Mammalian and Malaria Parasite Cyclase-associated Proteins Catalyze Nucleotide Exchange on G-actin through a Conserved Mechanism*

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Background: CAP recycles actin monomers from ADF/cofilin and catalyzes their nucleotide exchange through an unknown mechanism.

Results: The β-sheet domain of mouse CAP1 and a "mini-CAP" from the malaria parasite promote nucleotide exchange on actin.

Conclusion: The most conserved function of CAPs is re-charging actin monomers with ATP.

Significance: Actin dynamics from mammals to apicomplexan parasites rely on CAP-catalyzed nucleotide exchange.

Cyclase-associated proteins (CAPs) are among the most highly conserved regulators of actin dynamics, being present in organisms from mammals to apicomplexan parasites. Yeast, plant, and mammalian CAPs are large multidomain proteins, which catalyze nucleotide exchange on actin monomers from ADP to ATP and recycle actin monomers from actin-depolymerizing factor (ADF)/cofilin for new rounds of filament assembly. However, the mechanism by which CAPs promote nucleotide exchange is not known. Furthermore, how apicomplexan CAPs, which lack many domains present in yeast and mammalian CAPs, contribute to actin dynamics is not understood. We show that, like yeast Srv2/CAP, mouse CAP1 interacts with ADF/cofilin and ADP-G-actin through its N-terminal α-helical and C-terminal β-strand domains, respectively. However, in the variation to yeast Srv2/CAP, mouse CAP1 has two adjacent profilin-binding sites, and it interacts with ATP-actin monomers with high affinity through its WH2 domain. Importantly, we revealed that the C-terminal β-sheet domain of mouse CAP1 is essential and sufficient for catalyzing nucleotide exchange on actin monomers, although the adjacent WH2 domain is not required for this function. Supporting these data, we show that the malaria parasite Plasmodium falciparum CAP, which is entirely composed of the β-sheet domain, efficiently promotes nucleotide exchange on actin monomers. Collectively, this study provides evidence that catalyzing nucleotide exchange on actin monomers via the unknown mechanism.

The actin cytoskeleton is crucial for a number of cellular processes, including morphogenesis, migration, endocytosis, and cytokinesis. Actin filaments provide force for these processes by coordinated polymerization against cellular membranes and by serving as tracks for myosin motor proteins (1–3). Although the actin molecule itself and its role in these processes are very well conserved in eukaryotes from plants and fungi, the biochemical and functional properties of actin in apicomplexa parasites, which include the etiological agents of malaria and toxoplasmosis, are somewhat different. This is because apicomplexan actins share only 75–80% amino acid identity to mammalian actins, compared with >90% sequence identity between yeast and animal actins. Furthermore, apicomplexan actins form only unstable filaments. This is despite the fact that the actin cytoskeleton is essential for myosin-based movement, a process that is critical for parasite infection of host cells and life cycle progression (4).

The structure, dynamics, and the three-dimensional organization of actin filaments in cells are regulated by a large array of actin-binding proteins (1). Interestingly, only six groups of actin-binding proteins are conserved between mammalian cells and apicomplexan parasites. From these, the actin filament nucleating proteins formins, together with ATP-G-actin-binding protein profilin, drive actin filament assembly, whereas the heterodimeric capping protein inhibits actin filament assembly at the filament barbed ends. Actin-depolymerizing factor (ADF)/cofilins drive rapid actin dynamics by promoting filament severing/demembranization, although the malaria parasite ADF/cofilins appear to display significant biochemical differences compared with their mammalian counterparts (5–10).
The exact functions of the two other actin-binding proteins that are conserved between mammals and apicomplexan parasites, namely coronin and cyclase-associated protein (CAP), are currently less well understood.

CAP is a ubiquitous regulator of actin dynamics, which was first identified as a protein associated with adenylyl cyclase and a suppressor of an activated Ras allele (11, 12). The N-terminal region of the yeast CAP homologue (named Srv2) interacts with adenylyl cyclase, whereas the C-terminal region of this protein binds monomeric actin with high affinity (13–15). Subsequent studies showed that the ability to interact with actin in vitro and regulate actin dynamics in vivo are conserved functions of CAPs in all eukaryotes (16). Inactivation of CAP in mammalian, Caenorhabditis elegans, Drosophila, Dictyostelium, yeast, and plant cells results in severe defects in the organization of the actin cytoskeleton, abnormal accumulation of filamentous actin, and consequent problems in many actin-dependent processes such as endocytosis and cell migration (17–24). Inactivation of the CAP homologue from Plasmodium berghei demonstrated that this protein is essential for malaria parasite oocyst development in the mosquito midgut (25). Because oocyst development is not traditionally seen as an actin-dependent process, the direct role of CAP in regulating actin dynamics in apicomplexan parasites (for example in parasite cell motility) remains unknown.

Yeast, animal, and plant CAPs are large oligomeric proteins composed of multiple protein-protein interaction motifs. They are believed to promote rapid actin dynamics by releasing ADP-actin monomers from ADF/cofilin and recharging them to the assembly-competent ATP-bound state, and by accelerating actin monomers from ADF/cofilin and recharging them to the assembly-competent ATP-bound state, and by accelerating nucleotide exchange on actin monomers together with the WH2 domain (26–29). CAP is composed of an N-terminal α-helical domain, followed by a proline-rich region, a WH2 domain, and a C-terminal β-sheet domain (1A) (30–32). The C-terminal half of the protein binds ADP-G-actin with high affinity and promotes nucleotide exchange on actin monomers (14, 26, 31, 33). In budding yeast Srv2/CAP, the main ADP-G-actin binding region is located at the β-sheet domain; also, additional regions toward the N terminus from the domain are required for high affinity ADP-G-actin. However, mutations in the adjacent WH2 domain did not affect ADP-G-actin binding (15). Instead, the WH2 domain of yeast Srv2/CAP binds ATP-G-actin with moderate affinity (K_D ~1.5 μM) and was reported to promote the nucleotide exchange on actin monomers together with the β-sheet domain (31). Budding yeast Srv2/CAP also interacts with profilin through its proline-rich region, and with the ADF/cofilin-G-actin complex through specific residues at the N-terminal α-helical domain (34, 35). Whether these interactions are conserved in CAPs from other species is not known. Interestingly, CAPs from apicomplexan parasites are entirely composed of a β-sheet domain and lack other protein-protein interaction domains present in yeast and mammalian CAPs. Although they bind G-actin, the mechanism by which these “mini-CAPs” contribute to actin dynamics remains unknown (25).

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The C-terminal half of mouse CAP1 (C-CAP1) encoding residues 216–474 and β-sheet domain of budding yeast CAPs (ScSrv2(328–526)) were cloned into pGAT2 vector (36) to yield plasmids pPL304 and pPL690, respectively. The N-terminal half of mouse CAP1 (N-CAP1) encoding residues 1–221 and full-length Plasmodium falciparum CAP were cloned into the pHAT2 vector (36) to yield plasmids pPL357 and pPL590, respectively. The mouse CAP1 fragment containing the WH2 domain (WH2-CAP1) encoding residues 216–322 was cloned into pGAT2 (pPL379) vector for tryptophan fluorescence assays and into pHAT2 (pPL589) vector for NBD-actin fluorescence assays, actin polymerization assays, and nucleotide exchange assays. Specific mutations were introduced into CAP1 fragments by site-directed mutagenesis using the mutagenic oligonucleotides in PCR amplification to produce C-CAP1 constructs mutβ (pPL539) and mutWH2 (pPL505) and N-CAP1 construct mutα (pPL542). Alanine substitutions of prolines at the polyproline region of the WH2-CAP1 expressing vector resulted in constructs mutPP1 (pPL366), mutPP2 (pPL368), and mutPP1 + mutPP2 (pPL496). For detailed information about the mutated residues see Fig. 1A.

**Protein Expression and Purification**—All mouse CAP1, yeast Srv2/CAP, PfCAP, cofilin, and profilin constructs were expressed in Escherichia coli BL21 cells as described previously for mouse MIM-CT (37), except that wild-type and mutant mouse C-CAP1 and ScSrv2(328–526) constructs were grown at 16 °C for 16–20 h instead of 37 °C. Wild-type and mutant C-CAP1 proteins and ScSrv2(328–526) were purified with Ni-NTA Superflow beads (Qiagen). First, cell pellets were resuspended to 20 mM Tris, pH 8, 250 mM NaCl, 20 mM imidazole, 0.5 mM N-dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), lysed by sonication, and centrifuged 11,500 × g for 30 min. Cell lysates were added to Ni-NTA Superflow beads and incubated for 1–2 h at 4 °C in rotation. Beads were washed several times with buffer 20 mM Tris, pH 8, 250 mM NaCl, 20 mM imidazole, 0.5 mM DTT, and the proteins were eluted by increasing the concentration of imidazole to 250 mM. Imidazole was removed by dialyzing eluates against 2 liters of buffer (20 mM Tris, pH 8, 250 mM NaCl, 0.5 mM DTT) at 4 °C overnight. Protein solutions were concentrated in 10-kDa cut-off tubes (Millipore) to volumes of 100–500 μl.

For profilin binding assays, GST fusion proteins WH2-CAP1 WT, WH2-CAP1 mutPP1, WH2-CAP1 mutPP2, and WH2-CAP1 mutPP1 + mutPP2 were purified by glutathione-agarose beads followed by a thrombin digestion as described previously for mouse MIM-CT (37). After thrombin digestion, WH2-CAP1 protein solutions were diluted to buffer 30 mM Tris, pH 6.8, 150 mM NaCl and loaded onto a cation exchange column. Proteins were eluted from the column by a linear 0.15–1 mM NaCl gradient, and the buffer was exchanged to 30 mM Tris, pH 7.5, 50 mM NaCl in a concentration device. Mouse profilin-1 was purified as described previously for yeast cofilin (38), with the exception that buffers contained 20 mM Tris, pH 8.0.

PfCAP and WH2-CAP1 (in PHAT2 vector) were purified with Ni-NTA Superflow beads (Qiagen) as described for C-CAP1 proteins, except that DTT was not included in buffers, and 1% Triton X-100 was added to lysis buffer. The absence of DTT in the purification buffer did not contribute to oligomerization of PfCAP in SDS-PAGE (see Fig. 5A), because PfCAP
How CAPs Promote Nucleotide Exchange on Actin

purified in the presence of DTT also oligomerized on SDS-PAGE. The protein elutes were dialyzed against 20 mM Tris, pH 7.5, 50 mM NaCl. After dialysis, the protein solutions were concentrated and loaded into Superdex 75 or Superdex 200 gel filtration column (GE Healthcare). PfCAP eluted from Superdex 200 16/60 gel filtration column as a single peak at 75–85 ml, suggesting that the protein is either a monomer or a dimer in solution. In case of PfCAP, the fractions containing the protein were pooled and concentrated in a 10-kDa concentration tube, whereas WH2-CAP1 was further purified by a cation exchange column with buffer 30 mM Tris, pH 8.8, 50 mM NaCl. WH2-CAP1 was eluted from the column by a linear 0.05–1 M NaCl gradient, and the buffer was changed to 20 mM Tris, pH 7.5, 50 mM NaCl.

N-CAP1 WT and mutant were purified by resuspending the bacterial pellets to 20 mM Tris, pH 8, 250 mM NaCl, 20 mM imidazole, 0.2 mM PMSE, sonicating, and centrifuging at 11,500 × g for 30 min. The supernatant was further centrifuged at 100,000 × g for 35 min and loaded into a metal ion affinity chromatography column packed with chelating Sepharose chromatographic medium. N-CAP1 was eluted by a linear 20–250 mM imidazole gradient, and the eluate was dialyzed against 20 mM Tris, pH 8, 50 mM NaCl overnight. Protein solution was further purified by an anion exchange column packed with Q-Sepharose HP-agarose (GE Healthcare) by eluting N-CAP1 using a linear 0.05–1 M NaCl gradient.

Rabbit skeletal muscle actin was prepared as described previously (39). ADP-G-actin was prepared by incubating ATP-G-actin on hexokinase beads with glucose for 2–3 h at 4°C as described previously (40). Hexokinase beads were prepared by coupling hexokinase (Sigma) to CNBr-activated Sepharose 4B beads (GE Healthcare).

Actin Monomer Binding and Profilin Binding Assays—Interactions of C-CAP1 with ADP-G- and ATP-G-actin were measured by recording the change in the fluorescence of NBD-labeled G-actin as described previously (34, 41). The reaction buffer contained 2 mM Tris, pH 8, 0.1 mM CaCl₂, 1 mM MgCl₂, 100 mM KCl, 0.1 mM DTT, 0.2 mM ATP or ADP, 0.5 mg/ml BSA. ADP-G-actin-binding experiments were carried out as competition assays by using a twofold in construct, which binds ADP-G-actin with a $K_d$ of 0.03 μM at physiological salt (42). The data were fitted and analyzed by using the equations described in Ojala et al. (42). Interaction of CAP1 with profilin-1 was measured by trypsin fluorescence spectroscopy as described previously (34).

Native PAGE—The competition of C-CAP1 and profilin for ADP-G-actin binding was studied by native PAGE. The ADP-G-actin was prepared as described previously (34). The proteins were mixed in buffer (5 mM Tris, pH 8, 0.1 mM CaCl₂, 0.2 mM DTT, 0.2 mM ADP) and incubated at 4°C overnight. The final concentrations were as follows: 5 μM for both ADP-G-actin and profilin and 0, 0.1, 0.5, 1.0, 2.5, and 5.0 μM for C-CAP1. Proteins were mixed in 4× loading buffer (125 mM Tris, pH 8.8, 250 mM NaCl, 2.5 mM DTT, 50% glycerol, 0.1 mM ADP, and bromphenol blue), and the samples were run on 6% PAGE prepared without SDS at 4°C in a buffer (25 mM Tris, pH 8.3, 195 mM glycine, 0.5 mM DTT, 0.1 mM ADP, 0.1 mM CaCl₂).

Nucleotide Exchange Assay—The rate of nucleotide exchange from ADP-β-γ-G-actin to ε-ATP-G-actin (Invitrogen) was examined by mixing 20 μl of rabbit skeletal muscle actin alone or in the presence of CAP proteins in reaction buffer (20 mM Hapes-KOH, 8 mM Tris-HCl, 2 mM MgCl₂, 80 mM KCl, 0.1 mM EGTA, and 0.1 mM DTT, pH 6.9) (26) with 30 μl of ε-ATP. The final concentrations of ADP-G-actin and ε-ATP were 0.5 and 50 μM, respectively, whereas the concentrations of CAPs were varied as described in the legends to Figs. 4 and 6. The reaction was monitored with excitation of 365 nm and emission 410 nm at 25°C. The inverse of the half-time values was used for determining the $K_d$ value for the PfCAP-actin complex. The values were normalized from 0 to 1, and the binding curve was fitted to values by using equations described in Ojala et al. (42). Assays to measure the exchange of ε-ATP to ATP in monoclonal actin were carried out as described previously (37).

Actin Polymerization Assay—Actin polymerization in the presence of spectrin-F-actin seeds was measured by following the change in the fluorescence of pyrene-labeled actin (Cytokeleton). Unlabeled rabbit skeletal muscle actin and pyrene-labeled actin were first centrifuged 350,000 × g for 30 min to remove aggregates/oligomers. Unlabeled and pyrene-labeled actins were mixed together (90% unlabeled rabbit skeletal muscle actin and 10% pyrene-labeled rabbit skeletal muscle actin) and added to the reaction buffer with WH2-CAP1 construct or twinfilin and spectrin-F-actin seeds, resulting in a final concentration of 2.5 μM actin. The reaction buffer contained 5 mM Tris, pH 7.5, 0.2 mM CaCl₂, 2 mM MgCl₂, 100 mM KCl, 1 mM EGTA, 0.2 mM ATP, 0.2 mM DTT. The reaction was recorded at an excitation wavelength of 365 nm and emission wavelength of 407 nm. Spectrin-F-actin seeds were prepared as described previously (43). The apparent polymerization rates were determined from the slopes of polymerization curves.

Analytical Ultracentrifugation—Sedimentation velocity experiments were carried out at 22°C in a model E analytical ultracentrifuge (Beckman) equipped with absorbance optics, a photoelectric scanner, a monochromator, and an on-line computer. A four-hole rotor An-F Ti and 12-mm double sector cells were used. Rotor speed was 52,000 rpm. The sedimentation profile of PfCAP was recorded by measuring the absorbance at 280 nm. All cells were scanned simultaneously. The sedimentation coefficients were estimated from the differential sedimentation coefficient distribution ($c(s)$ versus $s$) using the SEDFIT program (44).

Supernatant Depletion Pulldown Assay—The supernatant depletion pulldown assay for examining the interaction between the N-terminal α-helical domain of CAP1 and GST-cofilin-1/ADP-G-actin complex was carried out as described previously (35). 2 μM ADP-actin and 1 μM N-CAP1 were incubated with GST or GST-cofilin-1 coupled to glutathione-agarose beads, and the amount of N-CAP1 in supernatant fractions was quantified from Coomassie-stained SDS-polyacrylamide gels by Quantity One program (Bio-Rad).

RESULTS

Mammalian CAP1 Binds ADP-G-actin with High Affinity through Its β-Sheet Domain—Yeast Srv2/CAP binds ADP-G-actin with high affinity ($K_d$ = 0.02 μM) through a site that was
mapped to specific residues (Glu-382, Glu-385, Asp-461, Lys-462, Lys-472, and Glu-473) in the C-terminal β-sheet domain (15). To reveal whether the high affinity ADP-G-actin interaction is conserved between yeast and mammalian CAPs, a C-terminal fragment of mouse CAP1 comprising residues 216–474 and containing the WH2 domain, polyproline regions PP1 and PP2, and the β-sheet domain (C-CAP1; Fig. 1, A and B) was expressed in E. coli and tested for binding to NBD-labeled G-actin by a fluorometric assay. Because interaction between C-CAP1 and NBD-ADP-G-actin resulted only in a very small change in the NBD fluorescence, C-CAP1 binding to G-actin was examined by a competition assay with another actin-binding protein, twinfilin-1. The twinfilin construct used in the assay binds ADP-G-actin with a $K_D$ of 0.03 μM and results in an increase in the NBD fluorescence when bound to G-actin (42). Addition of the C-terminal fragment of mouse C-CAP1 resulted in a decrease of NBD fluorescence of twinfilin-saturated actin monomers indicating that the two proteins compete for G-actin binding. This was further supported by a native PAGE assay, which demonstrated that C-CAP1 indeed can dissociate the twinfilin/ADP-G-actin complex (Fig. 2B). The NBD fluorescence data were fitted as described previously (42) by using the $K_D$ value of 0.03 μM for a twinfilin fragment and gave an equilibrium dissociation constant of 0.05 μM for the C-CAP1/ADP-G-actin complex at physiological ionic conditions (Fig. 2B). Importantly, mutations in the WH2 domain (mutWH2) did not decrease the affinity of C-CAP1 for ADP-G-actin (Fig. 2C), whereas mutations in the β-sheet domain (mutβ) corresponding to the surface-exposed residues that are critical for ADP-G-actin binding in yeast Srv2/CAP (15) efficiently diminished the ADP-G-actin binding in mouse C-CAP1 (Fig. 2D). Thus, these experiments demonstrate that high affinity ADP-G-actin binding through the C-terminal β-sheet domain is conserved between yeast and mammalian CAPs.

Interaction of the WH2 Domain of Mouse CAP1 with ATP-G-actin—WH2 domain is a ubiquitous ATP-actin monomer-binding motif that is present in many regulators of the actin cytoskeleton (45). Yeast, plant, and animal CAPs harbor a WH2 domain at their central region. However, studies on yeast Srv2/CAP revealed that this domain binds ATP-G-actin only with modest affinity ($K_D$ = 1.5–1.9 μM (15, 31)). Fluorometric assays revealed that the C-terminal fragment of mouse CAP1 increased the NBD-ATP-G-actin fluorescence by ~40%, although it has only minor effects on NBD-ADP-G-actin fluorescence. Assuming a 1:1 complex formation, these data show that C-CAP1 binds ATP-G-actin with a relatively high $K_D$ of ~0.4 μM (Fig. 3A). Interestingly, mutations at the β-sheet domain, which inactivate the ADP-G-actin binding of CAP1

**FIGURE 1. Sequence alignment of cyclase-associated proteins.** A, amino acid sequences of *Saccharomyces cerevisiae* Srv2/CAP (P17555), *P. falciparum* CAP (Q8I288), and *Mus musculus* CAP1 (P40124). The positions of different domains in CAP are marked above the sequences in white boxes. PP1 and PP2 correspond to the two polyproline regions of CAP. Alanine substitution mutations generated in this study are indicated below the sequences. B, schematic representation of CAP fragments used in this study. Alanine substitution mutations are marked on the domains with arrows (the exact amino acids mutated are visible in A). The numbers above the domains correspond to amino acid residues of each construct.
How CAPs Promote Nucleotide Exchange on Actin

FIGURE 2. Interaction of mouse CAP1 with ADP-G-actin. A, native gel assay for measuring competition between twinfilin and C-CAP1 for ADP-G-actin binding. 1st lane, actin. 2nd lane, twinfilin. 3rd lane, actin and twinfilin. 4th lane, C-CAP1. 5th lane, actin and C-CAP1. 6th to 10 lanes, actin, twinfilin, and increasing amounts of C-CAP1 from 0.1 to 5.0 μM. The concentrations of actin and twinfilin were both 5.0 μM. ADP-G-actin forms a complex with twinfilin (indicated by an asterisk) and with C-CAP1 (indicated by two adjacent asterisks). In the competition experiment (6th to 10 lanes), the ADP-G-actin/C-CAP1 complex band becomes visible when C-CAP1 concentration is increased, and this is accompanied by the disappearance of the ADP-G-actin/twinfilin complex band. Thus, C-CAP1 and twinfilin compete for binding to ADP-G-actin. B–D, NBD-actin fluorescence competition assay demonstrating that C-CAP1 binds ADP-G-actin with high affinity through its β-sheet domain. The binding of CAP1 for actin was examined by adding different concentrations of C-CAP1 to a mixture of 0.2 μM ADP-G-actin and 0.5 μM twinfilin. Twinfilin alone results in an increase in the NBD fluorescence of ADP-G-actin (42), whereas addition of CAP1 constructs resulted in quenching of the NBD fluorescence of the twinfilin/ADP-G-actin complex indicating that C-CAP1 and twinfilin compete with each other for binding to ADP-G-actin. The measured decrease of fluorescence was normalized; the binding curves (solid line) were fitted, and Kd values for the C-CAP1 ADP-G-actin complexes were calculated. Whereas wild-type C-CAP (B) and the C-CAP construct harboring a mutation in the WH2 domain (C) bind ADP-G-actin with high affinity under physiological ionic conditions, mutations in the β-sheet domain inhibit this interaction (D). n.d., not determined.

(see Fig. 2D), did not significantly affect the binding of C-CAP1 for ATP-G-actin (Fig. 3C), whereas a single point mutation in the WH2 domain (K271A, mutWH2) efficiently disrupted this interaction (Fig. 3B). These results suggest that CAP1 harbors separate binding sites for ADP-G-actin and ATP-G-actin located in C-terminal β-sheet domain and WH2 domain, respectively.

To confirm that the WH2 domain of CAP1 is indeed sufficient for ATP-G-actin binding, we expressed and purified the WH2 domain containing region of mouse CAP1 (residues 216–322) and measured its interaction with NBD-ATP-G-actin. This fragment, lacking the C-terminal ADP-G-actin binding β-sheet domain, resulted in an ~30% increase of NBD fluorescence. The data were fitted, assuming a 1:1 complex formation, and gave an equilibrium dissociation constant of 0.7 μM for the CAP1 WH2 domain-ATP-G-actin interaction (Fig. 3D). Moreover, the WH2 domain of mouse CAP1 did not affect the polymerization of pyrene-labeled G-actin from spectrin seeds (Fig. 3E), in contrast to the corresponding region of yeast Srv2/CAP that was reported to inhibit actin filament assembly (31) suggesting that binding of the CAP1 WH2 domain to ATP-G-actin allows actin polymerization. As a positive control, an actin monomer sequestering protein twinfilin (46) was included in the assay and showed to efficiently inhibit the polymerization of actin filaments from ATP-G-actin. Although the CAP1 WH2 domain did not affect actin polymerization, the domain inhibited nucleotide exchange (from e-ATP to ATP) of actin monomers in a concentration-dependent manner (Fig. 3F). Together, these data show that the WH2 domain of mouse CAP1 binds ATP-G-actin with high affinity and inhibits the nucleotide exchange on actin monomers, but it does not affect the incorporation of ATP-actin monomers into filament barbed ends.

ADP-G-actin-binding Site at the β-Sheet Domain of Mouse CAP1 Is Sufficient for Catalyzing Nucleotide Exchange on Actin Monomers—Human CAP1 and budding yeast Srv2/CAP accelerate the nucleotide exchange of ADP-actin to ATP-actin, and this activity has been assigned to the C-terminal halves of the proteins (26, 31). To elucidate the roles of the ADP-G-actin binding β-sheet domain and the ATP-G-actin binding WH2 domains of mouse CAP1 in nucleotide exchange, we examined the exchange of ADP to e-ATP on actin monomers in the presence of wild-type and mutant C-terminal halves of the protein (mutWH2 and mutβ) and WH2 domain alone. As expected, wild-type C-CAP1 accelerated nucleotide exchange on actin monomers (Fig. 4A). Interestingly, a mutation inactivating the WH2 domain (mutWH2) did not affect the nucleotide exchange activity of C-CAP1, whereas point mutations decreasing the affinity of the β-sheet domain for ADP-G-actin...
How CAPs Promote Nucleotide Exchange on Actin

FIGURE 3. ATP-G-actin binding of mouse CAP1 and its WH2 domain. A–C, change in the fluorescence of 0.2 μM ATP-G-actin was measured at different concentrations of wild-type (A), mutWH2 (B), and mutβ (C) mouse C-CAP1 proteins. Relative NBD fluorescence is shown on the y axis, and the concentration of C-CAP1 was divided by the concentration of actin on the x axis. Wild-type C-CAP1 and mutβ bound ATP-G-actin with high affinity, whereas the C-CAP1 construct harboring a mutation in the WH2 domain did not display detectable binding to ATP-G-actin under physiological ionic conditions. D, WH2 domain-containing fragment of mouse CAP1 binds ATP-G-actin with relatively high affinity as detected by the concentration-dependent change in the fluorescence of NBD-labeled actin. E, WH2-CAP1 construct does not affect the polymerization of pyrene-labeled actin from spectrin-F-actin seeds. 2.5 μM pyrene actin (10% pyrene actin, 90% unlabeled actin) in buffer (5 mM Tris, pH 7.5, 0.2 mM CaCl2, 0.2 mM ATP, 0.2 mM DTT) was polymerized by the addition of 0.1 mM KCl, 2 mM MgCl2, and 1 mM EGTA in the presence of spectrin-F-actin seeds and different concentrations (0–6 μM) of WH2-CAP1. The polymerization of filaments was followed by the increase in pyrene fluorescence, and the rates were normalized to the rate in the absence of WH2-CAP1. Actin monomer sequestering/barbed end capping protein twinfilin was used as a positive control in the assay. F, WH2-CAP1 construct decreases the rate of nucleotide exchange from e-ATP to ATP. The exchange of e-ATP for ATP was monitored by the decrease in the e-ATP fluorescence. 0.65 μM e-ATP-G-actin was mixed with 0, 0.25, 0.5, 1.0 or 1.5 μM WH2-CAP1, and the decrease in the fluorescence was followed after the addition of 1 mM ATP. The *K*~obs~ rates obtained from the data were normalized to the rate in the absence of WH2-CAP1 and plotted in the graph.}

(mutβ) diminished the nucleotide exchange activity of C-CAP1 (Fig. 4B). To re-examine the possible role of WH2 domain of budding yeast Srv2/CAP in nucleotide exchange (31), we generated a Srv2/CAP deletion construct, which lacks all critical actin-binding residues of the WH2 domain (Sc Srv2(328–526)). This protein also promoted nucleotide exchange from ADP-G-actin to ATP-G-actin (Fig. 4C). Thus, ATP-G-actin binding activity of the WH2 domain does not contribute to the nucleotide exchange in mouse CAP1 and budding yeast Srv2/CAP, whereas the ADP-G-actin binding β-sheet domain is required for this activity.

Malaria Parasite CAP Catalyzes the Nucleotide Exchange on Actin Monomers—In comparison with CAP proteins from yeast, plants, and animals, those found among Apicomplexa, a phylum of ancient protozoan pathogens, including the malaria parasite *P. falciparum*, is composed of only the C-terminal β-sheet domain (Fig. 1, A and B). Although *Cryptosporidium parvum* CAP has been shown to bind G-actin, the possible effects of CAP on actin dynamics, from either *C. parvum* or any other apicomplexan parasite, have not been reported (25). To explore the activity of an apicomplexan CAP in detail, a His-tagged *P. falciparum* CAP (PfCAP) was expressed in *E. coli* and purified by nickel beads and subsequent gel filtration. Surprisingly, the protein of the peak fractions from the gel filtration column did not migrate at the expected position on SDS-PAGE. Instead, a strong band was visible on the top of the gel, and only a minor portion of protein was visible in the expected position corresponding to a migration of an ~18-kDa protein (Fig. 5A, left panel). However, the same fractions migrated as a single band on nondenaturing PAGE (Fig. 5A, right panel), and the identity of the protein as PfCAP was confirmed by tandem mass spectrometry (MS/MS) ion search method using MALDI TOF/TOF analyzed as described previously (47). Analytical ultracentrifugation demonstrated that at a concentration of 0.2 mg/ml, the major peak was at 3.7 S, corresponding to a molecular mass of ~40 kDa (Fig. 5B). Thus, PfCAP appears to have a tendency to aggregate on SDS-PAGE, although it is predominantly dimeric in solution.

We could not detect clear binding of PfCAP to rabbit muscle G-actin by pulldown or NBD assays (data not shown). This is probably due to low sequence identity between rabbit and *P. falciparum* actins (~76%) compared with yeast, plant, and animal actins (>90%). However, PfCAP accelerated the nucleotide exchange on rabbit muscle G-actin by ~10-fold (Fig. 6, A and B). Although 0.1 μM mouse CAP1 was sufficient to efficiently catalyze the nucleotide exchange under these conditions (Fig. 4), higher concentrations of PfCAP were required to reach similar levels of nucleotide exchange (Fig. 6). We then exploited the nucleotide exchange assay to determine the affinity of PfCAP to rabbit muscle actin. By fitting the *t*~1⁄2~ values of the nucleotide exchange at different PfCAP concentrations as described under “Experimental Procedures,” we revealed that PfCAP binds rabbit muscle ADP-G-actin with an affinity of ~0.37 μM (Fig. 6B). In conclusion, PfCAP binds rabbit muscle actin with lower affinity compared with mouse and yeast CAPs, but when bound
to an actin monomer, it can efficiently catalyze nucleotide exchange to recharge ADP-actin monomers with ATP.

Mouse CAP1 Has Two Independent Profilin-binding Sites—

Human CAP1 and yeast Srv2/CAP bind the G-actin/cofilin complex through the N-terminal α-helical domain, with yeast Srv2/CAP also interacting with profilin through its polyproline region (26, 34, 35). Specific surface-exposed residues (Phe-198, Trp-199, Arg-202, Leu-204, Lys-205, and Glu-206) at the α-helical domain of yeast Srv2/CAP were identified to be critical for cofilin/G-actin binding as well as for cofilin-mediated filament severing (35, 29), and thus we tested whether this site is conserved in mammalian CAPs. A supernatant depletion pulldown assay provided evidence that the N-terminal domain of mouse CAP1 (residues 1–221; N-CAP) interacts with cofilin-1/G-actin complexes (Fig. 7). However, it is important to note that this assay does not provide direct information about the affinity of the interaction.

FIGURE 4. β-Sheet domain of mouse CAP1 catalyzes the nucleotide exchange from ADP to ATP on actin monomers. A, representative curves obtained when ε-ATP was added to ADP-G-actin solution in the presence (open circles) and absence (closed circles) of C-CAP1. Relative fluorescence of ε-ATP is shown on y axis and time in seconds is on x axis. The concentrations of C-CAP1 and actin were 0.1 and 0.5 μM, respectively. B, relative rates of the nucleotide exchange. The y axis shows the inverse of the half-time of the reaction. Although a mutation in the WH2 domain did not inhibit the nucleotide exchange activity of C-CAP1, point mutations in the β-sheet domain decreasing its affinity for ADP-G-actin inhibited the nucleotide exchange activity of C-CAP1. The concentrations of CAP1 constructs and actin were 0.1 and 0.5 μM, respectively. Error bars represent S.E. of at least three independent experiments. C, relative rates of the nucleotide exchange of yeast Srv2/CAP construct lacking the functional WH2 domain (ScSrv2(328–526)). The y axis shows the inverse of the half-time of the reaction. Concentrations of 0.1 and 0.5 μM of ScSrv2(328–526) was used, and the concentration of actin was 0.5 μM. Error bars represent S.E. of at least three independent experiments.

FIGURE 5. Purification of PfCAP. A, fractions collected from the gel filtration column run on denaturing SDS-PAGE (left panel) and nondenaturing PAGE (right panel). Although the protein does not migrate efficiently on SDS-PAGE, it migrates as a single band on native PAGE. B, differential sedimentation coefficient distribution (c(s) versus s) of PfCAP from sedimentation velocity experiments with 0.2 mg/ml PfCAP. A major peak of 3.7 S corresponds to the molecular mass of ~40 kDa, thus demonstrating PfCAP to be predominantly a dimer in solution.

FIGURE 6. PfCAP binds ADP-G-actin and catalyzes the nucleotide exchange from ADP to ATP on actin monomers. A, representative curves obtained when ε-ATP was added to ADP-G-actin solution in the presence (open circles) or absence (closed circles) of PfCAP. Relative fluorescence of ε-ATP is shown on the y axis and time in seconds is on the x axis. The concentrations of PfCAP and actin were 2.0 and 0.5 μM, respectively. Error bars represent S.E. of at least three independent experiments. B, KD value for the PfCAP-actin complex was determined by plotting the relative rates of nucleotide exchange with different concentrations of PfCAP. The inverse of the half-time of each reaction was calculated, and the values were normalized, and the binding curve (solid line) was fitted to obtain the KD value for the PfCAP-actin complex. For clarity, the y axis shows the absolute inverse of half-time values instead of normalized values.
binding to G-actin in the absence of cofilin suggesting that N-CAP1 interacts only with actin when it is in complex with cofilin. Mutations in the four conserved amino acids in N-CAP1 (mutα, Fig. 1A), which correspond to the residues that are crucial for cofilin/G-actin binding in budding yeast (35), efficiently diminished the cofilin-G-actin interactions of mouse CAP, suggesting that yeast and mammalian CAPs interact with cofilin/G-actin complexes through a conserved interface.

Most CAPs harbor two proline-rich regions. The first polyproline region (PP1) of yeast Srv2/CAP was shown to bind profilin, but whether other CAPs can bind profilin has not been reported (34). We expressed and purified a mouse CAP1 fragment containing the two proline-rich regions (residues 216–322) and examined its interaction with purified mouse profilin-1 by measuring tryptophan fluorescence of profilin. Fluorometric assays revealed that this CAP1 fragment indeed binds profilin with similar affinity (Kd ~1 μM, see Fig. 8A) to the one previously reported for the yeast Srv2-CAP profilin interaction. Interestingly, the PP1 region in mammalian CAPs is slightly longer than in yeast Srv2/CAP (GPPPPPGPPIPPIP versus PAPPAPPPAPPP), Whereas replacement of single proline residue in yeast Srv2/CAP by alanine completely abolished profilin binding (34), a triple replacement of the second, third, and fourth proline following either the first or second glycine residue in this region did not reduce the affinity of CAP1 for profilin (Fig. 8, B and C). Importantly, combining these two three-residue substitutions abolished profilin binding by mouse CAP1 almost completely (Fig. 8D). Together, these data show that mammalian CAPs can also bind profilin. However, in contrast to yeast Srv2/CAP, which harbors only one profilin-binding site, mouse CAP1 contains two adjacent profilin-binding sites.

### DISCUSSION

CAPs are important regulators of actin dynamics in all eukaryotes studied so far. Here, we provide evidence that the mechanisms by which CAP interacts with cofilin/ADP-G-actin complex through its N-terminal α-helical domain and with ADP-G-actin through its C-terminal β-sheet domain are conserved in evolution from yeast to mammals. However, the polyproline region of mouse CAP1 harbors two profilin-binding sites compared with one in yeast Srv2/CAP and the WH2 domain of mouse CAP1 binds ATP-G-actin with ~4-fold higher affinity than the yeast WH2 domain and allows actin filament assembly. Most importantly, our biochemical and mutagenesis analyses demonstrate that mouse CAP1 harbors distinct binding sites for ADP- and ATP-G-actin, which can be individually inactivated by point mutations. The high affinity ATP-G-actin site located mainly at the C-terminal β-sheet domain of mouse CAP1 is sufficient in promoting the nucleotide exchange on actin monomers. We also show that this activity is conserved in the mini-CAPs of apicomplexan parasites. A previous study provided evidence that at least in budding yeast Srv2/CAP, both the β-sheet and WH2 domains are required for promoting nucleotide exchange on actin monomers. It was therefore proposed that through separate interactions with actin, these two domains would work together to promote nucleotide exchange (31). However, our studies on mouse CAP1, yeast Srv2/CAP, and malaria parasite CAP show that, in these distant members of the family, the ATP-G-actin binding activity residing in the WH2 domain is dispensable for catalyzing nucleotide exchange on actin monomers and that only the ADP-G-actin binding function, which mostly resides...
in the β-sheet domain, is required for this activity. The differences between this study and the work by Chaudhry et al. (31) concerning the role of WH2 domain of yeast Srv2/CAP in nucleotide exchange most likely result from different experimental setups. The nucleotide exchange activity of yeast Srv2/CAP in Ref. 31 was examined by using the full-length protein, whereas our experiments were carried out using the isolated C-terminal half of the protein without possible interference of the N-terminal profilin/ADP-G-actin-binding site to the reaction. Furthermore, the study by Chaudhry et al. (31) measured nucleotide exchange from profilin/G-actin complexes, whereas we studied nucleotide exchange on actin without the presence of cofillin. Finally, instead of mutating charged amino acids in the WH2 domain, Chaudhry et al. (31) mutated hydrophobic residues, and thus these mutations may have either directly or indirectly affected the ADP-G-actin binding activity of Srv2/CAP. Thus, the ATP-G-actin binding WH2 domain is not needed for CAPs to promote nucleotide exchange from ADP-G-actin to ATP-G-actin per se, but at least in yeast Srv2/CAP this domain contributes to actin filament treadmilling and dissociation of cofillin-actin complexes in the context of full-length protein through a currently unknown mechanism.

Crystallographic and fluorometric studies have demonstrated that proteins inhibiting the nucleotide exchange on the actin monomer keep the cleft between actin subdomains 2 and 4 in a “closed” conformation, although those promoting the nucleotide exchange maintain this site in an “open” state, thus allowing nucleotide exchange on the bound actin monomer (48, 49). We thus propose that the β-sheet domain of CAP induces a conformational change in the actin molecule upon binding and that this would enhance the exchange of the nucleotide, which is located in the cleft between subdomains 2 and 4 of actin.

Apicomplexan CAPs lack all other protein-protein interaction domains except the β-sheet domain, which promotes nucleotide exchange on G-actin as shown here. Thus, catalyzing the nucleotide exchange appears to be the most highly conserved function of CAPs in all species. We therefore propose that CAP is the most central protein in re-charging ADP-actin monomers with ATP across the eukaryotic domain. Although profilin has been shown to catalyze nucleotide exchange on actin monomers, not all profilins display this activity. For example, budding yeast profilin displays only weak nucleotide exchange activity, and plant profilins examined so far do not display detectable nucleotide exchange activity (50–52). Thus, at least in these organisms CAP may be the main protein responsible for re-charging actin monomers. Furthermore, CAPs appear to be better suited for catalyzing the nucleotide exchange on newly depolymerized ADP-actin monomers compared with profilins. This is because yeast and mammalian CAPs display higher affinity (K₅₀ = 0.02–0.05 μM) for ADP-G-actin (this study and Ref. 15) compared with most profilins (K₅₀ ~0.5 μM) (53) and can thus more efficiently compete with ADF/cofilin for binding to ADP-G-actin.

Collectively, our data propose that apicomplexan CAPs are relatively simple actin-binding proteins, whose main function is to re-charge newly depolymerized ADP-actin monomers with ATP to facilitate their assembly into filament barbed ends (Fig. 9A). This nucleotide exchange function is conserved in mammalian CAPs, but these proteins are more complex and have acquired (or retained) other functions (Fig. 9B). The N-terminal α-helical domain of mouse CAP interacts with the cofillin/ADP-G-actin complex through a site that is conserved between yeast and mammalian CAPs. As proposed earlier (26, 35), this interaction may dissociate the cofillin-actin complex to facilitate the recycling of cofillin for new rounds of filament disassembly. Recent studies demonstrated that CAPs also enhance cofillin-mediated filament severing and that at least in yeast Srv2/CAP, this activity resides at the N-terminal α-helical domain (28, 29). The newly released ADP-actin monomer is delivered to the C-terminal β-sheet domain, which catalyzes the nucleotide exchange on the actin monomer similarly to the apicomplexan mini-CAPs. The “re-charged” ATP-actin monomer can subsequently be shuttled either to the WH2 domain or to profilin molecules that bind adjacent to the WH2 domain. Because the WH2 domain of mouse CAP1 allows actin filament assembly (Fig. 3E), the actin monomer can be directly delivered to the filament barbed end, assuming that the CAP1 protein is located in the vicinity of filament ends. However, the profilin-actin monomer complex can dissociate from CAP1 and be used for formin- or Ena/VASP-catalyzed filament polymerization (2, 54). We propose that whether the actin monomer is delivered
to the WH2 domain or profilin, CAP is precisely regulated by additional protein-protein interactions and/or post-translational modifications. Thus, future studies are needed to reveal how the actin monomer recycling by CAP is regulated and to elucidate the functions of the profilin and WH2 domain-actin interactions of CAP during various actin-dependent processes in animal cells. Furthermore, to understand the exact mechanisms by which the β-sheet domain of CAP catalyzes nucleotide exchange on actin, the structure of this domain in complex with actin monomers needs to be determined. Finally, structural information of the full-length CAP will be valuable for understanding how the distinct functions of the N-terminal α-helical domain, C-terminal β-sheet domain, WH2 domain, and profilin-binding sites are coordinated and to elucidate how the actin monomer shuttles between these sites.

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