2 failed to promote nucleotide exchange in the presence of UNC-60A (Figure 4, ninth bar) as well as in the absence of UNC-60A (K. Nomura and S. Ono, unpublished results). Therefore the C-terminal half of CAS-2 is necessary and sufficient for enhancement of the 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 the 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.

Table 1 Effects of CAS-2 on the Cc of actin

<table>
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<tr>
<th>Actin</th>
<th>MBP/MBP–CAS-2</th>
<th>Cc (μM)</th>
<th>Dissociation constant (μM)</th>
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<tr>
<td>ATP–actin</td>
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<td>1.2</td>
</tr>
</tbody>
</table>

Figure 4 Effects of CAS-2 on the exchange of actin-bound nucleotides in the presence of UNC-60A

ADP–G-actin (1 μM) was incubated with etheno-ATP in the absence or presence of 1 μM UNC-60A with or without 0.2–1 μM MBP, MBP–CAS-2, MBP–CAS-2N, MBP–CAS-2C or MBP–CAS-2CΔWH2, as indicated in the figure, and the fluorescence of etheno-ATP was monitored over time. Rates of exchange of nucleotides [kobs (s⁻¹)] were determined from the data and are shown in the histogram. Results are means ± S.D. for three independent experiments.

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

F-actin (10 μM) was pre-incubated with or without 20 μM UNC-60A for 30 min with 0.5 mM ATP (A and B), no ATP or ADP (C and D) or 0.5 mM ADP (E and 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-2CΔWH2 for 30 min. The mixtures were ultracentrifuged and the supernatants (s) and pellets (p) were analysed by SDS/PAGE. Experiments were performed at various concentrations of MBP and the MBP–CAS-2 variants. Representative results of gels with 0.2 μM of MBP or the MBP–CAS-2 variants are shown in (A), (C) and (E). Molecular mass markers in kDa (lane M) are shown on the left-hand side of the gels. A and U on the right-hand side of the gels indicate the 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). Results are means ± S.D. for three independent experiments. (G) Similar experiments were performed at various concentrations of ATP with pre-incubation with 10 μM F-actin and 20 μM UNC-60A, which were followed by the addition of 0.2 μM MBP (●), 0.2 μM MBP–CAS-2 (○), 0.2 μM MBP–CAS-2N (△), 0.2 μM MBP–CAS-2C (□) or 2 μM MBP–CAS-2CΔWH2 (○). Percentages of actin in the supernatants were quantified by densitometry and plotted as a function of ATP concentrations. Results are means ± S.D. for three independent experiments.

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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 μM) remained in the supernatant after ultracentrifugation (Figure 5A, lanes 1 and 2). UNC-60A (20 μM) promoted actin depolymerization and increased actin in the supernatant to ~60% of the total actin (Figure 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 (Figure 5A, lanes 5 and 6), the addition of 0.2 μM MBP–CAS-2 significantly decreased actin in the supernatant to ~30% of the total actin (Figure 5A, lanes 7 and 8). MBP–CAS-2N did not alter the amount of actin in the supernatants (Figure 5A, lanes 9 and 10), whereas MBP–CAS-2C had nearly equal activity as MBP–CAS-2 (Figure 5A, lanes 11 and 12). MBP–CAS-2CΔWH2 at a 0.2 μM concentration did not alter the distribution of actin (Figure 5A, lanes 13 and 14). By testing various concentrations of MBP–CAS-2, 0.1–1 μM of MBP–CAS-2 were sufficient to cause a decrease in actin in the supernatants in the presence of 10 μM actin and 20 μM UNC-60A (Figure 5B, ○), and MBP–CAS-2C exhibited a similar optimal concentration range (Figure 5B, □). At a high concentration (5 μM), the effects of MBP-CAS-2 and MBP-CAS-2C were diminished (Figure 5B), most likely due to their own actin-monomer-sequestering activities, as shown in Figure 3, thereby increasing actin in the supernatants. MBP–CAS-2CΔWH2 showed weak activity to decrease actin in the supernatants at high concentrations (2–5 μM) (Figure 5B, ○), 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 upon ATP (Figure 5). Under ATP/ADP-free conditions, neither MBP–CAS-2 nor MBP–CAS-2C decreased actin in the supernatants (Figures 5C and 5D). MBP, MBP–CAS-2N and MBP–CAS-2CΔWH2 also did not have significant effects (Figure 5C and 5D). Similarly, in the presence of 0.5 mM ADP, MBP and none of the MBP–CAS-2 variants reduced actin in the supernatants in the presence of UNC-60A (Figures 5E and 5F). 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 μM UNC-60A (Figure 5G, □ and ○). The weak activity by a high concentration (2 μM) of MBP–CAS-2CΔWH2 was not further enhanced by increasing ATP concentrations (Figure 5G, ○).

The effect of CAS-2 to antagonize UNC-60A was prevented by latrunculin A, an actin-monomer-sequestering drug (Figure 6A, lanes 5–8, and Figure 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 (Figure 6A, lanes 13–16, and Figure 6B). MBP and MBP–CAS-2N did not affect UNC-60A-mediated actin depolymerization in the absence or presence of latrunculin A (Figures 6A and 6B). These results indicate that MBP–CAS-2 and MBP–CAS-2C contribute to increasing F-actin under 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) (Figures 6C and 6D), suggesting that CAS-2-induced increase in pelletable actin depends upon the association of actin monomers with 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 the time course of depolymerization and polymerization was monitored by the light scattering assay (Figure 7). UNC-60A (10 μM) was mixed with F-actin (5 μM) at time zero, and actin depolymerization was detected by the decrease in light scattering.
Figure 7 Time-course monitoring of sequential UNC-60A-induced depolymerization and CAS-2-induced polymerization by light scattering measurement

F-actin (5.2 μM) was mixed with 10.3 μM UNC-60A at time zero 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 (indicated by arrows), the same reaction buffer without or with MBP or the MBP–CAS-2 variants was mixed (final concentrations are indicated in the Figure), and light scattering was continuously monitored. The final concentrations of actin and UNC-60A were 5 μM and 10 μM respectively. In all graphs, control experiments with buffer only are shown as grey plots with ● in (F) and (G), the added proteins are shown in the box on the right-hand side. Note that not all symbols are visible due to overlaps.

(Figure 7). After 30 min, MBP, MBP–CAS-2, MBP–CAS-2N, MBP–CAS-2C or MBP–CAS-2CΔWH2 was added and light scattering was continuously measured. In the presence of ATP, 0.5 μM MBP did not affect the light scattering (Figure 7A), whereas MBP–CAS-2 (at 0.2 and 0.5 μM) enhanced light scattering immediately after its addition indicating that actin polymerization was induced after depolymerization by UNC-60A (Figure 7B). MBP–CAS-2N did not have an effect (Figure 7C), whereas MBP–CAS-2C (>0.1 μM) triggered polymerization (Figure 7D). A high concentration (3.7 μM) of MBP–CAS-2CΔWH2 did not strongly induce polymerization (Figure 7E), which is consistent with its weak effect in the sedimentation assays (Figure 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 (Figure 7F) or in the presence of only ADP (Figure 7G), MBP and all of the 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 CAP 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 the actin monomer–filament equilibrium from UNC-60A-sequestered G-actin to increased F-actin in the presence of ATP. Importantly, the 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 the monomer–filament equilibrium by CAS-2 and UNC-60A (Figure 8). In the presence of ADP–G-actin, UNC-60A and CAS-2 (Figure 8A), ADP–F-actin can be formed, but will not be stable, as UNC-60A depolymerizes and sequesters ADP–G-actin. When the CAS-2 concentration is low, formation of the CAS–2–ADP–G-actin complex should be negligible, and CAS-2 does not contribute to monomer sequestration. ADP–G-actin will be rapidly converted into 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 the 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 the subsequent depolymerization and monomer sequestration by UNC-60A. Thus we predict that CAS-2 and UNC-60A co-operate to promote ATP-dependent actin filament dynamics (Figure 8A). However, if CAS-2 is absent, UNC-60A sequesters ADP–G-actin and inhibits nucleotide exchange on ADP–G-actin (Figure 8B). Therefore the cycle of actin dynamics will not be promoted.

The results of the present study 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 HFD 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 (K. Nomura and S. Ono, unpublished results) in similar experiments to that we reported previously for CAS-1 [18]. Therefore the 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 pH range of 6–8 (K. Nomura and S. Ono, 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 the dynamic reorganization of the actin cytoskeleton. In C. elegans, UNC-60A is widely expressed in non-muscle tissues and is 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 release actin monomers for the 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 owing to the 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. cas-2 mRNA 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 germine. We are currently investigating the in vivo functions of CAS-2 and its co-operation 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 the 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], whereas CAS-2C is sufficient for both G-actin binding and nucleotide exchange (as shown above). An additional C-terminal fragment of CAS-2 lacking the WH2 domain revealed that it is critical for its activity to promote nucleotide exchange (as shown above). 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 its N-terminal half for the nucleotide-exchange activity. The N-terminal halves of CAS-1 and CAS-2 bind to G-actin independently of the C-terminal halves [18]. However, additional studies, such as the chimeraic analysis of CAS-1 and CAS-2, are required to determine the differential mechanisms utilized by the two CAP isoforms to promote nucleotide exchange.

PFN has a similar function to CAP to promote the exchange of actin-bound nucleotides [2,10,38,39]. PFN also competes with ADF/cofilin for G-actin binding and promotes actin turnover synergistically with ADF/cofilin [6,7]. However, PFN preferentially binds to ATP–G-actin, and yeast PFN fails to promote nucleotide exchange on ADF/cofilin-bound actin.
ADP–G-actin [9]. Therefore, in yeast, a model of sequential processing of G-actin by ADF/cofilin, Srv2/CAP and PFN was proposed: Srv2/CAP dissociates ADF/cofilin from ADP–G-actin and promotes ATP/ADP exchange before PFN can bind to ATP–G-actin [17]. The functional relationship between PFN and CAP in animal cells is currently unknown. Mammalian PFNs have much higher activity to promote nucleotide exchange on G-actin than yeast PFN [38]. Therefore PFN and CAP could play independent roles to recharge G-actin with ATP in mammalian cells. C. elegans expresses three PFN isoforms [40], but their activities to catalyse nucleotide exchange have not been characterized. In C. elegans striated muscle, two PFN isoforms, PFN-2 and PFN-3, are expressed, but a double-null mutation for these PFNs 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 PFN in C. elegans muscle. PFN-1 is expressed in non-muscle cells [40] and is 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.

The present 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 pH values, and ADF has higher actin–monomer-sequestering activity than cofilin [4,42]. Indeed, ADF/cofilin plays a major role in increasing the 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, the phosphorylation of ADF/cofilin by LIM kinase or TESK (testis-specific 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 the exchange of actin-bound ADP for ATP. In contrast, if other CAPs have similar properties to C. elegans CAS-2, CAP can promote both the recharging of actin monomers with ATP and the 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 function primarily 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 orthologues (C-CAP) with homology to the C-terminal G-actin-binding (CAPR) 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 the 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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**REFERENCES**


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