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The C-terminal dimerization motif of cyclase-associated protein is essential for actin monomer regulation

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Abstract

Cyclase-associated protein (CAP) is a conserved actin-regulatory protein that functions together with actin-depolymerizing factor (ADF)/cofilin to enhance actin filament dynamics. CAP has multiple functional domains, and the function to regulate actin monomers is carried out by its C-terminal half containing a Wiskott-Aldrich Syndrome protein homology 2 (WH2) domain, a CAP and X-linked retinitis pigmentosa 2 (CARP) domain, and a dimerization motif. WH2 and CARP are implicated in binding to actin monomers and important for enhancing filament turnover. However, a role of the dimerization motif is unknown. Here, we investigated function of the dimerization motif of CAS-2, a CAP isoform in the nematode *Caenorhabditis elegans*, in actin monomer regulation. CAS-2 promotes ATP-dependent recycling of ADF/cofilin-bound actin monomers for polymerization by enhancing exchange of actin-bound nucleotides. A C-terminal half of CAS-2 (CAS-2C) has nearly as strong activity as full-length CAS-2. Maltose-binding protein (MBP)-tagged CAS-2C is a dimer. However, MBP-CAS-2C with a truncation of either one or two C-terminal β -strands is monomeric. Truncations of the dimerization motif in MBP-CAS-2C nearly completely abolish its activity to sequester actin monomers from polymerization and enhance nucleotide exchange on actin monomers. As a result, these CAS-2C variants, also in the context of full-length CAS-2, fail to compete with ADF/cofilin to release actin monomers for polymerization. CAS-2C variants lacking the dimerization motif exhibit enhanced binding to actin filaments, which is mediated by WH2. Together, these results suggest that the evolutionarily conserved dimerization motif of CAP is essential for its C-terminal region to exert the actin-monomer-specific regulatory function.

Summary Statement

The dimerization motif of cyclase-associated protein is comprised of two C-terminal β -strands. This motif of cyclase-associated protein is essential for actin-monomer sequestration, exchange of actin-bound nucleotides, and prevention of actin-filament binding.

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DECLARATION OF INTEREST

None.

AUTHOR CONTRIBUTION STATEMENT

Shohei Iwase performed the experiments, analyzed the data, and prepared figures. Shoichiro Ono developed the study, designed the experiments, analyzed the data, and wrote the manuscript.

Keywords

Actin depolymerizing factor (ADF)/cofilin; actin dynamics; nucleotide exchange

INTRODUCTION

The actin cytoskeleton is essential for many dynamic cellular functions. A number of actin-regulatory proteins control the rates of actin polymerization and/or depolymerization and contribute to spatiotemporal regulation of the cytoskeletal dynamics [1, 2]. In the polymerization-depolymerization cycles, ATP metabolism by actin is important for proper actin turnover. Actin is an ATPase that hydrolyzes ATP into ADP and Pi after actin is polymerized. When ADP-actin is depolymerized, actin-bound ADP is exchanged with free ATP or ADP. Since ATP-actin is highly competent for polymerization, in particular at the actin barbed ends, constant supply of free ATP to recharge actin monomers is essential for persistent cycling of actin polymerization and depolymerization. Actin depolymerizing factor (ADF)/cofilin promotes actin turnover by preferentially severing and depolymerizing ADP-actin filaments [3]. However, ADF/cofilin inhibits subsequent exchange of monomer-bound nucleotides [4]. To utilize ADF/cofilin-bound actin monomers for a new round of polymerization, profilin and cyclase-associated protein (CAP) are known to compete with ADF/cofilin and accelerate exchange of actin-bound nucleotides [5]. As a result, a combination of ADF/cofilin and profilin [6, 7] or CAP [8–10] significantly enhances actin turnover *in vitro*. CAP is more efficient than profilin to promote actin turnover due to its high-affinity interaction with ADP-actin [11, 12]. CAP and ADF/cofilin cooperate to regulate the dynamics of the actin cytoskeleton in yeast [9, 13], *C. elegans* [14], and mammalian cultured cells [15, 16]. Thus, further characterization of the biochemical properties of CAP will be important to understand the mechanism of actin cytoskeletal dynamics *in vivo*.

CAP interacts with both actin monomers and filaments and regulates multiple aspects of actin dynamics [5]. CAP promotes severing of actin filaments [17], which is mediated by direct interaction of the N-terminal helical-folded domain of CAP with actin filaments [18, 19]. CAP forms an oligomer *in vitro* and *in vivo* [9, 20, 21], and hexamer formation through the N-terminal putative coiled-coil region is important for enhancement of actin dynamics in budding yeast [18, 21]. In cooperation with twinfilin, CAP also promotes actin depolymerization from filament ends [22]. The effects of CAP on actin monomers, as described above, are primarily mediated by the C-terminal half of CAP containing a Wiskott Aldrich Syndrome protein homology 2 (WH2) domain, a β -helical CAP and X-linked retinitis pigmentosa 2 (CARP) domain, and a dimerization motif (see Fig. 1E for domain organization) [5]. Mutations in WH2 or CARP significantly reduce the affinity of CAP with actin monomers and abolish the ability of CAP to compete with ADF/cofilin to enhance nucleotide exchange on actin [11, 12, 19, 23], but how WH2 and CARP contribute to actin regulation is not clearly understood. In addition, function of the C-terminal dimerization motif remains uncharacterized. The CARP domain has a helical arrangement of β -strands and is dimerized by a “strand-exchange” of the dimerization motif containing two β -strands (β 8 and β 9) [24]. Apicomplexan C-CAP, which inherently has only CARP and the

dimerization motif, has the same structural arrangement [25], suggesting that this unique dimer configuration is functionally important. A previous study showed that dimerization-defective mutants of CAP fail to bind to actin as determined by immunoprecipitation from yeast lysates [26]. However, how defective dimerization affects the actin-regulatory function of CAP remains unknown.

Here, we specifically investigated a role of the dimerization motif of CAS-2, a non-muscle isoform of CAP in the nematode *Caenorhabditis elegans* [27] in actin regulation. CAS-2 has typical activities of CAP: it binds to monomeric actin and enhances nucleotide exchange. A C-terminal half of CAS-2 containing WH2, CARP, and the dimerization motif has nearly as strong activity as full-length CAS-2 and effectively compete with ADF/cofilin to recycle actin monomers for polymerization in an ATP-dependent manner [27]. Although WH2 and CARP are implicated in direct interaction with actin monomers [11, 12, 19, 23], truncation of the dimerization motif in *C. elegans* CAS-2 nearly completely abolishes its actin monomer-regulatory activities. In contrast, truncation of the dimerization motif enhances actin filament binding. Our results suggest that the dimerization motif of CAP is required for its actin-monomer-specific regulatory function.

EXPERIMENTAL

Proteins and materials

Rabbit muscle actin was purified from acetone powder (Pel-Freeze Biologicals) as described previously [28]. Pyrene-labelled rabbit muscle actin was prepared as described previously [29]. ADP-G-actin was prepared using hexokinase (Worthington Biochemical)-conjugated Sepharose beads as described [23]. UNC-60A was expressed in *Escherichia coli* and purified as described [30]. Etheno-ATP was purchased from Invitrogen. DyLight 680-labeled actin was prepared as described for preparation of DyLight 549-labeled actin [31] using DyLight 680 NHS ester (Thermo Fisher Scientific).

Homology modeling

A homology model of the structure of the CAS-2 C-terminus (residues 306 – 457) was generated by SWISS-MODEL [32] using the crystal structure of the C-terminal region of human CAP1 (residues 319 – 475) (Protein Data Bank accession number 1K8F) as a template. Molecular graphics in Fig. 1 were generated by UCSF Chimera [33].

Mutagenesis, expression, and purification of recombinant CAS-2C proteins

A bacterial expression vectors for CAS-2 (residues 1–457), CAS-2C WH2 (residues 275–475), and CAS-2C (residues 208–457 of CAS-2) as fusion proteins with histidine-tagged maltose-binding protein (MBP) were described previously [27]. To generate C-terminal truncations in MBP-CAS-2C, MBP-CAS-2C WH2, or MBP-CAS-2, the codon for Q439 or I444 was converted to a stop codon by a QuickChange Site-directed Mutagenesis kit (Agilent Technologies) to make MBP-CAS-2C β 8 β 9, MBP-CAS-2C β 9, MBP-CAS-2C WH2 β 8 β 9, or MBP-CAS-2 β 9. MBP was expressed from the original vector pDEST-HisMBP, which was developed by the group of David Waugh (National Cancer Institute, Frederick, MD, USA) [34] 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 and purification were performed essentially as described previously for MBP-CAS-2C.

Gel filtration chromatography

A Superose 6 10/300 GL gel filtration column (24 ml bed, GE Healthcare) was equilibrated with F-buffer [0.1 M KCl, 2 mM 2 mM MgCl₂, 0.2 mM DTT, 20 mM HEPES (pH 7.5)], and proteins at 1.0 mg/ml (500 µl) were applied and eluted with the same buffer. The eluates were collected in fractions of 0.5 ml each, and analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue R-250. Gels were scanned by an Epson Perfection V700 Photo Scanner at 300 dots per inch, and band intensity was quantified using Image J. Apoferritin (443 kDa) (A3660, Sigma-Aldrich), β -amylase (200 kDa) (A8781, Sigma-Aldrich), alcohol dehydrogenase (150 kDa) (A8656, Sigma-Aldrich), and Bovine serum albumin (66 kDa) (A8531, Sigma-Aldrich) were used as molecular mass standards.

Dynamic light scattering assays

MBP-CAS-2C, MBP-CAS-2C β 9, and MBP-CAS-2C β 8 β 9, which were freshly fractionated by Superose 6 gel filtration chromatography as described above, were analyzed by dynamic light scattering using DynaPro Plate Reader II (Wyatt Technology) at 25 °C for native molecular size estimation and 25 – 70 °C for assessment of conformational stability by heat denaturation. Hydrodynamic radii were calculated, and molecular weights as spherical particles were estimated using DYNAMICS 7.1 software (Wyatt Technology).

Determination of the apparent critical concentration of actin

Various concentrations of pyrene-labelled ATP-G-actin (20 % labelled) were polymerized in 0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, 20 mM HEPES, 0.2 mM DTT, 0.2 mM ATP for 18 h at room temperature. The pyrene fluorescence (excitation at 366 nm and emission at 384 nm) was measured with an F-4500 fluorescence spectrophotometer (Hitachi High-Technologies). Data were fitted to a linear correlation between total actin concentration and fluorescence intensity, and a critical concentration was determined as an inflection point of the actin concentration at which the slope changed. The equilibrium dissociation constant for CAS-2 binding to G-actin was calculated from the change in the apparent critical concentration as described [35].

Supernatant depletion assays for G-actin binding

DyLight 680-labeled ATP- or ADP-G-actin (0.2 µM, 10 % labeled) was incubated with various concentrations of MBP, MBP-CAS-2C, MBP-CAS-2C β 9, or MBP-CAS-2C β 8 β 9 in 0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, 20 mM HEPES, 0.2 mM DTT, 0.2 mM ATP or ADP, 0.1 mg/ml bovine serum albumin, 10 mM imidazole, pH 7.5 for 1 h at room temperature. Then, 20 µl of HisPur Ni-NTA Resin (Thermo Fisher Scientific) was added to the mixture and incubated for 1 h at room temperature with agitation (note that the MBP also has an N-terminal histidine-tag). The resin was removed by passing through a Micro-Spin Column (Thermo Fisher Scientific), and the flow-through fraction was subjected to SDS-PAGE (12 % acrylamide gel). The gel was scanned by an Odyssey Infrared Imaging

System (LI-COR) using a 700-nm channel. The fluorescence intensity of the actin band was quantified by ImageJ, and % G-actin depletion from the supernatants was calculated.

Assays for the exchange of actin-bound nucleotides

The exchange of actin-bound nucleotides was examined by monitoring the increase in the fluorescence of etheno-ATP. ADP-G-actin (1.3 μ M) in 150 μ l of G-buffer without ATP and 50 μ l of 160 μ M etheno-ATP with or without CAS-2C variants and/or UNC-60A were mixed. Then, the fluorescence of etheno-ATP (excitation at 360 nm and emission at 410 nm) was monitored for 20 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

For the experiments in Figures 6 and 8, F-actin (10 μ M) in F-buffer [0.1 M KCl, 2 mM 2 mM MgCl_2 , 0.2 mM DTT, 20 mM HEPES (pH 7.5)] with or without 0.5 mM ATP or ADP was preincubated with 20 μ M UNC-60A for 30 min at room temperature. Then, various concentrations of MBP or MBP-CAS-2 variants were added to the mixture and incubated for 30 min at room temperature. For the experiments in Figure 7, various concentrations of F-actin in F-buffer were incubated with 2 μ M MBP or MBP-CAS-2C variants for 1 h. The mixtures were ultracentrifuged at 42000 rev./min for 20 min at 4 $^{\circ}\text{C}$ using a Beckman 42.2Ti rotor. Supernatant and pellet fractions were adjusted to the same volumes and subjected to SDS-PAGE (12 % acrylamide gel) and staining with Coomassie Brilliant Blue R-250 (National Diagnostics). Protein Markers (29458-24, Nacalai USA) were used as molecular mass markers. Gels were scanned by an Epson Perfection V700 scanner at 300 dots per inch, and band intensity was quantified using ImageJ.

RESULTS

The C-terminal half of CAS-2 forms an oligomer through the C-terminal dimerization motif

To locate the dimerization motif of CAS-2, a non-muscle isoform of *Caenorhabditis elegans* CAP [27], we built a homology model of a C-terminal region of CAS-2 (residues 306 – 457) containing a CARP domain and a dimerization motif using the crystal structure of a C-terminal region of human CAP1 (residues 319 – 475) (PDB accession number 1K8F) [24] as a template (Fig. 1A–D). Quality of the homology model was assessed by QMEAN scores [36] (Supplementary Fig. 1). This fragment of human CAP1 has a helical arrangement of β -strands (β 1a to β 7) forming a homodimer *via* the two C-terminal β -strands (β 8 and β 9), which participate in a β -sheet of another monomer (Fig. 1C and D). Our homology model of the CAS-2 fragment was nearly identical to the structure of the human CAP1 fragment (Fig. 1A and B) and indicates that a dimerization motif of *C. elegans* CAS-2 is located at residues 439 – 457, which contains predicted β 8 and β 9. These “strand-exchanged” dimers of *C. elegans* CAS-2 and human CAP1 appear highly rigid and are stabilized by extensive monomer-monomer interfaces, suggesting that a point mutation would not allow us to disturb dimer formation. Previously, we showed that a C-terminal half of CAS-2 (CAS-2C: residues 208 – 457) has nearly as strong nucleotide exchange and actin-monomer recycling activities as full-length CAS-2 [27]. Therefore, we made two CAS-2C mutants with small C-

terminal truncations by introducing stop codons at positions of Q439 and I444, which are designated as CAS-2C β 8 β 9 and CAS-2C β 9, respectively (Fig. 1E). These CAS-2C variants were bacterially expressed and purified as fusion proteins with maltose-binding protein (MBP) (Fig. 1F). Although a recognition sequence for TEV protease is present at the junction between MBP and CAS-2C, cleavage of MBP by TEV protease was not successful. Therefore, we characterized CAS-2C variants as MBP-fusion proteins and used MBP in control experiments.

In analytical gel filtration chromatography, MBP-CAS-2C was eluted at 15 ml, whereas both MBP-CAS-2C β 9 and MBP-CAS-2C β 8 β 9 were eluted in a slower peak at 16.5 ml (Fig. 2A). Based on the elution profiles of molecular size markers (Fig. 2A and B, a–d), the size of MBP-CAS-2C was estimated as 300 kDa, whereas those of MBP-CAS-2C β 9 and MBP-CAS-2C β 8 β 9 were estimated as 78 kDa (Fig. 2B). Calculated molecular weights for monomers were MBP-CAS-2C (70.5 kDa), MBP-CAS-2C β 9 (69.1 kDa), and MBP-CAS-2C β 8 β 9 (68.4 kDa). These values suggest that, MBP-CAS-2C was estimated as a tetramer, whereas MBP-CAS-2C β 9 and MBP-CAS-2C β 8 β 9 were primarily monomeric. Since gel filtration can result in inaccurate estimation of molecular weight, we also analyzed hydrodynamic radii of these proteins in gel filtration fractions by dynamic light scattering measurements. Apparent hydrodynamic radii for MBP-CAS-2C, MBP-CAS-2C β 9, and MBP-CAS-2C β 8 β 9 were 4.67 nm, 3.47 nm, and 3.49 nm, respectively (Table 1). From these values, apparent native molecular weights were estimated as 124 kDa, 62 kDa, and 63 kDa, respectively (Table 1). Therefore, the dynamic light scattering assays suggest that MBP-CAS-2C is dimeric whereas MBP-CAS-2C β 9 and MBP-CAS-2C β 8 β 9 are monomeric. Conformational stability of these proteins were compared in a range of 25 – 70 °C by dynamic light scattering measurements. The three proteins were 50 % unfolded at 52 – 54 °C (Table 1) suggesting that the C-terminal truncations did not cause major alterations in the protein conformation. Thus, these results indicate that a C-terminal truncation of β 8 β 9 or β 9 specifically disrupts dimerization of CAS-2C.

The dimerization motif of CAS-2 is required for strong G-actin binding

The C-terminal half of CAP binds to G-actin and sequesters it from polymerization [24, 27]. Various concentrations of pyrene-labeled ATP-actin was polymerized in the presence of 2 or 5 μ M MBP-CAS-2C, and the pyrene fluorescence, which corresponds to the amounts of polymerized actin, was measured at a steady state (Fig. 3). MBP-CAS-2C effectively sequestered actin monomers and shifted the critical concentration from 0.10 μ M (actin only) to 1.0 and 3.9 μ M (2 and 5 μ M MBP-CAS-2C, respectively) (Fig. 3A). From these values, a dissociation constant for binding of MBP-CAS-2C with G-actin was estimated as 0.12 and 0.031 μ M (2 and 5 μ M, respectively). MBP alone did not have an effect on the critical concentration (Fig. 3A). Either MBP-CAS-2C β 9 or MBP-CAS-2C β 8 β 9 did not have a significant effect on the critical concentration (Fig. 3B and C). By using ADP-actin, MBP-CAS-2C effectively sequestered actin monomers as described previously [27], whereas either MBP-CAS-2C β 9 or MBP-CAS-2C β 8 β 9 did not (our unpublished data). However, the truncation mutants weakly enhanced pyrene fluorescence of polymerized ADP-actin (our unpublished data), which may be due to filament binding as described below.

Since actin monomer binding does not always result in actin monomer sequestration, we examined direct binding of CAS-2C to actin monomer by a modified supernatant depletion assay. G-actin was covalently labeled by DyLight 680 and incubated with MBP-CAS-2C variants with a polyhistidine tag. The MBP-fusion proteins and associated G-actin were pulled down by Ni-NTA resins. Then, DyLight 680-actin that remained in the supernatants was quantified by SDS-PAGE and an infrared imaging system (Fig. 4A), and percentages of G-actin depletion were calculated (Fig. 4B and C). The DyLight 680 label allowed quantitative measurements of G-actin with high specificity and sensitivity. The results show that MBP-CAS-2C bound to both ATP-actin ($K_d = 0.28 \mu\text{M}$) and ADP-actin ($K_d = 0.082 \mu\text{M}$) (Fig. 4A–C). However, MBP-CAS-2C $\beta 9$ and MBP-CAS-2C $\beta 8\beta 9$ poorly bound to G-actin (Fig. 4A–C). Therefore, these results indicate that the dimerization motif of CAS-2C is essential for its binding to actin monomer.

The C-terminal half of CAP is also responsible for enhancing exchange of G-actin-bound nucleotide [8, 23]. We previously showed that CAS-2C has nearly as strong nucleotide exchange activity as full-length CAS-2 [27]. MBP-CAS-2C strongly enhanced incorporation of etheno-ATP into ADP-G-actin, which is indicated by the rapid increase in the fluorescence of etheno-ATP (Fig. 5A). The rate of nucleotide exchange was enhanced 35-fold by $0.2 \mu\text{M}$ MBP-CAS-2C (Fig. 5C). UNC-60A (a *C. elegans* ADF/cofilin), at $1.0 \mu\text{M}$, slowed down exchange of actin-bound nucleotide (Fig. 5B) as described previously [27, 37], while $0.2 \mu\text{M}$ MBP-CAS-2C overrode UNC-60A's inhibitory effect and strongly enhanced the nucleotide exchange (Fig. 5B and C). However, either MBP-CAS-2C $\beta 9$ or MBP-CAS-2C $\beta 8\beta 9$ at $0.2 - 0.5 \mu\text{M}$ failed to enhance nucleotide exchange in the absence or presence of UNC-60A (Fig. 5A–C). Thus, the lack of the dimerization motif causes a strong defect in an important function of CAP as an actin nucleotide exchanger.

The dimerization motif of CAS-2 is required for ATP-dependent recycling of ADF/cofilin-bound actin monomers for polymerization

We previously reported that CAS-2 recycles UNC-60A-depolymerized actin monomers for polymerization in an ATP-dependent manner, and that CAS-2C is necessary and sufficient for this activity [27]. As expected from the effects on G-actin, CAS-2C with C-terminal truncations showed nearly no effects on actin-monomer recycling in the presence of UNC-60A (Fig. 6). We examined actin monomer-polymer equilibrium by sedimentation assays using ultracentrifugation. By actin alone in the presence of physiological salt concentrations (0.1 M KCl and 2 mM MgCl_2), most of actin was polymerized and precipitated (Fig. 6A, C, and E, lanes 1 and 2). UNC-60A promoted depolymerization by sequestering actin monomers, which resulted in a shift of $\sim 80\%$ actin into the supernatant (Fig. 6A, C, and E, lanes 3 and 4). In the presence of 0.5 mM ATP , $0.5 \mu\text{M}$ MBP-CAS-2C reduced UNC-60A-depolymerized actin to $\sim 50\%$ (Fig. 6A, lanes 7 and 8), indicating that MBP-CAS-2C induced recycling of a portion of UNC-60A-depolymerized actin for polymerization as described previously [27]. MBP-CAS-2C had a maximum activity above $0.1 \mu\text{M}$ in the presence of $20 \mu\text{M}$ UNC-60A and 0.5 mM ATP (Fig. 6B, white circles). However, in the absence of ATP or ATP (Fig. 6C, lanes 7 and 8) or presence of 0.5 mM ADP (Fig. 6E, lanes 7 and 8), MBP-CAS-2C had no detectable effects on UNC-60A-depolymerized actin (examined up to $1.0 \mu\text{M}$, Fig. 6D and F). MBP had no effects on

UNC-60A-depolymerized actin in the presence or absence of ATP or ADP (Fig. 6A, C, and E, lanes 5 and 6; Fig. 5B, D, and F, black circles). Either MBP-CAS-2C β 9 or MBP-CAS-2C β 8 β 9 also had no detectable effects on UNC-60A-depolymerized actin in the presence or absence of ATP or ADP (Fig. 6A, C, and E, lanes 8–12; Fig. 6B, D, and F). These results demonstrate that the dimerization motif of CAS-2C is critical for its actin-monomer recycling activity, which is suggested to be an important function of CAP to enhance actin filament turnover [5].

Truncation of the dimerization motif of CAS-2 promotes F-actin binding

The C-terminal half of CAP, including CAS-2C, primarily binds to G-actin [5]. However, we found that CAS-2C with C-terminal truncations exhibited enhanced binding to F-actin (Fig. 7). MBP-CAS-2C (2.0 μ M) was incubated with various concentrations of F-actin and examined by co-sedimentation assays. A portion of MBP-CAS-2C co-sedimented with F-actin and appeared to saturate at 17 % with an estimated dissociation constant of 5.3 μ M (Fig. 7B and G, white circles) suggesting a weak association with F-actin. MBP alone showed negligible co-sedimentation with F-actin (Fig. 7A and G, black circles). However, greater portions of MBP-CAS-2C β 9 and MBP-CAS-2C β 8 β 9 co-sedimented with F-actin (Fig. 7C and D). Binding of MBP-CAS-2C β 9 and MBP-CAS-2C β 8 β 9 with F-actin was saturated at similar values (39 % and 37 %, respectively) (Fig. 7G). Interestingly, MBP-CAS-2C β 8 β 9 (estimated K_d of 1.8 μ M) (Fig. 7D and G, white triangles) bound to F-actin more strongly than MBP-CAS-2C β 9 (estimated K_d of 6.2 μ M) (Fig. 7C and G, black triangles). These results suggest that the C-terminal β -strands have incremental inhibitory effects on F-actin binding. Deletion of the WH2 domain abolished F-actin binding without (MBP-CAS-2C WH2, Fig. 7E and G, black squares) or with C-terminal truncation (MBP-CAS-2C WH2 β 8 β 9, Fig. 7E and G, white squares), suggesting that the WH2 domain mediates F-actin binding of the CAS-2 C-terminal half.

Truncation of the dimerization motif in full-length CAS-2 abolishes its actin-monomer regulatory activity

CAPs from other organisms are known to oligomerize *via* the N-terminal region [18, 20, 21]. However, we found that truncation of β 9 from full-length CAS-2 (Fig. 8A) is sufficient to impair its actin-monomer regulatory activity. In the F-actin sedimentation assays in the presence of ATP, UNC-60A depolymerized F-actin and increased actin in the supernatant, whereas increasing concentrations of MBP-CAS-2 (full-length) increased actin in the pellets (Fig. 8B and D black circles), indicating that CAS-2 promoted recycling of actin monomers for polymerization. In contrast, MBP-CAS-2 β 9 had no significant effects on the amounts of UNC-60A-depolymerized actin (Fig. 8C and D white circles). In the presence of UNC-60A, 2 μ M MBP-CAS-2 enhanced nucleotide exchange on G-actin, while 2 – 5 μ M MBP-CAS-2 β 9 did not (Fig. 8E). These results indicate that β 9, as a part of the dimerization motif, is required in the context of full-length CAS-2 for competing with UNC-60A to promote nucleotide exchange and recycling of actin monomers for polymerization.

DISCUSSION

In this study, we showed that the C-terminal dimerization motif of CAS-2, a *C. elegans* CAP, is essential for its interaction with G-actin. The C-terminal half of CAS-2 is necessary and sufficient for binding to G-actin, which causes monomer sequestration and enhancement of nucleotide exchange. Truncation of the dimerization motif abolishes these effects on G-actin. As a result, CAS-2 C-terminal fragments lacking the dimerization motif fails to compete with ADF/cofilin to promote ATP-dependent actin turnover. These results are somewhat surprising because the C-terminal fragment contains previously identified G-actin-binding sites in WH2 [12] and CARP [11, 19, 23], indicating that these G-actin-binding sites are not sufficient to regulate actin dynamics without the dimerization motif. In contrast, the dimerization motif appears to be inhibitory for F-actin binding. These results suggest that the role of the CAP dimerization motif is to maintain a conformation that is specifically suitable for binding to G-actin.

Both MBP-CAS-2C $\beta 9$ and MBP-CAS-2C $\beta 8\beta 9$ were predominantly monomeric (Fig. 2), indicating that $\beta 9$ is required to stabilize a CAS-2C dimer. In yeast Srv2/CAP, human CAP1, and apicomplexan C-CAP, $\beta 8$ and $\beta 9$ form a hairpin-like structure and participate in a strand-exchanged dimer with a number of inter-strand bonds [24, 25]. Although $\beta 8$ interacts with $\beta 6a$ of another monomer with extensive hydrogen bonding, additional strand-exchanged interactions involving $\beta 9$ should play important roles in stabilizing a CAP dimer. The right-handed β -helical CARP domain is also present in several other proteins including tubulin cofactor C (TBCC) and X-linked retinitis pigmentosa 2 (RP2) [24]. However, either TBCC [38] or RP2 [39] lacks a dimerization motif and binds to a ligand as a monomer. Therefore, CAP has adopted a unique dimerization motif to bind to actin monomers.

We hypothesize that the rigid dimer of the CAP C-terminal region has a stable conformation suitable for G-actin-binding and subsequent monomer sequestration and nucleotide exchange. The parallel β -sheets, PB1 and PB2, on the surface of the CARP domain are slightly curved such that the dimer shows an “S” shape when the dimer is viewed down the helical axes [24] (Fig. 1B and D). This curved surface of the CAP dimer may provide a wide G-actin-binding interface spanning the two CAP monomers. Thus, monomeric CAP may not be able to hold G-actin in a stable complex. In the absence of an atomic-level structure of a CAP-G-actin complex, we currently do not know how G-actin interact with the CARP domain. Previous yeast two-hybrid interaction analysis using mutant actin suggest that actin surface residues that are involved in interaction with yeast Srv2/CAP are relatively spread [40]. Additional biochemical investigation on the CARP-G-actin interaction is required to understand how CARP regulates G-actin and how dimerization is important for this function.

The CAS-2 C-terminal fragments lacking the dimerization motif showed increased binding to F-actin, which is mediated by WH2 (Fig. 7). These results suggest another function of the dimerization motif to inhibit F-actin binding. Truncation of $\beta 9$ and $\beta 8\beta 9$ incrementally enhanced F-actin binding. Removal of the dimerization motif may allow to adopt a conformation suitable for F-actin binding. These results preclude the possibility that the C-terminal truncations disrupted the overall conformation of WH2 and CARP because the

mutants retained ability to bind to actin molecules. In the F-actin co-sedimentation assay, binding of full-length or truncated CAS-2C to F-actin reached a plateau leaving constant concentrations of CAS-2C variants unbound. This suggests that CAS-2C is present in multiple conformations, and that C-terminal truncations shift the equilibrium to a conformation that favors F-actin binding. Interestingly, the N-terminal helical-folded domain of mouse CAP1 also binds to F-actin in a similar manner [19], but a precise mechanism of this binding mode is unknown. Alternatively, the dimerization motif may interfere with F-actin binding by physically blocking the access of WH2 and/or CARP with the exposed surface of F-actin. In either case, the dimerization motif appears to be important to promote the G-actin-specific regulatory function of CAP by restricting competitive interaction with F-actin. Taken together, our study suggests that the G-actin regulatory function of CAP depends on dimerization through the C-terminus, and that the unique dimer configuration of the CARP domain is specifically adapted for G-actin binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations List

ADF	actin depolymerizing factor
CAP	cyclase-associated protein
CARP	cyclase-associated protein and X-linked retinitis pigmentosa 2
DLS	dynamic light scattering
MBP	maltose-binding protein
WH2	Wiskott-Aldrich Syndrome protein homology 2

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Figure 1. Homology model of the C-terminal region of CAS-2 and truncation of the putative dimerization motif

(A–D) A homology model of the dimeric CAS-2 C-terminal region (residues 306 – 457) (A and B) and the crystal structure of the dimeric human CAP1 C-terminal region (Protein Data Bank accession number 1K8F) (C and D) are shown as side views perpendicular to the dimer axes (A and C) and top views down the dimer axes (B and D). Each polypeptide chain is colored differently. Designations of β -strands (1a – 9) and parallel β -sheets (PB1 and PB2) correspond to those in human CAP1 [24]. From the homology model, Q439 and I444

were identified as positions for introduction of stop codons to truncate $\beta 8\beta 9$ and $\beta 9$, respectively (indicated by red in **A** and **B**). (**E**) Schematic domain structures of CAS-2 and its C-terminal fragments used in this study. The highlighted domains are the helical-folded domain (HFD), the proline-rich region (P), the WH2 domain, the CARP domain, and the dimerization motif. Residue numbers are shown on top of each model. (**F**) Purified MBP-CAS-2C (lane 1), MBP-CAS-2C $\beta 9$ (lane 2), MBP-CAS-2C $\beta 8\beta 9$ (lane 3), and MBP (lane 4) were analyzed by SDS-PAGE (5 μ g/lane) and visualized by Coomassie Brilliant Blue staining. Molecular mass markers in kDa (lane M) are shown on the left-hand side of the gel.

Figure 2. Estimation of oligomerization states of CAS-2C variants by analytical gel filtration chromatography

(A) MBP-CAS-2C, MBP-CAS-2C $\beta 9$, and MBP-CAS-2C $\beta 8\beta 9$ were applied to Superose 6 gel filtration chromatography (24 ml bed), and the eluted fractions were analyzed by SDS-PAGE. Relative amounts of eluted proteins are plotted on the graph. Peak positions of molecular mass markers are shown as: **a**, apoferritin (443 kDa); **b**, alcohol dehydrogenase (200 kDa); **c**, β -amylase (150 kDa); **d**, bovine serum albumin (66 kDa). (B) Native molecular masses of MBP-CAS-2C, MBP-CAS-2C $\beta 9$, and MBP-CAS-2C $\beta 8\beta 9$ were

estimated by the elution profiles of the standard proteins. Known molecular masses (Da) of the standard proteins are plotted in a logarithmic scale as a function of elution volumes (ml), and a standard line was drawn by linear regression. Based on the peak positions, MBP-CAS-2C was estimated as 300 kDa, while MBP-CAS-2C β 9 and MBP-CAS-2C β 8 β 9 were estimated as 78 kDa.

Figure 3. Effects of CAS-2C variants on the critical concentration of actin

Various concentrations of pyrene-labelled actin were polymerized in the absence of any additional protein (A–C, control: open circles) or presence of 5 μM MBP (A, open triangles), 2–5 μM MBP-CAS-2C (A, closed triangles and squares), 2–5 μM MBP-CAS-2C $\beta 9$ (B, closed triangles and squares), 2–5 μM MBP-CAS-2C9 $\beta 8\beta 9$ (C, closed triangles and squares) for 18 h, and the pyrene fluorescence (arbitrary units) was measured and plotted as a function of total actin concentrations. The apparent critical concentrations

were determined as the inflection points of actin concentrations at which a linear increase of the fluorescence was initiated.

Figure 4. Binding of CAS-2C variants to G-actin analyzed by supernatant depletion assays

DyLight 680-labeled ATP- or ADP-G-actin ($0.2 \mu\text{M}$) was incubated with various concentrations of MBP, MBP-CAS-2C, MBP-CAS-2C $\beta 9$, or MBP-CAS-2C $\beta 8\beta 9$ for 1 h. The MBP-fusion proteins and associated G-actin were pulled down by Ni-NTA resins, and DyLight 680-actin in the supernatants were analyzed by SDS-PAGE and infrared imaging (A). Percentages of G-actin depletion from the supernatants were calculated and plotted as a function of total concentrations of MBP-fusion proteins for ATP-actin (B) and ADP-actin (C). Data are means $\pm$ standard deviations ($n = 3$).

Figure 5. Effects of CAS-2C variants on the exchange of actin-bound nucleotides in the presence or absence of UNC-60A

ADP-G-actin (1 μ M) was incubated with etheno-ATP in the absence (**A**) or presence of 1 μ M UNC-60A (**B**) with or without MBP, MBP-CAS-2C, MBP-CAS-2C $\beta 9$, or MBP-CAS-2C $\beta 8\beta 9$, and the fluorescence of etheno-ATP was monitored over time (**A** and **B**). MBP-CAS-2C $\beta 9$ and MBP-CAS-2C $\beta 8\beta 9$ are abbreviated to $\beta 9$ and $\beta 8\beta 9$ in **A** and **B**, respectively. Rates of exchange of nucleotides [k_{obs} (s^{-1})] were determined from the data and shown in the bar graph (**C**). Data are means $\pm$ standard deviations ($n = 3$).

Figure 6. Effects of CAS-2C variants on the amounts of pelletable F-actin in the presence of UNC-60A

F-actin (10 μ M) was pre-incubated with 20 μ M 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 incubated with buffer only or buffer with various concentrations of MBP, MBP-CAS-2C, MBP-CAS-2C $\beta 9$, or MBP-CAS-2C $\beta 8\beta 9$ for 30 min. The mixtures were ultracentrifuged and the supernatants (S) and pellets (P) were analyzed by SDS-PAGE. Representative results of gels with 0.5 μ M MBP, MBP-CAS-2C, MBP-CAS-2C $\beta 9$, or MBP-CAS-2C $\beta 8\beta 9$ are

shown in **A**, **C**, and **E**. Molecular mass markers in kDa (lane M) are shown on the left-hand side of the gel. Percentages of actin in the supernatants were quantified by densitometry and plotted as a function of concentrations of the CAS-2 variants in **B**, **D**, and **F**. Data are means $\pm$ standard deviations ($n = 3$).

Figure 7. Binding of CAS-2C variants to F-actin

Various concentrations of F-actin were incubated with 2 μ M MBP (A), MBP-CAS-2C (B), MBP-CAS-2C $\beta 9$ (C), MBP-CAS-2C $\beta 8\beta 9$ (D), MBP-CAS-2C WH2 (residues 257 – 457) (E), or MBP-CAS-2C WH2 $\beta 8\beta 9$ (residues 257 – 438) (F) for 1 h and ultracentrifuged. The supernatants (S) and pellets (P) were analyzed by SDS-PAGE (A–F), and portions (%) of MBP or MBP-CAS-2C variants that co-sedimented with F-actin were plotted as a function of total F-actin concentrations (G). Positions of actin (A) and MBP or MBP-CAS-2C variants (M) are indicated on the right-hand side of the gels. Sedimented

portions in the absence of actin were subtracted from the data as non-specific sedimentation. Data are means $\pm$ standard deviations (n = 3).

Figure 8. Effects of a C-terminal truncation in full-length CAS-2 on actin-monomer regulation
(A) Schematic structures of full-length CAS-2 and CAS-2 β 9. Residue numbers are shown on top of each model. **(B–D)** Effects of MBP-CAS-2 and MBP-CAS-2 β 9 on the amounts of pelletable F-actin in the presence of UNC-60A were analyzed by F-actin sedimentation assays. F-actin (10 μ M) was pre-incubated with 20 μ M UNC-60A and 0.5 mM ATP for 30 min, and then incubated with buffer only or buffer with various concentrations of MBP-CAS-2 **(B)** or MBP-CAS-2 β 9 **(C)** for 30 min. The mixtures were ultracentrifuged and the supernatants (S) and pellets (P) were analyzed by SDS-PAGE **(B, C)**. Molecular mass markers in kDa are shown on the left-hand side of the gel. Positions of actin (A) and

UNC-60A (U) are indicated on the right of each gel. Percentages of actin in the supernatants were quantified by densitometry and plotted as a function of concentrations of the CAS-2 variants in **D**. Data are means $\pm$ standard deviations ($n = 3$). (**E**) Effects of MBP-CAS-2 and MBP-CAS-2 $\beta 9$ on the exchange of actinbound nucleotides in the presence of UNC-60A. ADP-G-actin (1 μ M) was incubated with etheno-ATP in the presence of 1 μ M UNC-60A with or without MBP-CAS-2 or MBP-CAS-2 $\beta 9$, 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 bar graph. Data are means $\pm$ standard deviations ($n = 3$).

Estimation of oligomerization states of CAS-2C variants by gel filtration chromatography and dynamic light scattering (DLS).

Table 1

Protein	Estimated molecular mass (kDa)				r_h (nm)*	T_m (°C)**
	Gel filtration	DLS	Calculated			
MBP-CAS-2C	300	124	71		4.67±0.24	54
MBP-CAS2C β9	78	62	69		3.47±0.41	53
MBP-CAS2C β8β9	78	63	68		3.49±0.36	52

* Hydrodynamic radius (r_h) was determined by dynamic light scattering (DLS) measurements. Data are means ± standard deviations, n = 10.

** Melting temperature (T_m) was determined by measuring r_h in a range of 25 – 70 °C. T_m represents a temperature at which 50 % of the protein was unfolded.