Published in final edited form as:

Nat Rev Mol Cell Biol. 2014 October ; 15(10): 677–689. doi:10.1038/nrm3869.

Capping Protein Regulators Fine-Tune Actin Assembly Dynamics

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Abstract

Capping protein (CP) regulates actin polymerization by binding the barbed end of an actin filament, which blocks addition and loss of actin subunits. Recent structural and biochemical studies provide new insight into how cells control the actin capping activity of CP. Several molecules indirectly regulate CP by interacting with filament barbed ends and preventing binding of CP; others bind directly to CP and sterically block its interaction with an actin filament. A diverse and otherwise unrelated set of proteins contains a motif for CP regulation termed the “Capping Protein Interaction” (CPI) motif. These proteins bind directly to CP, recruit or target CP to a subcellular location, and modulate its actin-capping activity via allosteric effects.

Introduction

The assembly of actin filament networks is important for the formation of many subcellular structures and performance of many cell functions ^{1,2}. Individual filaments polymerize and depolymerize, filaments connect with each other to form crosslinked branching networks and bundles of straight filaments, and those filament assemblies interact with other molecules, especially with membranes, to form subcellular components, such as muscle sarcomeres, filopodia, lamella, ruffles, neuronal growth cones and dendritic spines. Numerous phenomena related to cell shape and movement depend on dynamic actin filament networks, such as migration, contraction, adhesion and protrusion. Membrane trafficking and transport often involve actin assembly, with endocytosis and phagocytosis as examples.

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The Authors declare no competing interests

Polymerization of actin filaments involves actin subunit nucleation coupled with filament elongation at free barbed ends. Nucleation factors stabilize small actin oligomers and filaments elongate by adding actin subunits to the faster-growing barbed ends and the slower-growing pointed ends. Filaments associate with each other to form bundles and branching networks. Actin cytoskeleton disassembly occurs by breaking links between filaments and severing filaments. ATP hydrolysis fuels filament dynamics; actin subunits bind ATP for polymerization, and filaments convert ATP to ADP as they age. The bound nucleotide influences the assembly / disassembly state.

The barbed ends of the actin filaments play key roles in filament dynamics at cellular structures because subunits add and leave filaments at barbed ends and because barbed ends interact with structures such as membranes and sarcomere Z-disks. Spontaneous formation of a new filament from subunits is rare; the rate depends on the subunit concentration, which is generally low. Cells use three mechanisms to create free barbed ends for filament elongation: nucleation of actin subunits by proteins such as Arp2/3 complex and formins^{3,4}, severing existing filaments by proteins such as ADF/cofilin^{5,6}, and removal of proteins that block, or cap, barbed ends^{7,8}. Once new free barbed ends exist, elongation factors promote their growth and, conversely, filament capping proteins limit their growth.

The heterodimeric actin capping protein, referred to here as CP, is an essential factor that restricts actin filament elongation by binding to free actin filament barbed ends. A number of other proteins, including gelsolin-family members^{9,10}, adducins¹¹ and Eps8^{12,13}, possess barbed-end capping activity. We focus here on CP as the most ubiquitous and abundant capper. Other barbed-end cappers have critical but more specialized roles, with restricted cell and tissue expression patterns and distinct modes of regulation. Recent biochemical and cell biological studies have identified several molecules capable of binding to CP, some of which influence actin capping activity⁷. The physiological relevance of the interactions of these molecules with CP in cells is an important question and a current focus of study. Potential physiologic roles relate to spatial and temporal regulation of CP via mechanisms that recruit or target active CP, inhibit capping activity of CP, and uncap actin filaments that are capped by CP⁷.

One class of CP regulators contains a motif necessary and sufficient for binding CP, termed CPI for “Capping-Protein Interaction” and includes a diverse set of otherwise unrelated multi-domain scaffold proteins - CARMIL, CD2AP/CIN85, CKIP-1, FAM21 and CAPZIP^{14,15}. CARMIL proteins have a second CP-binding motif termed CSI for “CARMIL-Specific Interaction”¹⁵. Here we discuss how the filament capping activity of CP is regulated, directly or indirectly, by CPI-containing proteins and other molecules, including polyphosphoinositides, V-1 / myotrophin, Ena/VASP proteins, and formins.

Capping Protein and Actin Dynamics - the Basics

CP is found in nearly all eukaryotic organisms, from humans to fungi to plants and in nearly all cells and tissues of every organism⁷. In many settings, CP is a critical determinant of the behavior and interactions of actin filament barbed ends⁷. In the sarcomere of striated muscle cells, CP is an essential component of the Z-disk (leading to its other name CapZ), where it

caps the barbed ends of the actin-based thin filaments¹⁶⁻¹⁸. In non-muscle cells, CP is important for the assembly of cortical actin and for actin-based motility^{7,8}, including membrane protrusions in migrating cells.

CP is required for the Arp2/3 complex-mediated assembly of actin, based on reconstitution of actin-based motility from purified proteins *in vitro*¹⁹, and on studies of the assembly of cortical actin networks at the leading edge of motile cells²⁰⁻²³. According to the dendritic nucleation model for Arp2/3 complex-based assembly, activation of Arp2/3 complex leads to the creation of a branched actin filament network via the formation of new daughter filaments on the sides of mother filaments (Figure 1). The daughter filaments nucleated by Arp2/3 complex have free barbed ends, which grow for a time and then become capped by CP^{1,21}. In this model, filament capping is proposed to ensure that the network consists of short filaments with a high density of branches, thought to provide the mechanical stiffness needed for efficient generation of force by barbed-end growth at the membrane^{20,23}. An alternative and non-exclusive proposed role for capping is to prevent the growth of barbed ends that are older and no longer in contact with the membrane, which serves to “funnel” the available pool of actin subunits to the locations where filament elongation most effectively pushes on the membrane²⁴.

The biochemical properties of CP were reviewed previously⁷. CP is a ~64 kDa heterodimer of alpha and beta subunits. One CP molecule binds to one filament barbed end, and the presence of CP is sufficient to block the addition and loss of actin subunits at that end^{25,26}. The K_d for binding of CP to the barbed end is sub-nanoMolar, and the half-time for dissociation *in vitro* is ~30 min²⁷. The alpha and beta subunits are extensively intertwined in the mushroom-shaped structure²⁸ (Box 1); thus, the heterodimer behaves as a single protein in terms of its physical properties, including stability and denaturation²⁹. From N- to C-terminus, the two subunits have a similar set of secondary structural elements, and the tertiary arrangement of those elements in the heterodimer is similar for the two subunits²⁸.

Structural and biochemical studies led to a model for barbed end capping by CP^{25,28,30,31}. A basic patch on the flat top of the mushroom-shaped CP interacts with an acidic patch on the barbed end of the filament, at the interface between the two terminal actin subunits (Site 1, Figure 2A). In addition, the hydrophobic side of the amphipathic alpha helix at the C-terminus of the beta subunit of CP (Site 2, Figure 2A), binds to the hydrophobic cleft of the last actin subunit of the filament^{25,31}. How the two CP-actin sites function in the binding mechanism is an interesting question. Ensemble experiments suggested a kinetic model in which the basic / acidic patch interactions (Site 1) form first, followed by the hydrophobic interaction of the beta tentacle with the actin hydrophobic cleft²⁵; however a more complete understanding of the binding mechanism remains to be elucidated. The two sites may have some degree of independence, allowing for the existence of states in which only one of the sites is occupied. The nature of these states may be important for understanding how CP regulators function in cells.

Several molecules affect the interaction of CP with the barbed end of the actin filament via various mechanisms. Barbed end binding is inhibited indirectly by proteins that compete with CP for binding barbed ends. In addition, a number of molecules bind directly to CP and

sterically block its interaction with a barbed end⁷. Finally, a diverse group of proteins bind directly to CP and inhibit its capping activity by an allosteric mechanism (see below).

Indirect Regulators of CP

Indirect regulators bind to the actin filament, not to CP. Their interactions with actin have implications for how CP binds to the barbed end.

Formins

Formins generate F-actin networks consisting of long filament bundles, such as actin cables, filopodia, and cytokinetic rings for processes as varied as cell motility, cytokinesis, polarized cell growth, vesicle transport and tissue morphogenesis³². Formins nucleate unbranched actin filaments and promote filament elongation at barbed ends. Formins associate directly with barbed ends and function as processive or 'leaky' caps that promote the addition or loss of actin subunits at the barbed end while antagonizing the binding of CP³³. These effects on F-actin are due interaction of the two formin-homology domains, FH1 and FH2, which define formins, with a barbed end. Outside of their FH1 and FH2 domains, formins are extremely diverse, containing a series of regulatory domains that provide for membrane association^{34,35}, activation by Rho-family GTPases^{36,37}, autoinhibition³⁸ and dimerization.

The presence of the FH2 domain at the filament barbed end inhibits the binding of CP, which would otherwise terminate elongation³⁹ (Figure 1B). Many formins exist, and often several different ones are expressed in a single cell; the anti-capping and filament elongation properties of individual formins vary widely^{32,40-43}.

Ena/VASP Proteins

The Ena/VASP family of proteins are implicated in a variety of fundamental cellular processes, including cell migration, axon guidance, and the movement of the bacterial pathogen *Listeria monocytogenes*⁴⁴. In cells, VASP overexpression leads to increased migration rate and actin filament length at the leading edge – effects similar to those of CP depletion⁸. Like formins, Ena/VASP proteins associate with barbed ends in a processive manner, actively delivering monomers to the growing barbed end while antagonizing filament capping by CP⁴⁵⁻⁴⁷. In contrast to formins, Ena/VASP proteins lack nucleation activity⁴⁸.

Ena/VASP proteins share a tripartite domain organization, consisting of N- and C-terminal Ena/VASP homology 1 and 2 (EVH1 and EVH2) domains and a central Pro-rich region. The EVH1 domain targets Ena/VASP proteins to focal adhesions, filopodia and lamellipodia via interactions with proteins containing the consensus sequence 'FPPPP'⁴⁴. The EVH2 domain comprises globular (monomeric) and filamentous actin-binding sites (GAB and FAB), as well as a C-terminal coiled-coil (CC) region that mediates tetramerization^{49,50}. Both the GAB and FAB domains are required for VASP to associate with the barbed end and compete with CP⁴⁶. The central Pro-rich region binds profilin-actin, which is a critical reservoir for active actin subunits, accelerating barbed-end elongation. Structural and biochemical studies show that profilin-actin binds simultaneously to the Pro-rich and GAB

domains of VASP, with an affinity greater than that of either profilin or actin alone, placing the actin subunit in a state that promotes its direct addition to the barbed end⁵¹. Together, these findings suggest a model whereby filament-bound Ena/VASP prevents binding of CP to the barbed end (Figure 2B).

Steric Regulators

Steric regulators directly bind CP, weakening its interactions with filament barbed ends.

V-1 / Myotrophin

V-1, also known as myotrophin, is a 13-kDa protein comprised of three and one-half ankyrin repeats⁵². V-1 binds CP with high affinity ($K_d \sim 40$ nM) and inhibits its barbed-end capping activity⁵³. Originally identified as a protein upregulated in the cerebellum during mouse development^{54,55}, V-1 was independently linked to cardiac hypertrophy and thus called myotrophin⁵⁶⁻⁵⁹. V-1 / myotrophin also interacts with NF-kappaB and co-translocates to the nucleus with p65, which is involved in inducing cardiac hypertrophy^{60,61}.

Biochemical studies found that V-1 inhibited barbed-end capping only when mixed with CP prior to exposure to actin filaments^{53,54}. V-1 could not remove CP already bound to a barbed end. Structural approaches, including NMR spectroscopy⁶² and x-ray crystallography⁶³ showed that V-1 interacts with the basic patch (Site 1) on CP through a concave surface formed by the short inner helices of the ankyrin repeats and the variable inter-repeat loops of V-1³¹ (Figure 1C). Consistent with the proposed steric mechanism of CP regulation by V-1, point mutations in the basic patch of CP disrupt both V-1 binding and barbed-end capping⁶². The binding sites on V-1 / myotrophin for CP and for NFkappaB are essentially identical^{53,60}, suggesting that V-1 / myotrophin might interact with CP or NFkappaB, but not both. If so, competition between CP and NFkappaB for binding V-1 / myotrophin could direct cross-talk between signaling and control of actin assembly.

Polyphosphoinositides

Several cytoskeletal proteins are regulated by lipids, especially anionic phospholipids and polyphosphoinositides^{64,65}. CP is one such protein, interacting with and being inhibited by anionic phospholipids^{27,66-68}. PIP₂, either in the form of micelles or when diluted with neutral lipids in liposomes, binds and inhibits CP⁶⁶. PIP₂ appears to weaken the interactions between the alpha and beta subunits of CP and to selectively interact with the alpha subunit⁶⁶. PIP₂-mediated inhibition may require CP binding to a cluster of PIP₂ molecules in the membrane.

Computational and biochemical approaches, combined with mutagenesis, indicated that anionic lipids bind to the basic patch on CP, similar to V-1^{67,68}. Free barbed ends formed rapidly when PIP₂ was added to capped actin filaments in ensemble polymerization assays, suggesting that PIP₂ might cause uncapping^{66,67}. However, a study using TIRF microscopy to visualize actin filaments failed to observe uncapping and suggested that PIP₂ functions via a steric mechanism similar to that of V-1 (Figure 1C)⁶⁸. That study suggested that the appearance of free barbed ends in the ensemble assays might have resulted from filament breakage during mixing; however, one would expect the same effect to be observed in

experiments with V-1, which was not the case. Additional experiments should help resolve the controversy.

One possible explanation to account for the functional difference of PIP₂ compared to V-1, with respect to uncapping activity, is that the basic patch (Site 1) on CP bound to a barbed end may be partially accessible to the smaller-sized PIP₂ molecule(s) but not accessible to V-1. This explanation is more likely if, as discussed above, Site 1 dissociates from the barbed end independently of Site 2, allowing space for small, but not large, molecules to approach Site 1.

Allosteric Inhibition by CARMIL Proteins

CARMIL proteins bind directly to CP via CPI and CSI motifs (Box 1). This interaction has an allosteric effect on the actin-binding site, which lowers the affinity of CP for the barbed end and promotes dissociation of CP from the barbed end, i.e. “uncapping”.

Acan125, the first identified CARMIL, was discovered in *Acanthamoeba* in a biochemical search for binding partners of the SH3 domain of myosin-IC⁶⁹. Subsequent work uncovered the *Dictyostelium* homolog⁷⁰. This protein, initially called p116, co-purified with CP and Arp2/3 complex, leading to its being renamed “CARMIL,” for Capping protein Arp2/3 complex Myosin-I Linker⁷⁰. In *Dictyostelium*, the protein localized with regions of dynamic actin, and a haploid knockout strain exhibited multiple phenotypes related to altered actin dynamics, including loss of macropinocytosis.

Amoeba species have single genes for CARMIL, but vertebrates have three. Murine CARMIL1 was the first vertebrate CARMIL characterized⁷¹. Murine CARMIL1 “uncapped” actin filaments capped by CP, based on the rapid appearance of free barbed ends when it was added to capped filaments⁷¹. The CARMIL1-CP complex retained a low level of capping activity, and CARMIL1 and CP co-localized in cultured cells. As discussed in Box 1, CARMIL1 CBR binds CP at a site distinct from its actin-binding site; thus, CARMIL1 might target the capping activity of CP in cells, not simply inhibit it.

CARMIL Proteins and Actin Dynamics

CARMIL1 has an important role in regulating actin dynamics at the plasma membrane. CARMIL1 localizes to dynamic actin in lamellipodia, ruffles, macropinosomes, and the leading edge of lamella in migrating cultured cells^{71,72}. CARMIL1 appears to be de-localized in retracting edges⁷³, suggesting selective targeting to advancing membranes. Depletion of CARMIL1 in cultured cells leads to loss of lamellipodia, ruffles and macropinosomes^{71,72}. CARMIL1 depletion has only a modest effect on cell migration in a cultured-cell wound healing model, despite the nearly complete loss of lamellipodia^{71,72,74}. This result is consistent with a number of studies showing that lamellipodia are dispensable for migration under these conditions⁷⁴. Increased expression of CARMIL1 enhances lamellipodia formation⁷¹, and high-level expression causes formation of abnormal spikes and club-shaped protrusions that contain lamellipodial molecular markers⁷².

The ability of CARMIL1 to interact with CP has physiological relevance for dynamic actin assembly, based on studies of two mutant CARMIL1 proteins. Expression of a CARMIL1 mutant with two of the conserved residues in the CPI motif, Lys 987 and Arg 989, changed to Ala, failed to restore lamellipodia, ruffling and macropinocytosis in human cells depleted of endogenous CARMIL1⁷⁴. The mutant partially rescued modest defects in cell migration in a wound-healing model. Similar results were obtained with expression in CARMIL1-depleted cells of CARMIL1 containing an internal deletion of 123 aa residues that removed the CPI and CSI motifs, except that cell migration was not at all restored⁷¹. In this mutant CARMIL1, the deleted region also included a novel membrane-targeting motif, the BH (basic hydrophathy) motif⁷⁵. The lack of the BH motif may account for the discrepancy in the phenotypic rescue results in the two studies.

Far less is known about CARMIL2, but its function and localization are distinct from those of CARMIL1 in human cultured cells that simultaneously express both isoforms⁷². Like CARMIL1, CARMIL2 contains conserved CPI and CSI motifs, which inhibit CP *in vitro* (Kim and Cooper, 2014, unpublished observations). Depletion of CARMIL2 leads to a nearly complete loss of lamellipodia, ruffling and macropinocytosis, in a wound-healing model of cultured cells, similar to the effects of loss of CARMIL1⁷².

One striking feature of CARMIL2 is its co-localization with the vimentin intermediate filament network⁷², which is not observed for CARMIL1. Vimentin has been implicated in actin-based protrusions near the cell edge⁷⁶. The localization of CARMIL2 with vimentin networks raises the possibility that CARMIL2 is a molecular link between vimentin filaments and dynamic actin assembly.

Another striking feature is that CARMIL2-depleted cells display a multipolar shape characterized by the extension of leading edges in several directions, when plated at low density⁷². Live-cell movies reveal that CARMIL2-depleted cells randomly extend new processes without retracting or redirecting existing ones, resulting in loss of coordinated movement. Such behavior could result from loss of intrinsic polarity because the microtubule-organizing center and the Golgi in CARMIL2-depleted cells are generally found on the side of the nucleus away from the leading edge, opposite their positions in control cells. CARMIL2-depleted cells also exhibit decreased levels of myosin-IIB⁷², depletion of which produces a similar multipolar morphology in single cells⁷⁷. These observations raise the possibility that the effects of CARMIL2-depletion on cell morphology and migratory behavior involve decreased myosin-IIB.

The most convincing evidence that CARMIL1 and CARMIL2 have distinct non-overlapping functions is that the expression of CARMIL1 did not rescue the phenotypic effects of CARMIL2 depletion and vice-versa⁷². To be clear on the semantics of “overlapping” functions, we emphasize that whereas CARMIL1 and CARMIL2 likely have similar biochemical activities on CP and resulting general effects on actin filament dynamics, we predict that the CARMIL isoforms exert specific spatial and temporal control on distinct pools of actin filaments within the cytoplasm, consistent with their distinct subcellular localizations⁷².

Very little is known about the biology of CARMIL3. The primary amino acid sequence of CARMIL3 is more similar to that of CARMIL1 than CARMIL2. One study found that LRRC16B (the human gene encoding CARMIL3) is an oncofetal gene, whose overexpression leads to increased proliferation and tumorigenicity of transformed cells ⁷⁸.

CARMIL Domains and Structure

Structures of N-terminal fragments of mouse CARMIL1 were characterized by x-ray crystallography (668-aa) and small angle x-ray scattering (SAXS) (878-aa), respectively ⁷⁹. The crystal structure revealed the presence of a non-canonical pleckstrin homology (PH) domain at the N-terminus (Figure 3A and 3B). The PH domain is integrated with a 10-aa amphipathic alpha-helix at the N-terminus and a beta-strand and alpha-helix at the C-terminus, named the Linker region. The PH domain contributes to membrane localization; the domain is distinctive in binding preferentially to phosphatidylserine and monophosphorylated lipids, as opposed to signaling lipids such as PIP₂.

The presence of a membrane-binding PH domain in CARMIL suggests a mechanism for CP regulation at the interface between filament-barbed ends and the plasma membrane. CARMIL proteins are localized at cellular membranes, and deletion of the PH domain impairs but does not abrogate plasma membrane localization ⁷⁹. The membrane-binding BH motif ⁷⁵, noted above, and association with myosin-I may also target CARMIL to the plasma membrane.

Following the Linker region is the N-cap, a helix-loop-helix shielding the LRR domain from exposure to solvent at its N-terminal end, stabilizing its structure (Figure 3A and 3B) ⁸⁰. CARMIL1's LRR domain consists of 16 repeats with an overall planar horseshoe shape characteristic of this fold. The LRR consensus sequence (LxxLxLxxN/CxL) consists of a beta-strand, which occupies the concave inner side of the horseshoe, connected by an "ascending loop" to an alpha-helix on the convex surface of the domain. Adjacent repeats are connected to each other by "descending loops". The LRR domain is a classical protein-protein interaction fold, and the inner side of the horseshoe and ascending loops frequently mediate such interactions ⁸¹. No binding partner of CARMIL's LRR domain has been identified, but its large size offers a platform for multiple interactions to be discovered. The C-cap, another helix-loop-helix motif, caps the C-terminal end of the LRR domain.

Following the C-cap, residues 689-878 comprise the helical dimerization (HD) domain. The crystal structure did not include the HD domain, but low-resolution structural analysis and biochemical analysis indicated that the HD mediates antiparallel homodimerization (Figure 3A and 3B). In cells, homodimerization but not heterodimerization was observed for human CARMIL1 and CARMIL2 ⁷². Approximately 100-aa C-terminal to the HD domain are the CPI and CSI motifs that mediate binding to CP. Homodimerization has important consequences for CARMIL biochemistry; interactions of CARMIL1 with CP and with membranes, are enhanced by dimerization. Low-resolution SAXS analysis of the CARMIL dimer suggest the two PH domains are positioned such that they might simultaneously interact with the plasma membrane ⁷⁹.

The C-terminal region of mouse CARMIL1 (Lys 1079 to Val 1371) is rich in proline residues, with six canonical SH3 domain-binding motifs (PxxP). The SH3 domain of myosin-I isoforms has been shown to bind this sequence in studies with amoebae and human cells ^{70,72,82,83}. The functional significance of this interaction remains to be explored. Other SH3 domain-containing proteins that bind this region of CARMIL are likely to emerge. In amoebae, the Pro-rich region is upstream of the CPI motif, and the CSI motif is absent (Box 1).

Allosteric Inhibition by Non-CARMIL Proteins

A diverse set of other proteins contain CPI motifs, allowing them to bind to and allosterically inhibit CP. These proteins are involved in various cellular functions mediated by other domains. While these functions are distinct, they highlight common themes for CP regulation, suggesting potential common mechanisms, including membrane interaction, dimerization, SH3-domain interactions, and auto-inhibition.

CKIP-1

Casein kinase 2 interacting protein-1 (CKIP-1) is a 46 kDa molecular scaffold, identified as a binding partner of the alpha subunit of casein kinase 2 (CK2) ⁸⁴ (Figure 3B and Box 1). CKIP-1 contains a CPI motif through which it interacts with CP ⁸⁵. Overexpression of CKIP-1 increased cellular beta actin levels, increased the amount of transverse actin filaments, and induced cell spreading ⁸⁵, and these effects depended on CKIP-1's ability to bind CP, based on experiments with CPI-motif mutants ⁸⁶. CKIP-1 inhibits the capping activity of CP, and addition of CK2 results in formation of a ternary complex that exhibits more potent inhibition. CK2 phosphorylates CP on Ser 9 of the alpha subunit, *in vitro* and *in vivo* ⁸⁵, raising the possibility that CKIP-1 promotes the phosphorylation of CP by CK2, providing another means to regulate its capping activity.

CKIP-1 shuttles between the plasma membrane and the nucleus ⁸⁷. CKIP-1 interacts with a number of targets in addition to CP, including AP-1/c-Jun ⁸⁸, Akt ⁸⁹, ATM ⁹⁰, IFP35/Nmi ⁹¹ and Smurf1 ⁹². Based on these interactions, CKIP-1 may contribute to signaling pathways involved in cell growth, cytoskeleton organization, apoptosis, muscle differentiation, immune response and bone formation ⁹³.

Except for the CIP motif, CKIP-1 is unrelated to CARMIL. However, CKIP-1 and CARMIL share several common structural and functional features. Like, CARMIL, CKIP-1 forms dimers ^{91,94}, mediated by a C-terminal putative leucine zipper domain. CKIP-1 contains an N-terminal PH domain that mediates plasma membrane localization, and which, like the PH domain of CARMIL1, binds preferentially to monophosphorylated lipids and phosphatidylserine ^{84,94}. The CKIP-1 PH domain has also been implicated in protein-protein interactions ^{89,94}. CKIP-1 translocates from the membrane into the nucleus upon cleavage by Caspase-3 during apoptosis ⁹⁴. To our knowledge, nuclear translocation has not been examined for other CPI-motif proteins.

FAM21

FAM21 is one of the subunits of the heteropentameric WASH regulatory complex, which links Arp2/3 complex-dependent actin polymerization to endosome-to-Golgi transport⁹⁵⁻⁹⁸ and endosome-to-plasma membrane sorting of integrins⁹⁹ (Figure 4 and Box 1). FAM21 has a CPI motif near its C-terminus; CP interacts with the WASH complex via this motif^{100,101}, and the FAM21 interaction inhibits the capping activity of CP¹⁰⁰. FAM21 contains an N-terminal globular domain and a C-terminal tail featuring several repeats of a novel “L-F” motif¹⁰². The L-F motif repeats facilitate the interaction and recruitment of FAM21 and WASH complex to retromer, via a direct interaction with the retromer subunit VPS35.

In *Dictyostelium*, FAM21 is implicated in recycling the WASH complex to acidic lysosomes¹⁰³. Recycling involves dynamic actin assembly and depends on the interaction of FAM21 with CP, based on the phenotype of FAM21 mutants deficient for binding CP.

CD2AP / CMS / CIN85 Family

The human gene CD2AP encodes CD2-Associated Protein, a multi-functional scaffold that links actin assembly to the formation of the immunological synapse¹⁰⁴ and the kidney podocyte slit diaphragm (Figure 3B and Box 1)^{105,106}. CD2AP has been called p130^{Cas} ligand with multiple SH3 domains (CMS)¹⁰⁷ and mesenchyme-to-epithelium transition protein with SH3 domains (METS-1)¹⁰⁸. In addition, CIN85 (C-Cbl-Interacting Protein of 85 kDa) is a homologue of CD2AP.

CD2AP binds tightly to CP and cortactin by mass spectrometry analysis¹⁴. Mapping the interaction site led to identification of the CPI motif and appreciation that the CPI motif was sufficient to inhibit CP¹⁴. From N- to C-termini, CD2AP and CIN85 contain three SH3 domains, a Pro-rich region and a C-terminal coiled coil domain. The CPI motif lies within the Pro-rich region. The SH3 domains interact with CD2, ALIX, and c-Cbl^{109,110}. In cells, CD2AP localizes to the plasma membrane and recruits both CP and cortactin, contributing to actin assembly¹¹¹.

CapZIP

CapZ-interacting protein (CapZIP) was identified in immune and muscle cells as a CP-associated protein and a substrate of several stress-activated protein kinases (SAPKs)¹¹². CapZIP contains a CPI motif¹⁵ and a putative coiled coil sequence; it is also rich in proline residues (Figure 3B and Box1). Certain cellular stresses result in phosphorylation of CapZIP by MAP kinase, and the dissociation of CP from CapZIP¹¹², suggesting that interactions of CPI-motif proteins with CP might be generally regulated by phosphorylation.

Coordination Between CP Regulators

Proteins containing the CPI motif constitute a large and diverse group of allosteric CP regulators. Some CPI-motif proteins have been implicated in interactions of actin filaments with membranes. In many cases, by targeting or decreasing capping activity, these proteins promote actin assembly that drives changes in cell shape and causes membranes to move

(Figure 4 and Box 1). The various functions of CPI motif-containing proteins likely reflect the broad diversity of cellular functions driven by actin assembly, revealing a critical role for CP as a ubiquitous and important regulator of actin filament dynamics.

The common ability of CPI-motif proteins to interact with specific cellular membranes and to bind to CP suggests two hypotheses for their function in cells. One possibility is that the interaction of CP with CPI-motif proteins recruits or targets actin capping activity to sites of actin assembly; the other is that CPI-motif proteins decrease the actin-capping activity of CP or promote uncapping. These two hypotheses are not necessarily exclusive of one another. We envision that the cell uses CPI-motif proteins to target CP and tune its capping activity in order to function at different locations and at different times. Given the density of actin filaments and abundance of CP and its regulators, complexes of CP and a CPI-motif protein exhibit a level of capping activity that is likely to be significant in cells. In addition, the multiple signaling and scaffolding domains of CPI-motif proteins provide for a number of possible cooperative or multivalent interactions that likely determine the spatial and temporal regulation of actin capping.

Summary and Outlook

The actin-capping activity of CP is important for regulating the availability of free barbed ends in cells, which is central to the control of actin assembly. Competition between CP and indirect regulators, such as formins and Ena/VASPs, for binding to the barbed end, helps to shape the architecture, organization and dynamics of cellular actin filaments. Other proteins regulate capping activity by binding CP directly and inhibiting its interaction with the barbed end via either steric blocking or allosteric mechanisms.

Looking ahead, important questions include the physiological relevance of the CP regulators and the interaction among the modes of regulation. Whether or not these regulators alter actin dynamics, filament architecture and the connections of actin filaments to membranes are important questions to explore. High-resolution light and electron microscopy will be important tools to uncover the structure of filaments, their organization and their connections, along with live-cell analysis at the level of single molecules and *in vitro* to determine mechanisms of action.

Acknowledgements

We thank our lab colleagues for comments and assistance with the project and the manuscript. We thank Drs. Richard Cheney and Robert Insall for comments on the manuscript. This work was supported by NIH grants GM038542 and GM095509 to JAC and GM073791 and MH087950 to RD. ME was supported by NIH training grant 5T90DA02287104.

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Box 1

Allosteric Regulation by Capping Protein Binding Motifs – CPI and CSI

A diverse set of otherwise unrelated proteins, which includes CARMIL, CD2AP, CKIP-1, CapZIP, and CIN85, contain a conserved CPI motif, which binds directly to CP at a site distinct from its actin-binding surface¹⁵. The CPI motif is defined by the consensus sequence LxHxTxRPPKx6P¹⁴. Peptides containing this sequence, from CARMIL and CD2AP, inhibit actin capping and uncapping barbed ends^{14,15,71,113}. Structures of complexes of CP with the CPI motifs of three proteins, CARMIL1^{15,63,79}, CD2AP^{15,63}, and CKIP-1⁶³, reveal a conserved mode of interaction. In the co-crystal structures, the CPI motif, which in the unbound state is intrinsically disordered¹¹⁴, adopts a stable extended conformation, wrapping around the CP stalk, which is opposite the barbed end-binding site. The positively charged core region of the CPI motif binds an acidic groove formed by the N-terminal helical bundle of the CP beta subunit, which comprises half of the stalk region.

In the case of CARMIL, the co-crystal structure of a longer, 115-aa fragment (human CARMIL1 residues 964-1078) bound to CP revealed additional interactions mediated by a ~14-aa region C-terminal to the CPI motif¹⁵. This additional interacting region was named the CARMIL-specific interaction (CSI) motif¹⁵ (Figure 2A and B) because it is absent from other allosteric regulators of CP. The CSI motif binds CP on the opposite side of the stalk from the CPI motif. Together, the CPI (residues 968-1004) and CSI (residues 1021-1035) motifs of CARMIL constitute the capping protein-binding region (CBR)¹⁵.

In support of an allosteric mechanism^{15,63,115}, NMR studies show that CBR binding induces large chemical shift changes in the basic patch of CP that binds the barbed end¹¹⁴. Moreover, CP mutations that impair barbed end binding do not affect CBR binding to CP¹¹⁵. Additional evidence for an allosteric mechanism is that binding of the CPI motif to CP induces dissociation of V-1, which interacts with the basic patch (Site 1) of CP that binds the filaments^{73,63}. Finally, computational studies reveal changes on the barbed end-binding surface of CP upon CBR binding^{115,116}.

An important point relevant to cellular function is that the CBR-CP complex has weak capping activity ^{71,113}. The affinity of CP for the barbed end may have physiological relevance despite the ~100-fold decrease in affinity, when one considers the high concentrations of the reactants in the cytoplasm. The potential physiological role for regulation of capping by CBR is supported by the observation of similar lifetimes for the CARMIL-CP complex at the barbed end measured by TIRF microscopy *in vitro* (~10.5 s) ¹¹⁷, and that of CP at barbed ends in cells (~30 s) ¹¹⁸.

Box 2**Updated Phylogenetic Analysis of the CARMIL Family**

We present here an updated phylogenetic analysis of the CARMIL protein family. Vertebrates have three CARMIL genes; primitive eukaryotes, amoebozoans, and invertebrates have a single CARMIL gene; and fungi, plants and apicomplexans do not have one. The sea squirt is the earliest chordate with two CARMIL genes, probably resulting from a gene duplication event preceding the large genomic expansion that led to the appearance of vertebrates ¹¹⁹.

The three vertebrates CARMIL genes are highly conserved ^{72,113}. Although all isoforms have similar lengths and domain organizations, their protein sequences differ substantially, with each isoform characterized by a distinct set of similar residues. The isoform-specific sequences suggest the existence of isoform-specific functions in cells; indeed, such cases have been discovered, as discussed in the text.

Figure 1. Capping protein regulates actin filament dynamics during several cellular processes
 As predicted by the dendritic nucleation model, CP regulates Arp2/3 complex-dependent actin assembly at various cellular membranes, including lamellipodial protrusions, at sites of endocytosis and pinocytosis and associated with endosomal compartments undergoing fission and fusion. Capping protein also regulates assembly of actin filaments of filopodia, which can arise from dendritic actin networks.

Figure 2. Modes of CP Inhibition

A) No inhibition of barbed-end capping. CP binds to the barbed end of an actin filament via electrostatic interactions at Site 1 (basic patch) and hydrophobic interactions with the beta tentacle (Site 2). Capping protein is depicted as a heterodimer composed of an alpha (green) and beta (light blue) subunit; an actin filament barbed end is depicted in blue. B) Competition with elongation factors. Formins and Ena/VASP proteins compete with CP for binding to the barbed end of an actin filament. C) Steric inhibition. V-1 / myotrophin interacts with CP on its actin-binding surface (Site 1), sterically blocking the interaction of

CP with the barbed end. D) Allosteric Inhibition. CPI and / or CSI motifs bind CP in the stalk region of the CP heterodimer and induce a conformational change that promotes dissociation of CP from the barbed end. The capping protein structure is depicted as a heterodimer composed of an alpha (green) and beta (light blue) subunit.

Figure 3. CPI-motif proteins

A) Model of CARMIL domain organization and structure. The crystal structure of CARMIL1 residues 1-668 is shown as a dimer, mediated by the HD domain (light purple). Individual domains are labeled and colored as in panel B. B) common and diverse motifs of the CPI family of proteins. The CPI motif common to this family is highlighted in the dashed box (magenta). Common oligomerization domains are shaded light purple; PXXP motifs, cyan; PH domains, marine. Other domains are indicated. Binding partners for each CPI protein are indicated at downward arrows oriented toward the domain with which each

interacts. Regulatory interactions (dimerization and autoinhibition) are indicated by upward arrows. Amino acid residue numbering corresponds to human isoforms.

Figure 4. Cellular functions of CPI Motif Proteins

Diverse actin-dependent processes are regulated by CPI-motif proteins. CPI-motif proteins target CP to different cellular membranes and modulate actin assembly. CARMIL and CKIP-1 directly bind the plasma membrane via interactions of a PH domain. CARMIL may also target to membranes via interactions with some myosin-I isoforms. CD2AP is enriched at the slit-diaphragm in kidney podocytes, where it interacts with cortactin, nephrin, and podocin and helps to maintain slit diaphragm integrity. Fam21 is a component of the WASH

regulatory complex associated with sorting and recycling endosomes. The CP-binding activity of CAPZIP can be modulated by phosphorylation by MAP kinases.

Table 1
Diverse functions and binding partners of CPI family members

Protein	Subcellular localization	Functions	Binding Partners
CARMIL1	Leading edge/plasma membrane	Migration ^{71,72} ; Ruffling and macropinocytosis ^{72,74}	Myosin I ^{69,70,83,120,121} , CP ^{70,71,113,117,122} , Phospholipids ^{75,79} , Trio ^{72,123}
CARMIL2	Vimentin Intermediate Filaments	Motility/Polarity ⁷² /T-cell activation ¹²⁴	vimentin ⁷² , CP ¹²⁴
CARMIL3	unknown	<i>unknown</i>	<i>unknown</i>
CD2AP	Slit diaphragm, leading edge/plasma membrane	T-cell activation ^{104, 125} , Glomerular filtration ¹²⁶ , lamellipodial formation ¹²⁷	CD2 ¹⁰⁴ , Cbl-b/c ^{109,128,128,129} , p-130Cas ¹³⁰ , Cortactin ^{127,131} , Nephlin ¹³² , Podocin ¹³³ , Rab4 ¹²⁸ , AP-2 ¹³⁴ , PI3K ¹³⁵ , dendrin ¹³⁶ , ARAP3, p115Rho GEF, Hip1R, STAP1 ¹³⁷ , ASAP1 ^{137,138} , Rac1 ¹³⁹ , alpha-actinin 4 ¹²⁴ , actin ¹²⁴ , CP ^{125,140}
CKIP-1	Plasma membrane, nucleus	Cell morphology ^{86,94} , Muscle differentiation ^{141,142} , Apoptosis ⁸⁸ , Tumor growth ^{93, 143} , Bone formation ⁹² , T-cell activation ¹⁴⁴ , cytokine signaling ⁸⁹	CK2 ⁸⁴ , CARMA1 ¹⁴⁴ , HDAC4 ¹⁴⁵ , Rpt6 ¹⁴⁶ , Smurf1 ^{92,146} , CP ⁸⁶ , AP-1(c-Jun) ⁸⁸ , IFP35/Nmi ⁸⁹ , PI3K(ATM) ¹⁴⁷ , phospholipids ⁹⁴
CAPZIP	Plasma membrane	<i>unknown</i>	MAPKAP-K2/3 ¹¹²
Fam21	Recycling endosomes	Endosomal sorting ^{97,148}	CP ¹⁰⁰ , VPS35 ^{101,102,149} , FKBP15 ¹⁰¹ , WASH ⁹⁷ , Phospholipids ¹⁰⁰