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Structural characterization of a capping protein interaction motif defines a family of actin filament regulators

Maria Hernandez-Valladares^{1,3}, Taekyung Kim^{2,3}, Balakrishnan Kannan¹, Alvin Tung¹, Adeleke H Aguda¹, Mårten Larsson¹, John A Cooper², and Robert C Robinson¹

¹Institute of Molecular and Cell Biology, A*STAR, Proteos, Singapore.

²Department of Cell Biology and Physiology, Washington University Medical School, St. Louis, Missouri, USA.

Abstract

Capping protein (CP) regulates actin dynamics by binding the barbed ends of actin filaments. Removal of CP may be one means to harness actin polymerization for processes such as cell movement and endocytosis. Here we structurally and biochemically investigated a CP interaction (CPI) motif present in the otherwise unrelated proteins CARMIL and CD2AP. The CPI motif wraps around the stalk of the mushroom-shaped CP at a site distant from the actin-binding interface, which lies on the top of the mushroom cap. We propose that the CPI motif may act as an allosteric modulator, restricting CP to a low-affinity, filament-binding conformation. Structure-based sequence alignments extend the CPI motif-containing family to include CIN85, CKIP-1, CapZIP and a relatively uncharacterized protein, WASHCAP (FAM21). Peptides comprising these CPI motifs are able to inhibit CP and to uncap CP-bound actin filaments.

Actin polymerization provides force and organization to drive and shape many cellular processes. Protrusion of membranes in animal cells, for example, is proposed to be driven by the addition of actin subunits to free barbed ends of actin filaments. The barbed end is kinetically and thermodynamically favored over the pointed end for polymerization. Thus, the innate ability of actin polymerization to create force requires that, in general, actin-filament barbed ends should be capped to prevent unwanted polymerization, which would lead to the exhaustion of the actin monomer pool. Under these capped-filament conditions, polymerization can be initiated through three mechanisms: *de novo* nucleation, filament severing or filament uncapping. Nucleation and severing have been studied in depth, but the molecular details of uncapping mechanisms remain largely unexplored.

The universal actin-capping protein in eukaryotic cells is capping protein (CP), a heterodimer of structurally related α - and β -subunits. CP is found in a range of cellular actin-containing structures and has an affinity of 0.1–1 nM for filaments^{1,2}. CP is present in

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Correspondence should be addressed to R.C.R. (rrobinson@imcb.a-star.edu.sg).

³These authors contributed equally to this work.

AUTHOR CONTRIBUTIONS

M.H.-V., T.K., B.K. and A.T. carried out the work; A.H.A. created reagents used in the work; M.H.-V., T.K., M.L., J.A.C. and R.C.R. designed the work and interpreted the data; R.C.R. wrote the paper.

Accession codes. Protein Data Bank: The coordinates and merged reflection data for CARMIL and CD2AP CPIs bound to CP $\Delta\beta$ Tent and the CBR115–CP complex have been deposited with accession codes 3LK2, 3LK4 and 3LK3, respectively.

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relatively stable structures such as the sarcomere of striated muscle, where CP defines and anchors the barbed end of thin filaments at the Z-disc, leading to the alias CapZ. Similarly, CP appears to bind to the end of the actin-related protein 1 (Arp1) minifilament in the mammalian dynactin complex (reviewed in ref. 3). CP is also involved in highly dynamic actin structures. Lamellipodia of moving cells contain a mixture of uncapped and CP-capped actin filaments close to the leading edge⁴. The uncapping of these filaments and their subsequent elongation may provide force during lamellipodia protrusion.

The structural basis of actin-filament capping by CP has been established at low resolution. First, the X-ray structure of CP revealed a mushroom-shaped molecule comprising two symmetrically and structurally related subunits⁵. The stalk of the mushroom is formed from the α -helical N termini from both subunits. The cap of the mushroom consists of a common intersubunit β -sheet, across which the C-terminal regions form antiparallel helices terminating in unrelated structures termed ‘tentacles’. Both α - and β -tentacles are well ordered in the structure; however, the β -tentacle is bound to a neighboring symmetry-related molecule and is likely to be mobile in solution. Second, the CP crystal structure was oriented into 23-Å resolution cryo-electron microscopy data obtained from capped filaments⁶. This resulted in a capping model in which the α -tentacle and β -subunit of the mushroom cap provide the majority of the interaction surface with the base of the filament, physically impeding the addition of actin monomers. The β -tentacle is speculated to reorient to provide a second actin contact⁶.

Protein and lipid inhibitors of CP actin-filament capping are manifest. The protein CP Arp2/3 myosin I linker (CARMIL) is a multifunctional actin regulator consisting of an N-terminal domain that includes leucine-rich repeats and a 51-residue region near the C terminus, which comprises a CP-binding and uncapping motif that includes the CARMIL homology domain 3 (CAH3, residues 965–1038)^{7–10}. CARMIL has also been proposed to contain a WH2 domain, an acidic domain and a polyproline region. CARMIL-like CP binding motifs exist in a range of proteins that have diverse modular architectures (**Fig. 1a**). CK2-interacting protein 1 (CKIP-1) consists of an N-terminal pleckstrin homology domain followed by the CP binding motif. CKIP-1 is able to inhibit CP from interacting with an actin filament^{11,12}. A 22-residue peptide corresponding to a fragment of the CARMIL CAH3 (residues 984–1005) competes with CKIP-1 in binding CP and has a direct effect on the activity of CP. CD2-associated protein (CD2AP) and its homolog CIN85 both comprise three SH3 domains, the CP binding motif and a C-terminal coiled-coil domain^{13–15}. The CD2AP CP binding motif, consisting of residues 474–513, is sufficient to bind CP with $K_d = 6$ nM and has some uncapping activity. The CP binding motif is defined as LXHXTXXRPK(6X)P (ref. 13).

Actin-filament capping by CP is also inhibited by the proteins CapZ-interacting protein (CapZIP) and V-1 (myotrophin). CapZIP is a 416-residue protein of unknown structure that is subject to stress-induced phosphorylation. The phosphorylation leads to the dissociation of the CapZIP–CP complex, suggesting a possible role in regulating actin remodeling¹⁶. V-1, a protein involved in the differentiation of muscle, is an ankyrin repeat protein that binds to CP, inhibiting the interaction with actin. V-1 has been proposed to interact through two loops, which protrude from the ankyrin-repeat back bone, with a CP binding site that includes the β -tentacle¹⁷. The CP-interacting motif on CapZIP is currently unknown. The phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) has also been reported to inhibit CP^{18,19}. The PIP₂ binding site overlaps with the actin binding site and includes Lys256 and Arg260 from the α -subunit and Arg225 from the β -subunit. Conflicting data exist as to whether PIP₂ can remove CP from an actin filament^{18,19}.

Here we have investigated the poorly understood mechanism of CP-filament uncapping by the CP binding motifs from CARMIL and CD2AP. Sequence-alignment and truncation experiments led to the confirmation of a short CPI motif that retained some uncapping activity. We report the crystal structures of CARMIL and CD2AP CPI motifs (*Homo sapiens*) bound to chicken CP (*Gallus gallus*) and a longer fragment of CARMIL that also includes a second unique CP-interacting motif. Structural features and sequence comparisons allow expansion of the CPI motif-containing family from CARMIL, CD2AP, CIN85 and CKIP-1 to also include CapZIP and the newly characterized protein FAM21 (here termed WASHCAP, WASH- and capping protein-associated protein). *In vitro* assays confirm that peptides comprising each CPI motif possess the predicted CP inhibition and uncapping activities. The structures suggest consideration of an allosteric mechanism of filament uncapping by the CPI motif as a potential alternative to direct competition for actin-binding residues on CP.

RESULTS

Functional definition of the CARMIL CPI motif

To verify a minimal binding and uncapping fragment of CARMIL, we constructed a series of CARMIL truncations and tested them in pyreneactin polymerization assays. In this assay, we inferred an increase in bulk-solution polymeric actin from an increase in pyrene fluorescence²⁰. First, in capping inhibition assays, we simultaneously added CARMIL truncations (**Fig. 1**) and CP to growing actin filaments that were nucleated from spectrin-actin seeds, which have free barbed ends and capped pointed ends. CP binding region 71 (CBR71) was slightly less effective than CBR115 in preventing CP from capping the barbed end of the filaments (**Fig. 2a** and **Supplementary Fig. 1a**). CBR49 showed a markedly reduced, yet measurable, anti-capping activity. We observed a similarly reduced level of activity for CBR37, a peptide derived from sequence-alignment analysis and later shown to comprise the CP binding region determined by crystallography (see below) flanked by two extra residues at each terminus.

Second, in uncapping assays, we added CARMIL truncations to actin filaments that were capped at both ends, at the pointed end by spectrin seeds and at the barbed end by CP, in the presence of excess free actin subunits. The CARMIL truncations all showed concentration-dependent increases in pyrene fluorescence, in line with CP-uncapping activity (**Fig. 2b** and **Supplementary Fig. 1b**). The active concentrations for the CARMIL truncations in this uncapping assay were similar to those in the CP-binding assay (**Fig. 2a** and **Supplementary Fig. 1a**), suggesting that the properties of sequestration and uncapping are manifested in the same interaction between CARMIL and CP. We further substantiated the validity of the assay by showing that CBR37 is able to uncap CP-bound actin filaments in the absence of spectrin seeds. In this experiment, we included profilin in order to prevent the excessive formation of CP-stabilized actin nuclei (**Supplementary Fig. 1c**). We also removed a CP truncation that lacked the β -tentacle (CP $\Delta\beta$ Tent) and had reduced capping activity from the filaments by CBR37 (**Supplementary Fig. 1d**). Hence, the minimal peptide CBR37 shows both CP sequestration and uncapping activities and is referred to henceforth as the CP interaction (CPI) motif. The CAH3 domain from CARMIL includes peptide sequences that flank the CPI motif, leading to stronger uncapping activity¹⁰. However, an even larger region leads to the most potent uncapping observed in this study (CBR115). There is no clear homology between CARMIL, CD2AP and CKIP-1 outside the CPI motif; hence, the CPI motif is a more general CP interaction region that exists within CAH3 domain, which includes a more extensive CP binding region.

To assess the affinity of the CPI motif in binding CP, we labeled the CBR37 peptide (which contains a C-terminal cysteine residue) with the fluorescent probe Alexa Fluor 488 C₅

maleimide. We titrated Alexa488-CBR37 with CP and observed a concentration-dependent increase in fluorescence anisotropy consistent with slowed molecular tumbling upon association with CP (**Fig. 2c**). Curve-fitting analysis suggests a K_d of 0.16 μM for this interaction, with 95% confidence interval for the range 85–244 nM. CP $\Delta\beta$ Tent and CP showed indistinguishable association kinetics with respect to Alexa488-CBR37, as did wild-type CP in the presence of G-actin (1:1 ratio) in low-salt buffer (**Supplementary Fig. 2a,b**). Hence, the CPI motif interacts with CP at a site that does not include the β -tentacle. This differentiates CP binding of the CPI motif from the CP interaction proposed for V-1 (ref. 17). The Alexa488-CBR37–CP complex could be dissociated by competition with unlabeled CBR37 ($K_d = 0.10 \mu\text{M}$, 95% confidence interval for 10–186 nM) or by F-actin but not by G-actin as monitored by a decrease in anisotropy (**Fig. 2d,e**).

We observed the effect of CP and the CPI motif on single actin filaments using total internal reflection fluorescence (TIRF) microscopy. We polymerized BODIPY tetramethylrhodamine C_5 maleimide–labeled actin (red actin) in the presence of excess profilin and then capped it with CP. We simultaneously added CBR115, CBR37 or the CD2AP CPI motif with BODIPY FL C_1 iodoacetamide–labeled G-actin (green actin) under polymerizing conditions while maintaining constant profilin and CP concentrations. Maintenance of profilin levels prevented the formation of CP-stabilized actin nuclei, and preserving a constant CP concentration was necessary to ensure competent filament capping during solution exchange due to its fast off rate. In these experiments, we observed red filaments acting as nuclei for green filaments in the presence of CBR115, CBR37 or CD2AP CPI motif or in the absence of CP (**Fig. 3** and **Supplementary Videos 1–4**). However, green actin alone could not elongate from the red-actin CP-capped filaments in the absence of CARMIL truncations (**Fig. 3e** and **Supplementary Video 5**). Taken together, these data show that the CPI motif from CARMIL binds CP ($K_d \sim 0.1\text{--}0.2 \mu\text{M}$) and partially retains the uncapping and sequestration activities of CBR115 ($K_d \sim 1.5 \text{ nM}$)⁸. Single-molecule TIRF microscopy experiments suggest that CPI motifs will form ternary complexes with CP at the barbed end of filaments¹⁰. This behavior is consistent with the efficiency of uncapping by CBR37 (**Fig. 2b**) relative to the CBR37–CP dissociation constant ($K_d \sim 0.1\text{--}0.2 \mu\text{M}$). Hence, CPI motifs may have a role in protein localization, in targeting either CP or the CPI motif-containing protein to specific cellular regions, as well as modulating the affinity of CP for actin filaments.

Structures of the CARMIL and CD2AP CPI complexes with CP

To understand the nature of the uncapping interaction, we determined the crystal structure of the CP $\Delta\beta$ Tent–CARMIL CPI motif complex (**Fig. 4a–d**). The CPI motif wraps around the stalk at the underside of the cap of the mushroom-shaped CP. The CPI motif is in an extended conformation, which half encircles the α -helices of the CP stalk. The N-terminal one-third of the CPI motif contacts the α -subunit of CP and the C-terminal two-thirds contact the β -subunit. The binding position for the CPI motif is distant to the actin binding interface (viewer's perspective in **Fig. 4**) and to the PIP₂ binding interface¹⁸. The structure of CP $\Delta\beta$ Tent in this complex is essentially identical, with the exception of the lack of the β -tentacle, to that when CP was crystallized alone⁵. A second structure of the CD2AP CPI motif bound to CP $\Delta\beta$ Tent showed the common interaction of the CPI motifs with CP (**Fig. 5a**). The C-terminal portion, which includes the LXHXTXXRPK(6X)P motif, shares an interaction mode with CARMIL in binding the CP β -subunit¹³; however, the N-terminal region takes a divergent path across the CP α -subunit.

Unique interactions of CARMIL with CP

In order to understand the interactions that distinguish CBR115 from CBR37, the CBR115–CP structure was elucidated (**Fig. 5b**). This structure reveals two ordered CARMIL regions

that lie within the CAH3_{965–1038} (ref. 10), which roughly correspond to the CBR71_{964–1034} region studied here that has similar activity to CBR115 (**Fig. 2a,b** and **Supplementary Fig. 1a,b**). The first ordered CARMIL region is the CPI motif, Ile971–Cys1004, which is essentially identical to that observed in the CPΔβTent–CARMIL CPI motif complex (**Fig. 5a**). The second ordered CARMIL region, Arg1021–Thr1035, binds to the under-side of the CP mushroom cap on the opposite side of the CP mushroom stalk to which the CPI motif binds. This region appears not to be present in other CPI-motif proteins from sequence analyses and is termed here the CARMIL-specific interaction (CSI) motif. Together, these two regions resemble a finger (CPI) and thumb (CSI) encircling the stalk on the underside of the mushroom cap. The CSI motif has two prominent phenylalanine residues (Phe1029 and Phe1030) that are buried at the mushroom cap–stalk junction. We observed no interpretable electron density for the β-tentacle.

Sequence contributions to the interactions

The extended nature of the CARMIL CPI and CSI motifs leads to multiple interactions with CP (**Figs. 4a** and **5** and **Supplementary Fig. 3**). In these conformations, the CPI and CSI motifs have no domain structure. Hence, sequence conservation across species in the CPI (**Fig. 4e**) and CSI motifs generally correlate with critical CP-interacting residues. To determine the contribution of charged residues within to the uncapping process, alanine mutations of structurally important interacting residues within the CPI–CP complex were shown to have complementary lowering of activity in the pyrene-actin polymerization assay (**Supplementary Fig. 4a–d**). The widespread distribution of the interacting residues throughout the CPI motif suggests that these residues play a role in the association between the CPI motif and CP rather than providing individual elements to the uncapping process.

The CPI motif family of proteins

The structural and functional definition of the CPI motif prompted reinvestigation of sequence databases to determine the extent of the CPI motif–containing family. The known family members (CARMIL, CD2AP, CIN85 and CKIP-1) show homology within the CPI motif to CapZIP and a recently characterized protein, FAM21 (NCBI NP_001005751) (**Figs. 1b** and **6a**). We observed no obvious homo-logy between the CP binding regions of CARMIL and either V1 or the actin monomer– and CP-binding protein twinfilin. We subjected peptides comprising the CPI motifs from each protein to the pyrene-actin uncapping and inhibition assays. All six peptides showed uncapping and inhibitory activities, functionally verifying the extended CPI-motif family (**Fig. 6b** and **Supplementary Fig. 4e**). Sequence alignment reveals that FAM21 and CapZIP have highest residue identity with CD2AP (**Fig. 6a**), whereas CKIP-1 is more similar to CARMIL in the N-terminal one-third of the CPI motif. Notably, full-length CapZIP showed a similar level of uncapping activity toward CP as measured for the isolated CapZIP CPI motif (**Supplementary Fig. 1e** and **Fig. 6b**). This starkly contrasts to the CPI motif from CARMIL, which shows a markedly reduced uncapping activity in comparison to that of CBR115, which has a second CP binding region in the CSI motif (**Figs. 2b** and **5b**). Considering this variation in uncapping activities, the balance between CP capping and uncapping as the predominant mode of action may differ among the CPI-motif proteins.

Direct competition model of CP uncapping

To determine the mechanism by which the CPI motif removes CP from an actin filament, we constructed a series of models. The cryo–electron microscopy structure of the CP-capped actin filament (**Fig. 7a**) provided the template for superimposing the CPI motif onto the capped filament⁶ (**Fig. 7a,b**). There is no direct steric clash between the CPI motif and the terminal actin protomer in the model (**Fig. 7a,b**). However, at the resolution of the electron microscopy structure (23 Å), errors in the CP placement or nonmodeled conformational

changes in either CP or the terminal actin require that this mechanism be considered. The side chains of Lys980, Glu983 and Phe985 from the CARMIL CPI motif approach the side chains of Ile287 (7.5 Å), Arg290 (9 Å) and Met283 (8 Å) from actin, respectively. That these residues are not well conserved either within the CARMIL family (**Fig. 4e**) or, more generally, in the CPI motif (**Fig. 6a**) argues that direct competition for close or overlapping binding sites may not be the mechanism of uncapping, particularly given the high affinity of CP for F-actin ($K_d = 0.1\text{--}1\text{ nM}$). The longer, more potent CP binding fragment of CARMIL CAH3 (ref. 10) includes the CSI motif that is also distant from the actin-binding surface (**Fig. 7b**). The disordered residues between the CPI and CSI motifs may be sufficient in length to reach toward the actin binding site on the topside of the CP mushroom cap. However, this region shows lower sequence identity across species relative to the CPI and CSI motifs. In particular, chicken CARMIL contains a two-residue deletion, CBR49, which includes eight residues from the disordered region, shows little change in uncapping activity with respect to CBR37 (**Fig. 2** and **Supplementary Fig. 1b**). Thus, the disordered region may not be involved in direct competition.

β -tentacle sequestration model of CP uncapping

The current model of CP binding to an actin filament involves the movement of the β -tentacle to contact the terminal actin protomer between subdomains 1 and 3 (ref. 6) (cyan versus orange in **Fig. 7a**). The CARMIL CPI motif binds the α -subunit of CP in close proximity to the β -tentacle (compare **Fig. 7a**, cyan, to **Fig. 7b**, yellow). Hence, a second possible mechanism for CPI motif-induced uncapping may be through the creation of a β -tentacle binding site at the interface of the α -subunit of CP and the CARMIL CPI motif to compete with the actin- β -tentacle interaction (**Fig. 7c**). To test this possibility, we assayed either the β -tentacle alone or the β -tentacle as a glutathione-S-transferase (GST) fusion protein by gel filtration, pulldown and fluorescence anisotropy studies for interaction with CP $\Delta\beta$ Tent in the presence of CBR37 or CBR115. We observed no interaction between the β -tentacle and the CP $\Delta\beta$ Tent complexes for either the β 1-subunit and β 2-subunit isoforms of CP, which differ by four residues in the tentacle region (**Supplementary Figs. 5** and **6**). Moreover, CBR37 showed uncapping activity for CP $\Delta\beta$ Tent (**Supplementary Fig. 1d**), and β -tentacle was not ordered in the CBR115-CP complex structure, effectively ruling out competition for the β -tentacle as a sole mechanism for uncapping.

Allosteric model of CP uncapping

A final possibility for an uncapping mechanism is that the CPI motif locks CP in a less favorable conformation for interaction with a filament. This mechanism requires that CP undergo a conformational change in binding to the actin filament (**Fig. 7d**). In support of this mechanism, the two molecules in the asymmetric unit of the original CP structure⁵ shows variation in the curvature of the common β -sheets. When the alternate conformation was included in the capped filament model (**Fig. 7e**), we observed discernable differences at the actin interface. Hence, we propose that the CPI motif may function as a clasp around the stem of mushroom-shaped CP, restricting the conformation of the filament-binding cap and preventing flexing of the common β -sheet. Thus, upon binding CP at the barbed end of a capped filament, the CPI motif may restrict the CP conformation, stabilizing it in the structure determined here, which is less compatible with filament interaction, leading to the allosteric uncapping of the filament. The uncapping activity of CBR37 is approximately three orders of magnitude lower than that of CBR115 (**Fig. 2b**). The superior binding of CBR115 (1.5 nM (ref. 8)) to CP through the CSI motif relative to that of CBR37 (0.1–0.2 μM) accounts, at least in part, for the increased uncapping activity. This suggests that the allosteric mechanism should also be considered for CAH3 uncapping activity as a possible alternative to direct competition. High-resolution structures of the actin-CP complex are

required to categorically distinguish between the direct competition and allosteric mechanisms of CPI motif-induced uncapping.

DISCUSSION

The CPI motif family of proteins

Here we have structurally and functionally characterized the CPI motifs from CARMIL and CD2AP, and we have shown that CIN85 and CKIP-1 contain bona fide uncapping motifs in addition to those reported for CARMIL and CD2AP. The CPI motif family has been extended to include the known CP binding partner CapZIP and the largely uncharacterized protein FAM21 (**Fig. 1**). The architectures of this family of proteins place the CPI motif in a wide range of multiple-domain settings. Members of this family of proteins are known to integrate signaling pathways to control actin dynamics through interactions with CP and other actin regulators. CKIP-1 links the protein kinase CK2, which is involved in regulating cell polarity, morphology and differentiation, to actin remodeling^{11,12}. The homologous proteins CD2AP and CIN85 are known to be associated with endocytic components, CP and cortactin²¹. CIN85 also binds WIP, WIRE, N-WASP and CARMIL, whereas CD2AP is indirectly linked to N-WASP through PSTPIP1 (refs. 21–23). Thus, CD2AP and CIN85 tie endocytosis and cadherin-based junctions to actin remodeling and tissue patterning²⁴. Duboraya, a zebrafish homolog of CapZIP, has been shown to be needed for actin organization and cilia formation in Kupffer's vesicle for proper left-right patterning^{16,25}. CARMIL integrates actin-filament uncapping, monomer binding, Arp2/3-mediated filament nucleation and myosin binding within its own architecture for use at the leading edge and in pinocytosis⁷. These data suggest that the CPI motif is likely to be a general actin-filament regulator that is incorporated into diverse proteins in order to harness actin polymerization for a wide variety of cellular processes. The precise role of any CPI motif will be dependent on the larger protein architecture. Indeed, flanking sequences around the CARMIL CPI motif that include the CSI motif, which does not appear to be conserved in the other CPI motif members, greatly increased the uncapping potency (**Figs. 2a** and **5b**), whereas CapZIP appeared to solely rely on the CPI motif (**Fig. 6b** and **Supplementary Fig. 1e**). This situation is analogous to that of the unstructured T β 4/WH2 motif, which contributes to G-actin sequestering and actin-filament nucleating machineries in different protein settings^{26–28}.

FAM21, a protein with four isoforms, has recently been characterized at the cellular level. In two reports, FAM21 (ref. 29) (also referred to as KIAA0592 (ref. 30)) has been found to be a component of the WASH (Wiskott-Aldrich syndrome protein and SCAR homolog) complex that activates Arp2/3 nucleation of actin filaments. FAM21-WASH was shown to locate to endosomes, where it controls fission and retrograde trafficking through Arp2/3-controlled actin polymerization and microtubule interactions^{29,30}. The larger WASH complex contains WASH, FAM21, CP, tubulins, coiled-coil domain containing 53 proteins, strumpellin (KIAA0196 (ref. 31)), uncharacterized protein KIAA1033 and hsp70s. Here we have shown a direct interaction between CP and FAM21, which is likely to be reproduced in the WASH complex. The WASH complex showed reduced filament capping with respect to CP, which is in line with the properties of the FAM21 CPI motif. Hence, we propose that FAM21 be renamed WASHCAP (WASH- and capping protein-associated protein) to reflect its known interacting partners (**Fig. 1b**). Components of the WASH complex have also been implicated in pathogen entry into cells. WASHCAP was identified as the virus penetrating factor that is critical for fluid-phase vaccinia virus cellular uptake through endocytosis³². Similarly, WASH has been implicated in *Salmonella typhimurium* invasion³³.

WASHCAP has been observed to be highly phosphorylated by MS-based proteomics and to be present both in the cytoplasm and in the nucleus (<http://www.phosida.com/>). The acidic

nature (pI 4.8) of this poly-LF(D/E)_nLF-containing protein (**Fig. 1b**) is enhanced through phosphorylation. Several of the phosphorylation sites show consensus sequences for CK1 or CK2, and approximately half are sensitive to epidermal growth factor stimulation. The poly-LF(D/E)₄₋₆LF motifs are followed by basic regions. Reoccurring sequences are suggestive of repetitive structure and/or multiple binding motifs. The acidic stretches in WASHCAP are reminiscent of Arp2/3-binding 'A' motifs and potentially may help locate Arp2/3 to the WASH complex. Similarly, WASHCAP basic regions may be useful in targeting the WASH complex to membranes. Yeast two-hybrid screens have identified two transcriptional repressors, ataxin 1 and TRIM27, as potential binding partners (<http://www.h-invitational.jp/>). Hence, WASHCAP may be a phosphorylation- dependent actin regulator with a transcriptional feedback role.

Implications for the biological role of the CPI motif

The data presented here indicate that the CPI motif may be important in recruiting CP or CPI motif-containing proteins and in changing the dynamics of capped filaments. Superimposition of the X-ray structures onto the electron microscopy model of a CP-capped actin filament⁶ suggests that the CPI and CSI motifs may regulate the affinity of CP for actin filaments through an allosteric mechanism (**Fig. 7**). In cells, CP dissociates from the actin-filament network relatively quickly³⁴ compared to the dissociation rate seen with pure proteins *in vitro*³⁵, which suggests that uncapping may occur in cells. Four of the six CPI motif-containing proteins (CARMIL, CD2AP, CIN85 and WASHCAP) are used in endocytotic processes and link CP to Arp2/3 through diverse architectures or complexes. The functional linkage of Arp2/3 to CP has been shown to increase actin-based force generation through enhancing filament nucleation³⁶. Physical association of CP and Arp2/3 raises a possible scenario whereby Arp2/3 complexes may be targeted, through the CPI motif, to the ends of CP-capped actin filaments. Such interactions have the potential to uncapping the barbed end of a mother filament and simultaneously nucleate a daughter filament.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ONLINE METHODS

Plasmids, proteins and peptides

We expressed chicken full-length CP ($\alpha 1/\beta 1$) and CP $\Delta\beta$ Tent ($\alpha 1/\beta 1$ lacking the final 34 residues) in BL21(DE3) *E. coli* using pET-3d- $\beta I'/\beta II$, kindly provided by T. Obinata (Chiba University) and purified them as described previously³⁷ with the following modifications. We replaced the cation-ion exchange chromatography step with size-exclusion chromatography using a Superdex 200 HiLoad 16/60 column (GE Healthcare). We

supplemented all the buffers except the gel-filtration buffer with 0.2 mM PMSF, 0.5 mM benzamidine and 20 units per ml aprotinin. We treated protein samples with 4 mM Pefabloc SC accompanied by the protector solution (Roche) before injection onto the gel-filtration column.

We amplified the CBR115 fragment of human CARMIL1a (GenBank FJ009082) residues Glu964–Ser1078 from cDNA by PCR and cloned it into the pGEX-6P-3 vector (GE Healthcare). We expressed the GST fusion protein in BL21(DE3) *E. coli* and purified it with glutathione fast-flow Sepharose resin (GE Healthcare). We grew and induced cultures with IPTG at 23 °C. After elution from the glutathione resin, we mixed GST-CBR115 with PreScission protease (GE Healthcare) and dialyzed the mixture into S-Sepharose buffer (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 1 mM NaN₃) overnight (16 h). To purify CBR115 from GST, we applied the mixture to an S-Sepharose column and eluted it with a 10- to 700-mM KCl gradient. For storage, we dialyzed CBR115 into 10 mM Tris-HCl, pH 8.0, 40 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 1 mM NaN₃ and kept it on ice. We made His-tagged CBR115 residues Glu964–Ser1078 by cloning the region of interest into the pRSFDuet-1 vector (Novagen) and purified it using the Talon metal affinity resin (Clontech). We determined concentrations of CBR115 fragments based on the far UV absorbance at A₂₈₀ or A₂₁₅ and A₂₂₅.

We purified actin from rabbit skeletal muscle³⁸. We cloned human profilin 1 into the pSY5 vector, a modified version of pET-21d(+), and expressed it with an 8×His tag³⁹. We purified the protein with a HisTrap column followed by gel filtration with a Superdex 75 HiLoad 16/60 column (GE Healthcare). We purchased CBR52, the reported fragment of the CARMIL domain that inhibits CP and uncaps actin filaments (Leu961–Asp1012)⁹, CBR37 (Ser968–Cys1004), defined from the structural analysis of the CPΔβTent–CPI motif complex, CPI peptides (**Fig. 6a**) from CD2AP, CIN85 and CKIP-1 (Auspep Pty. Ltd) and WASHCAP and CapZIP CPI peptides (Neo peptide) at >95% purity.

Actin polymerization assays

We prepared spectrin-actin seeds (spectrin-band 4.1-actin complex) as described⁴⁰ with minor modifications. We collected 100 ml of fresh whole blood in acid citrate/dextrose. We extracted the red blood cell ghost pellet with 3–5 ml (per 10 ml of original cells) of extraction buffer (0.3 mM Na₂HPO₄) at 37 °C for 10 min. We centrifuged the extract for 1 h at 125,000g, made the supernatant in 2 mM DTT and added an equal volume of ethylene glycol for storage at –20 °C.

We performed pyrene-actin polymerization assays as described^{2,8} with minor modifications. For a total volume of 200 μl, we Mg²⁺-exchanged actin subunits (5% pyrene labeled) at 1.5 μM in G-buffer (2 mM Tris-HCl, pH 7.2, 0.2 mM ATP, 0.5 mM DTT, 1 mM NaN₃ and 0.2 mM CaCl₂). Then, we added 10 μl of 20× KME buffer (200 mM Tris-HCl, pH 7.5, 1 M KCl, 20 mM MgCl₂, 20 mM EGTA) and 20 μl of spectrin-actin seeds. For capping assays, we added 10 nM CP to the mixture at time zero. We varied the concentration of CBR fragments and CPI peptides, which were also added at time zero. For the uncapping assays followed by pyrene-actin polymerization, we polymerized actin from seeds in the presence of 10 nM CP for 200 s, added the CBR fragments of CARMIL and CPI peptides and then polymerized them for 300 s.

Peptide labeling and fluorescence anisotropy measurements

We labeled the C-terminal free sulfhydryl group of CBR37 with the thiol-specific fluorescence probe Alexa Fluor 488 C₅ maleimide (Molecular Probes). We removed the

excess of the probe with a PD-10 desalting column (GE Healthcare). We estimated the efficiency of labeling to be 50% based on an SDS-PAGE gel. We determined the concentration of the conjugated protein from absorption spectra assuming that the extinction coefficient of the probe at the lowest energy maximum ($72,000 \text{ M}^{-1} \text{ cm}^{-1}$ at wavelength 493 nm) is not affected by conjugation⁴¹. We dispensed and equilibrated (30 min) the reaction mixture, which consisted of 250 nM fluorescent conjugate and increasing concentrations of protein or protein complex, in a 96-well, black flat-bottomed plate (Nunc). We measured fluorescence anisotropy at wavelength 518 nm after correcting for instrument polarization bias (G-factor) using a Safire² fluorimeter (Tecan). We fit binding curves of anisotropy versus protein or protein complex concentrations to a single-site binding model⁴², and we fit competition curves as described⁴³ using GraphPad Prism4 (<http://www.graphpad.com/>).

Crystallography and TIRF assays

Details of X-ray crystallographic experiments combined with data collection, merging and refinement statistics are detailed in

References

1. Kovar DR, Wu JQ, Pollard TD. Profilin-mediated competition between capping protein and formin Cdc12p during cytokinesis in fission yeast. *Mol. Biol. Cell.* 2005; 16:2313–2324. [PubMed: 15743909]
2. Wear MA, Yamashita A, Kim K, Maeda Y, Cooper JA. How capping protein binds the barbed end of the actin filament. *Curr. Biol.* 2003; 13:1531–1537. [PubMed: 12956956]
3. Cooper JA, Sept D. New insights into mechanism and regulation of actin capping protein. *Int. Rev. Cell Mol. Biol.* 2008; 267:183–206. [PubMed: 18544499]
4. Mejillano MR, et al. Lamellipodial versus filopodial mode of the actin nanomachinery: pivotal role of the filament barbed end. *Cell.* 2004; 118:363–373. [PubMed: 15294161]
5. Yamashita A, Maeda K, Maeda Y. Crystal structure of CapZ: structural basis for actin filament barbed end capping. *EMBO J.* 2003; 22:1529–1538. [PubMed: 12660160]
6. Narita A, Takeda S, Yamashita A, Maeda Y. Structural basis of actin filament capping at the barbed-end: a cryo-electron microscopy study. *EMBO J.* 2006; 25:5626–5633. [PubMed: 17110933]
7. Jung G, Remmert K, Wu X, Volosky JM, Hammer JA III. The *Dictyostelium* CARMIL protein links capping protein and the Arp2/3 complex to type I myosins through their SH3 domains. *J. Cell Biol.* 2001; 153:1479–1497. [PubMed: 11425877]
8. Yang C, et al. Mammalian CARMIL inhibits actin filament capping by capping protein. *Dev. Cell.* 2005; 9:209–221. [PubMed: 16054028]
9. Urano T, Remmert K, Hammer JA III. CARMIL is a potent capping protein antagonist: identification of a conserved CARMIL domain that inhibits the activity of capping protein and uncaps capped actin filaments. *J. Biol. Chem.* 2006; 281:10635–10650. [PubMed: 16434392]
10. Fujiwara I, Remmert K, Hammer JA III. Direct observation of the uncapping of capping protein-capped actin filaments by CARMIL homology domain 3 (CAH3). *J. Biol. Chem.* 2010; 285:2707–2720. [PubMed: 19926785]
11. Canton DA, et al. The pleckstrin homology domain-containing protein CKIP-1 is involved in regulation of cell morphology and the actin cytoskeleton and interaction with actin capping protein. *Mol. Cell. Biol.* 2005; 25:3519–3534. [PubMed: 15831458]
12. Canton DA, Olsten ME, Niederstrasser H, Cooper JA, Litchfield DW. The role of CKIP-1 in cell morphology depends on its interaction with actin-capping protein. *J. Biol. Chem.* 2006; 281:36347–36359. [PubMed: 16987810]
13. Bruck S, et al. Identification of a novel inhibitory actin-capping protein binding motif in CD2-associated protein. *J. Biol. Chem.* 2006; 281:19196–19203. [PubMed: 16707503]

14. Gaidos G, Soni S, Oswald DJ, Toselli PA, Kirsch KH. Structure and function analysis of the CMS/CIN85 protein family identifies actin-bundling properties and heterotypic-complex formation. *J. Cell Sci.* 2007; 120:2366–2377. [PubMed: 17606992]
15. Hutchings NJ, Clarkson N, Chalkley R, Barclay AN, Brown MH. Linking the T cell surface protein CD2 to the actin-capping protein CAPZ via CMS and CIN85. *J. Biol. Chem.* 2003; 278:22396–22403. [PubMed: 12690097]
16. Evers CE, et al. The phosphorylation of CapZ-interacting protein (CapZIP) by stress-activated protein kinases triggers its dissociation from CapZ. *Biochem. J.* 2005; 389:127–135. [PubMed: 15850461]
17. Bhattacharya N, Ghosh S, Sept D, Cooper JA. Binding of myotrophin/V-1 to actin-capping protein: implications for how capping protein binds to the filament barbed end. *J. Biol. Chem.* 2006; 281:31021–31030. [PubMed: 16895918]
18. Kim K, et al. Structure/function analysis of the interaction of phosphatidylinositol 4,5-bisphosphate with actin-capping protein: implications for how capping protein binds the actin filament. *J. Biol. Chem.* 2007; 282:5871–5879. [PubMed: 17182619]
19. Kuhn JR, Pollard TP. Single molecule kinetic analysis of actin filament capping. Polyphosphoinositides do not dissociate capping proteins. *J. Biol. Chem.* 2007; 282:28014–28024. [PubMed: 17656356]
20. Cooper JA, Walker SB, Pollard TP. Pyrene actin: documentation of the validity of a sensitive assay for actin polymerization. *J. Muscle Res. Cell Motil.* 1983; 4:253–262. [PubMed: 6863518]
21. Dikic I. CIN85/CMS family of adaptor molecules. *FEBS Lett.* 2002; 529:110–115. [PubMed: 12354621]
22. Havrylov S, Rzhetsky Y, Malinowska A, Drobot L, Redowicz MJ. Proteins recruited by SH3 domains of Ruk/CIN85 adaptor identified by LC-MS/MS. *Proteome Sci.* 2009; 7:21–39. [PubMed: 19531213]
23. Badour K, et al. The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. *Immunity.* 2003; 18:141–154. [PubMed: 12530983]
24. Johnson RI, Seppa MJ, Cagan RL. The *Drosophila* CD2AP/CIN85 orthologue Cindr regulates junctions and cytoskeleton dynamics during tissue patterning. *J. Cell Biol.* 2008; 180:1191–1204. [PubMed: 18362180]
25. Oishi I, Kawakami Y, Raya A, Callol-Massot C, Izpisá Belmonte JC. Regulation of primary cilia formation and left-right patterning in zebrafish by a noncanonical Wnt signaling mediator, duboraya. *Nat. Genet.* 2006; 38:1316–1322. [PubMed: 17013396]
26. Hertzog M, et al. The β -thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. *Cell.* 2004; 117:611–623. [PubMed: 15163409]
27. Irobi E, et al. Structural basis of actin sequestration by thymosin- β 4: implications for WH2 proteins. *EMBO J.* 2004; 23:3599–3608. [PubMed: 15329672]
28. Dominguez R. The β -thymosin/WH2 fold: multifunctionality and structure. *Ann. NY Acad. Sci.* 2007; 1112:86–94. [PubMed: 17468236]
29. Gomez TS, Billadeau DDA. FAM21-containing WASH complex regulates retromer-dependent sorting. *Dev. Cell.* 2009; 17:699–711. [PubMed: 19922874]
30. Derivery E, et al. The Arp2/3 activator WASH controls the fission of endosomes through a large multiprotein complex. *Dev. Cell.* 2009; 17:712–723. [PubMed: 19922875]
31. Valdmanis PN, et al. Mutations in the KIAA0196 gene at the SPG8 locus cause hereditary spastic paraplegia. *Am. J. Hum. Genet.* 2007; 80:152–161. [PubMed: 17160902]
32. Huang CY, et al. A novel cellular protein, VPEF, facilitates Vaccinia virus penetration into HeLa cells through fluid phase endocytosis. *J. Virol.* 2008; 82:7988–7999. [PubMed: 18550675]
33. Hänisch J, et al. Molecular dissection of *Salmonella*-induced membrane ruffling versus invasion. *Cell. Microbiol.* 2010; 12:84–98. [PubMed: 19732055]
34. Miyoshi T, et al. Actin turnover-dependent fast dissociation of capping protein in the dendritic nucleation actin network: evidence of frequent filament severing. *J. Cell Biol.* 2006; 175:947–955. [PubMed: 17178911]

35. Schafer DA, Jennings PB, Cooper JA. Dynamics of capping protein and actin assembly *in vitro*: uncapping barbed ends by polyphosphoinositides. *J. Cell Biol.* 1996; 135:169–179. [PubMed: 8858171]
36. Akin O, Mullins RD. Capping protein increases the rate of actin-based motility by promoting filament nucleation by the Arp2/3 complex. *Cell.* 2008; 133:841–851. [PubMed: 18510928]
37. Soeno Y, et al. Generation of functional β -actinin (CapZ) in an *E. coli* expression system. *J. Muscle Res. Cell Motil.* 1998; 19:639–646. [PubMed: 9742448]
38. Burtnick LD, et al. Structure of the N-terminal half of gelsolin bound to actin: roles in severing, apoptosis and FAF. *EMBO J.* 2004; 23:2713–2722. [PubMed: 15215896]
39. Wang H, et al. Helix straightening as an activation mechanism in the gelsolin superfamily of actin regulatory proteins. *J. Biol. Chem.* 2009; 284:21265–21269. [PubMed: 19491107]
40. Casella JF, Maack DJ, Lin S. Purification and initial characterization of a protein from skeletal muscle that caps the barbed ends of actin filaments. *J. Biol. Chem.* 1986; 261:10915–10921. [PubMed: 3733738]
41. Rusinova E, et al. Alexa and Oregon green dyes as fluorescence anisotropy probes for measuring protein-protein and protein-nucleic acid interactions. *Anal. Biochem.* 2002; 308:18–25. [PubMed: 12234459]
42. Owen BA, Lang WH, McMurray CT. The nucleotide binding dynamics of human MSH2–MSH3 are lesion dependent. *Nat. Struct. Mol. Biol.* 2009; 16:550–557. [PubMed: 19377479]
43. Vinson VK, De La Cruz EM, Higgs HN, Pollard TP. Interactions of *Acanthamoeba* profilin with actin and nucleotides bound to actin. *Biochemistry.* 1998; 37:10871–10880. [PubMed: 9692980]

Figure 1.

Domain organization of CPI motif-containing proteins. **(a)** Previously known CPI family members. L, leucine-rich repeat; SH3, src homology domain 3; PH, pleckstrin homology domain; CC, coiled coil. The green bars above CD2AP, CIN85 and CKIP-1 signify reported CPI regions. Black bars above CARMIL detail the constructs used in this study. CBR37, CBR49, CBR71 and CBR115 comprise residues Ser968–Cys1004, Glu964–Asp1012, Glu964–Val1034 and Glu964–Ser1078, respectively. CBR71 is approximately equivalent to CAH3_{965–1038} (ref. 10). **(b)** Novel CPI family members. WASHCAP contains multiple repeats homologous to LF(E/D)_nLF (green) that show a high incidence of serine residues. The LF(E/D)_nLF motifs are generally followed by basic residue-rich regions.

Figure 2.

Functional definition of the CPI motif. **(a)** Inhibition of CP capping by histidine (His)-tagged CBR115 and CBR37 as monitored by the pyrene-actin polymerization assay. **(b)** Uncapping of CP-capped actin filaments. Data for His-CBR71 and His-CBR49 are included in **Supplementary Figure 2a,b**. Pyrene-labeled actin (1.5 μM , 7% pyrene) was polymerized in the presence of spectrin-actin seeds, and actin polymer concentration was monitored by pyrene fluorescence intensity. For the inhibition assay, actin was polymerized in the presence of CP with His-CBR115 or CBR37 at the indicated concentrations (nM). The concentration of CP (chicken $\alpha 1/\beta 1$) was 10 nM for CBR115 and 8 nM for CBR37. For the uncapping assay, actin was polymerized in the presence of CP (10 nM); fragments of CBR were added after 200 s. Actin polymerized in the absence of CP is included as a positive control in these and subsequent pyrene assays. These controls (labeled C) show the fastest polymerization in each experiment. **(c)** Fluorescence anisotropy analysis of the interaction of Alexa488-CBR37 with CP. **(d)** Fluorescence anisotropy measurements indicating that the Alexa488-CBR37-CP complex is dissociated upon competition with unlabeled CBR37 but not with G-actin. **(e)** Fluorescence anisotropy measurements suggesting dissociation of the Alexa488-CBR37-CP complex at high concentrations of F-actin.

Figure 3.

CARMIL- and CD2AP-induced uncapping of CP-capped actin filaments monitored by TIRF microscopy. **(a–c)** CBR115 (200 nM) **(a)**, CBR37 (250 μ M) **(b)** and CD2AP CPI peptide (200 μ M) **(c)** support green actin filament growth from the previously capped red actin filaments, indicating uncapping. **(d)** In the absence of CP, the red actin filaments act as seeds for the green actin. **(e)** The CP-capped actin filaments do not elongate in the absence of CARMIL or CD2AP truncations. Red actin (2 μ M) was polymerized and capped with CP (chicken α 1/ β 1, 50 nM), stabilized with phalloidin and diluted to 50 nM. CARMIL or CD2AP truncations were added to CP-capped red actin filaments concurrently with green actin (0.2 μ M) and CP (50 nM). Profilin was maintained throughout at a 1.5 molar ratio over actin. Time stamps (min:sec) for frames are given; “–” refers to before addition.

Figure 4.

The structure of the CPΔβTent-CARMIL CPI motif complex. **(a–c)** Three views of the CPΔβTent-CARMIL CPI complex. CARMIL CPI motif is shown as a surface (yellow) and CP α-subunit (red) and β-subunit (blue) as schematic representations. **(b)** 90° rotation around the *x* axis with respect to **a**. **(c)** 180° rotation around the *y* axis with respect to **a**. N and C, N and C termini, respectively. **(d)** Cartoon representation of **a**, depicting the mushroom-like CP structure with the CPI motif half-encircling the stalk on the underside of the mushroom cap. The β-tentacle, which was not included in the construct for the structure, is added for completeness. **(e)** Sequence homology of the CARMIL CPI across species displayed as a sequence logo (<http://weblogo.berkeley.edu/>) with colors red, blue, yellow and green signifying acidic, basic, hydrophobic and hydrophilic residues, respectively. The major CPI residues, α-subunit (red) and β-subunit (blue), are displayed above the logo. S denotes a residue that plays a structural role; X signifies a noninteracting, nonstructural residue in this complex. A more detailed analysis of the CPΔβTent-CARMIL CPI complex is given in **Supplementary Figure 3**.

Figure 5.

Structural differences between CARMIL and CD2AP in binding CP. **(a)** The structure of the CPΔβTent-CD2AP CPI motif complex with CARMIL CPI motif structure overlaid. The C-terminal half of the CD2AP CPI motif (cyan) generally follows the path of the CARMIL CPI motif (yellow) in binding the β-subunit of CP. The paths of N termini of the CD2AP and CARMIL CPI motifs differ slightly in binding to the α-subunit. **(b)** The structure of CBR115-CP. Two ordered CARMIL regions are observed: CPI (Ile971–Cys1004, yellow) and CSI (CARMIL-specific interaction; Arg1021–Thr1035, green). Phenylalanine residues (1029 and 1030) are shown in black. A more comprehensive analysis of the interactions within these structures is given in **Supplementary Figure 3**.

Figure 6.

The CPI motif family of proteins. **(a)** Structure-based sequence alignment of the CPI motif family. α and β refer to the interacting subunit of CP. **(b)** Pyrene-actin assays monitoring CP uncapping induced by the six members of the CPI motif family. Concentrations of CP and the 37-mer peptides for CPI motif family are as indicated. Actin monomer and spectrin seeds concentrations are as described for **Figure 2**. The associated inhibition assays can be found in **Supplementary Figure 4e**. FAM21 is here referred to as WASHCAP. AU, arbitrary units.

Figure 7.

Models of CPI motif uncapping. **(a)** Model of a CP-capped actin filament⁶. The cyan and orange helices and arrow denote the proposed repositioning of the β -tentacle. The CARMIL CPI (yellow surface) and CSI (green surface) motifs are superimposed on the model. **(b)** 180° rotation of **(a)** around the y axis. The β -tentacle is omitted for clarity. **(c)** A cartoon depicting a potential uncapping mechanism of β -tentacle sequestration by the CPI motif. **(d)** A cartoon depicting flexibility in the mushroom cap of CP. **(e)** Superimposition of two CP structures (α -subunits, red and orange; β -subunits, blue and cyan) showing the relative position of the CPI (yellow surface) and CSI (green surface). The CPI motif is speculated to alter CP flexibility, disfavoring the filament-bound conformation.