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Capping protein: new insights into mechanism and regulation

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Temporal and spatial control of the actin cytoskeleton are crucial for a range of eukaryotic cellular processes. Capping protein (CP), a ubiquitous highly conserved heterodimer, tightly caps the barbed (fast-growing) end of the actin filament and is an important component in the assembly of various actin structures, including the dynamic branched filament network at the leading edge of motile cells. New research into the molecular mechanism of how CP interacts with the actin filament *in vitro* and the function of CP *in vivo*, including discoveries of novel interactions of CP with other proteins, has greatly enhanced our understanding of the role of CP in regulating the actin cytoskeleton.

The assembly of actin filament structures in eukaryotes is essential for numerous biological processes and requires precise coordination [1–5]. In some cases, actin filaments are organized into regular and stable structures that turn over relatively slowly [6]. In protrusive motile processes such as lamellipodia, however, the remodeling of actin filaments is rapid – on the order of seconds. In addition to lamellipodia, which consist of branched filament networks, the leading edge of motile cells contains filopodia, which comprise unbranched, parallel filaments [7]. To achieve this diversity of actin filament assembly and organization, and to regulate assembly spatially and temporally, the cell uses a host of regulatory proteins to control polymerization and to direct the assembly of filaments into higher-order structures [8,9].

Actin filaments are polar double-helical polymers of globular subunits (Figure 1) that are arranged head to tail (Figure 2) [10]. The filaments have two ends, referred to as barbed and pointed. Specialized proteins coordinate filament turnover and remodeling by binding to the ends to regulate the addition and/or loss of monomers [8,9,11]. The barbed end has higher association and dissociation rate constants for actin subunits than does the pointed end [10] (Figure 2), and thus dominates the dynamics of filament assembly. *In vivo* barbed ends can be created when new filaments are nucleated by the Arp2/3 complex [5] or formins [12–14]. New barbed ends are often oriented toward a membrane [15], such that polymerization pushes the membrane forward.

The actin cytoskeleton of a living cell is in a steady state far from equilibrium. Subunits constantly flux through the system in an inexorable coupling of assembly and disassembly. The mechanism of filament disassembly is not well understood, but it seems to depend on the 'aging' of filaments, which occurs as the ATP molecules in subunits hydrolyze (Figure 2), promoting subunit dissociation [16]. Moreover, actin-depolymerizing factor or cofilin (ADF/cofilin) binds cooperatively to aging filaments, promoting filament severing and the dissociation of subunits from the pointed ends [17]. Actin subunit flux and recycling are facilitated by actin-monomer-binding proteins such as profilin, thymosin β4 [1] and twinfilin [18]. In addition, as filaments age their barbed ends become capped, which helps to regulate subunit flux.

Here, we discuss research from the past few years – notably an X-ray crystal structure – that has provided new insight into the molecular details of the interaction of capping protein (CP) with the barbed end of the actin filament, and the role(s) of CP in the control and regulation of actin filament dynamics *in vivo*. In addition, we also discuss recent discoveries of several new binding partners and antagonists for CP that have suggested new mechanisms – direct and indirect – for modulating the capping activity of CP that enables cells to generate
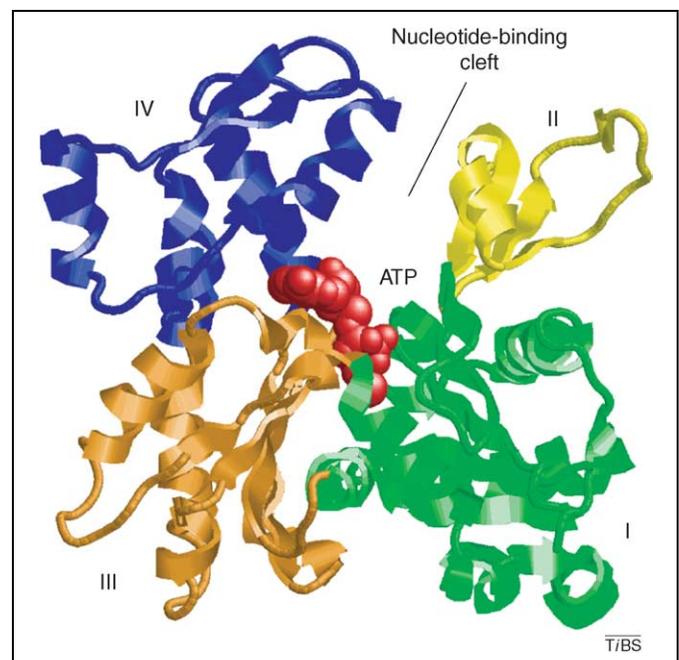
Figure 1. Structure of monomeric ATP-actin. Shown is a ribbon-chain trace of vertebrate α-actin (375 residues, ∼42 kDa) taken from the crystal structure of a complex between actin and DNase I [90]. Subdomains I (green), II (yellow), III (orange) and IV (blue), and the nucleotide-binding cleft with bound ATP in space-filling representation (red) are indicated.

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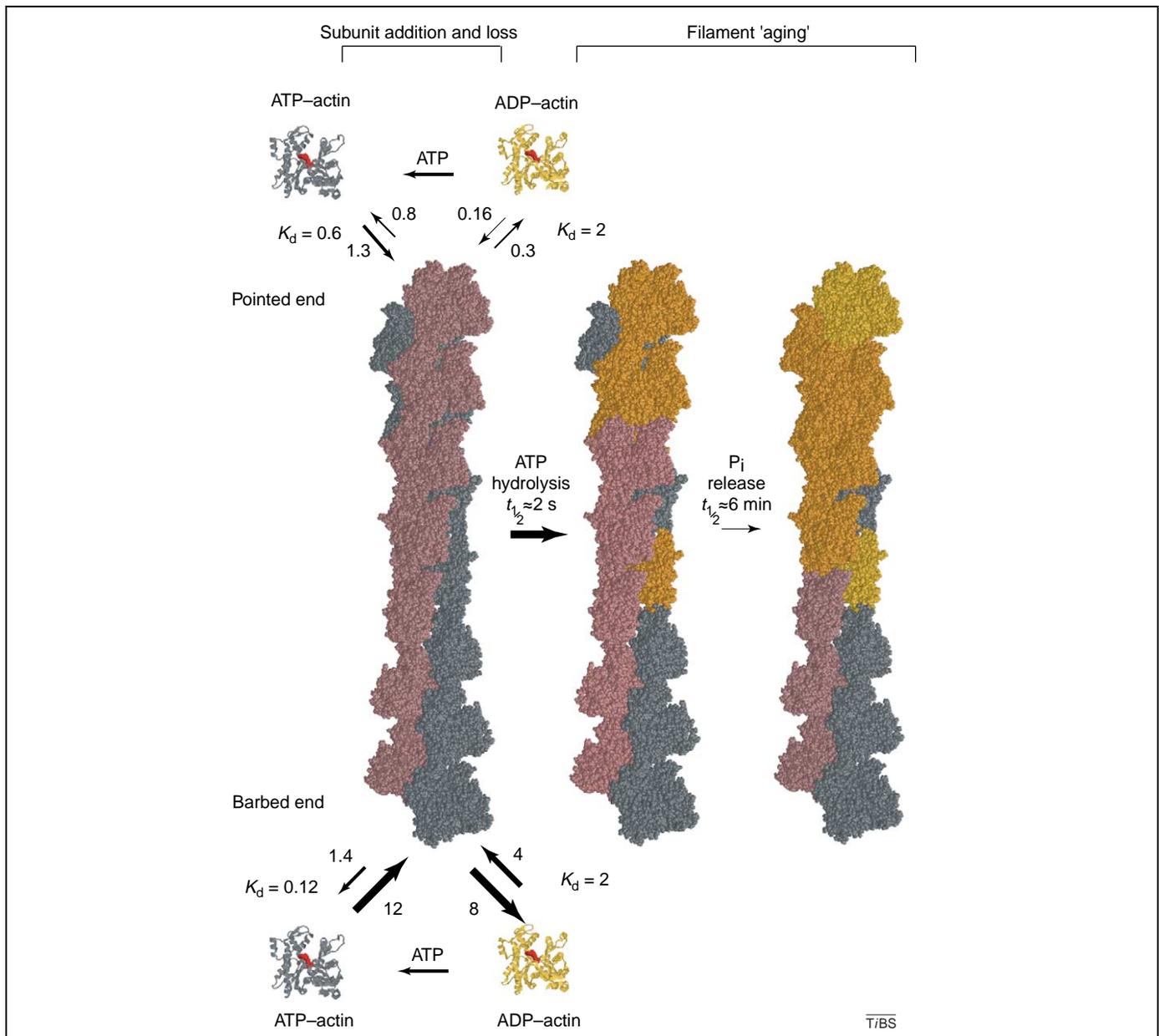
Figure 2. Salient features of actin filament structure and biochemistry. Three actin filaments are shown as space-filling representations of 14 actin subunits aligned in a right-handed two-stranded, 358-Å pitch helix, as described by Holmes *et al.* [91]. Each protofilament is colored differently (gray and rose) to highlight the helical structure of the filament and the staggered arrangement of the terminal subunits at the protofilament ends. Subunits in the filament are related to each other by a rotation of 166° and a translation up the filament long axis of 27.5 Å. Above the filament on the left are listed the association ($\mu M^{-1}\,s^{-1}$) and dissociation (s^{-1}) rate constants at the barbed and pointed ends for ATP-actin (gray) [90] and ADP-actin (yellow) [92] monomers, which are shown as ribbon-chain traces with the corresponding bound nucleotide in red. Also listed are the corresponding equilibrium dissociation constants (μM), which equal the critical concentrations for polymerization. The filaments in the center and on the right illustrate the process of ATP hydrolysis, which takes place on actin subunits on their incorporation into the polymer and which might function as an internal timer of filament 'aging' to trigger processes that disassemble actin filaments in cells. Shown are the rates of the fast hydrolysis of ATP bound to each subunit, which produces ADP•P_i-actin (gold), and the subsequent slow phosphate release of (P_i), which produces ADP-actin (yellow). A consequence of these different kinetic constants is that, at steady state, ATP-actin adds to the barbed end and ADP-actin dissociates from the pointed end. This leads to a slow treadmilling of subunits from the barbed end to the pointed end. *In vivo* regulatory proteins are required to enhance these processes, including the exchange of ADP for ATP, to account for the physiological rates of disassembly and filament turnover. Figure modified, with permission, from Ref. [5].

structurally and functionally distinct actin-filament architectures.

The CP family

Capping protein, which is also known as β-actinin, CapZ in skeletal muscle and Cap32/34 in *Dictyostelium*, is an αβ heterodimer with an α subunit of 32–36 kDa and a β subunit of 28–32 kDa. Highly conserved homologs of CP are found in nearly all eukaryotic cells, including fungi [19], higher plants [20] and various cells and tissues in vertebrates [19]. No proteins outside the CP family share substantial sequence similarity with CP. *In vitro*, CP caps actin filament barbed ends with high affinity, thereby preventing the addition or loss of actin subunits [19,20].

Tertiary structure of CP

The X-ray structure of chicken CP α1β1 reveals a pseudo-twofold rotational symmetry for the heterodimer [21]. Both subunits have extremely similar secondary and tertiary structures, despite lacking any amino acid
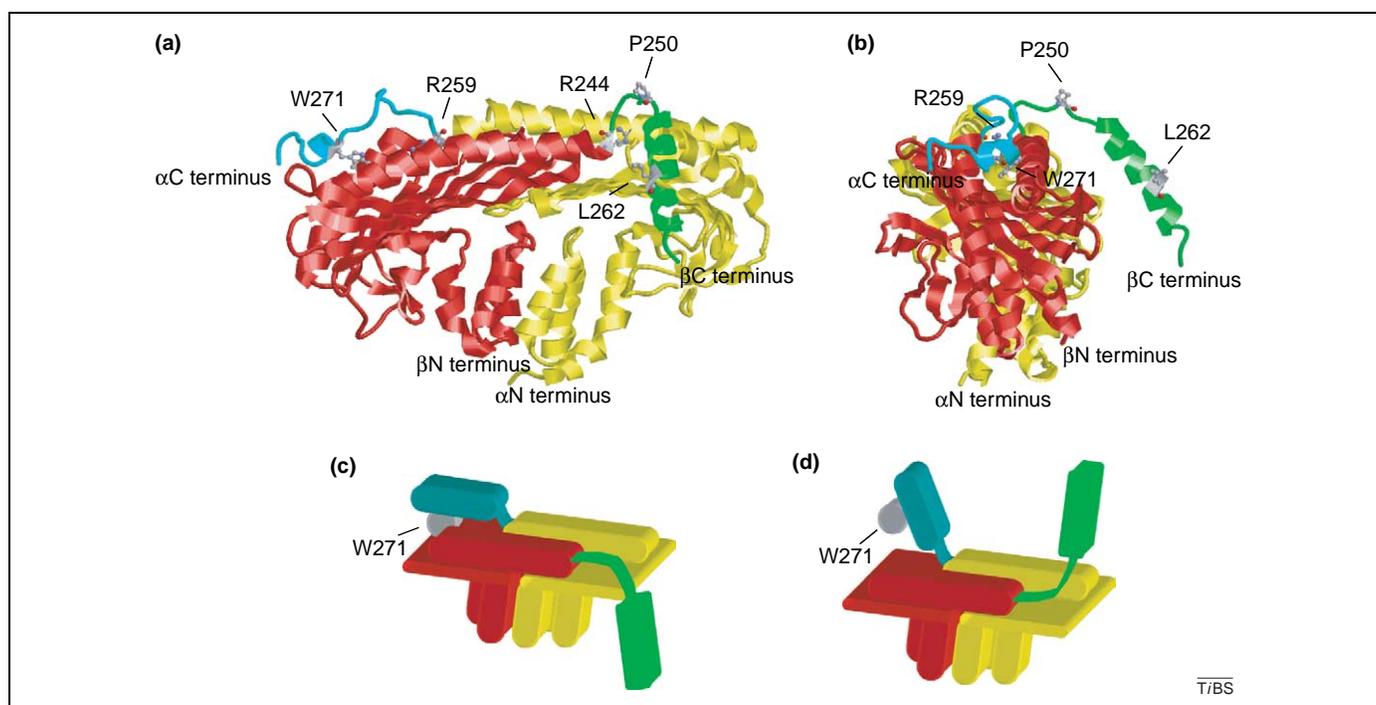
Figure 3. Three-dimensional structure of capping protein (CP). **(a,b)** X-ray crystal structure of chicken CP α1β1 [21]. The α subunit (yellow), its proposed C-terminal 28-residue tentacle (Arg259–Ala286; cyan), the β subunit (red) and its proposed C-terminal 34-residue tentacle (Arg244–Asn277; green) are shown as ribbon-chain traces. The positions of various important point mutations and structural boundaries are labeled and shown in ball-and-stick representation. In (b), the structure has been rotated 90° into the plane of the page to show how the C terminus of the β subunit extends from the body of the molecule. **(c,d)** Cartoons of the CP structure, highlighting differences between the orientations of the C-terminal regions observed in the X-ray structure (c), and the orientations predicted by the 'tentacle' model [21] (d). In the X-ray structure (c), the C terminus of the α subunit (cyan) lies along the upper surface of the heterodimer and is tethered by an apparent hydrophobic contact, to which the residue Trp271 (gray cylinder) contributes significantly, to the upper surface of the β subunit (red). The C terminus of the β subunit (green) is protruding from the body of the protein, in a similar orientation to that observed in the X-ray structure, and is apparently mobile and flexible. By contrast, the tentacle model predicts that in solution both of the C-terminal regions of CP are extended and mobile, as shown in (d).

sequence similarity (Figure 3a,b). No other protein structures in the Protein Data Bank resemble the CP structure.

Overall, CP resembles a mushroom. The stalk consists of the N-terminal regions of both subunits, organized into a six α-helix bundle (three α-helices from each subunit). The mushroom cap comprises a ten-stranded antiparallel β-sheet (five strands from each subunit), on top of which lie two long C-terminal α-helices (one helix from each subunit) running perpendicular to the β-sheet strands (Figure 3a,b). The extreme C terminus of the β subunit is a four-turn amphipathic α-helix that protrudes out from the body of the protein. The C terminus of the α subunit also contains an amphipathic α-helix; however, this helix is folded down onto the surface of the protein in an apparent hydrophobic contact [21] (Figure 3a,b). A homology model of budding yeast CP, based on the structure of chicken CP, yields a remarkably similar structure [22], which is not surprising given the high sequence similarity across organisms [19].

The twofold rotational symmetry of the CP structure, coupled with the fact that one molecule of CP binds to a barbed end comprising two actin subunits (Figure 2), has inspired the 'tentacle' model, which predicts that CP caps actin filaments through the following two properties [21]. First, the C-terminal regions of both CP subunits bind actin. Second, the C-terminal regions are mobile, extended and flexible in solution, acting like tentacles to reach out and grab the barbed end (Figure 3c,d). Despite the fact that both CP and the actin filament possess a twofold

rotational symmetry, there is significant mismatch between them to suggest that an unusual mechanism is involved in the interaction of CP with the barbed end. The second prediction of the tentacle model – namely, that the C-terminal actin-binding regions of CP are mobile and flexible – might reconcile this symmetry mismatch.

A mechanism for barbed end capping by CP

The tentacle model has been tested in structure–function analyses of chicken and budding yeast CP [22,23], using purified mutant proteins expressed in bacteria. On the basis of functional assays for barbed end assembly and disassembly *in vitro*, the C-terminal regions (~30 amino acids) of both subunits have been found to be necessary for binding actin, in support of the first main prediction of the tentacle model.

For chicken and yeast CP, removal of both proposed tentacles caused complete loss of actin-binding activity [22,23]. Loss of the C-terminal 28 amino acids from the α1 subunit reduced the capping affinity by 5,000-fold and the capping on-rate by 20-fold in chicken CP [23]. By contrast, removal of the C-terminal 34 amino acids from the chicken CP β1 subunit reduced the affinity by 300-fold with no effect on the capping on-rate [23]. Qualitatively similar results have been obtained for budding yeast CP, for which the C terminus of α subunit was found to be much more important in terms of capping affinity and kinetics [22]. In addition, replacing a single conserved amino acids (both hydrophobic and hydrophilic) in the

C-terminal regions also decreased capping affinity by between 10- and 150-fold [22,23] (Figure 3a,b). The stability and global structure of the CP truncation and point mutants appeared unchanged [22,23], suggesting that the amino acids deleted and substituted are indeed functionally important for the capping activity of CP.

In addition, the C-terminal 28 or 34 amino acids of the chicken CP β1 subunit (Figure 3a,b), prepared as glutathione *S*-transferase fusion proteins or synthetic peptides, were sufficient to cap the ends on their own with affinities remarkably similar to the capping affinity of the chicken CP α1 subunit C-terminal deletion mutant [23]. Thus, CP seems to use its two extreme C-terminal regions as independent actin-binding sites to cap the barbed end.

The hydrophobic sides of the amphipathic helixes in the C-terminal regions are candidates for the actin-binding sites, especially if both these regions are mobile and flexible in solution. For some actin-binding proteins, including gelsolin [24] and vitamin-D-binding protein [25], an α-helix with a patch of hydrophobic residues contributes to actin binding. In addition, hydrophobic patches are found on the actin surfaces at the barbed end of the filament, such as in the cleft between subdomains I and III. Consistent with this hypothesis, replacing single conserved residues on the hydrophobic faces of the amphipathic α-helices in the C-terminal sequences of both subunits, including Trp271 in the α1 and Leu262 in the β1 subunit of chicken CP, decreased the capping affinity by a significant (between 10- and 90-fold) amount [22,23].

For the α subunit, however, other data argue against the hydrophobic region being in direct contact with actin. In the crystal structure of chicken CP, the C terminus of the α1 subunit is folded down and the hydrophobic side of its amphipathic helix is oriented downwards, making hydrophobic contacts with the body of the heterodimer [21] (Figure 3a–c). This region had higher temperature factors than other regions of the protein in the crystallography study, suggesting that it might be mobile in solution [21], but studies of CP binding to the protein S100B argue otherwise [26].

S100B is a ubiquitous, symmetric homodimer of 21.5 kDa that requires a Ca^{2+}-dependent conformational change to enable it to bind its target proteins, which are often substrates of kinase-dependent phosphorylation reactions [27]. S100B has been found to bind tightly (dissociation constant, $K_d \approx 0.2$–1 μM) to a 12-residue peptide by phage-display studies, and the sequence of this peptide is present in the C-terminal region of the α subunit of vertebrate CPs [28]. In an NMR solution structure of S100B bound to the 12-residue peptide, the hydrophobic residues of the amphipathic α-helix in the peptide contact a hydrophobic binding pocket in S100B [29]. In particular, the tryptophan residue corresponding to Trp271 in the chicken CP α1 subunit is a central component of this hydrophobic interaction. In the CP crystal structure, Trp271 is a central component of the apparent hydrophobic contact between the C-terminal region and the surface of the main body of the protein (Figure 3a–c).

If the C terminus of the α subunit is flexible and extended in solution (compare Figures 3c and 3d), then S100B should bind to whole wild-type CP in solution. No such interaction has been observed in several *in vitro* physical binding and functional assays carried out with high concentrations of protein under conditions in which S100B binds tightly to the 12- and 28-residue α-subunit peptides and to denatured CP [26,28]. Relatively high concentrations of the non-ionic detergent Triton-X100 did allow S100B to bind weakly to CP without denaturing either protein, however, and this binding inhibited the capping activity of CP *in vitro* [26]. Thus, for the C terminus of the α subunit, these studies contradict the second main prediction of the tentacle model – namely, that the actin-binding regions are flexible and mobile, extending away from the body of CP to bind actin.

Nevertheless, we can envisage a scheme in which the orientation of the C terminus of the α subunit in free CP changes on binding to the end of the actin filament. Such a structural rearrangement might allow the highly conserved hydrophobic residues in this region, which are apparently occluded from the solution in free CP, to make direct contact with the barbed end. In addition, it should be noted that many of the residues that constitute the hydrophilic surface of the α-subunit C terminus are highly conserved [19], and these residues might be also important for the direct interaction between the α subunit and actin.

Because S100B binds to a peptide derived from the α subunit of vertebrate CP, it has been speculated that S100B might target or regulate CP in cells [28]. This seems unlikely, however, given the lack of binding between S100B and whole wild-type CP [26], the lack of colocalization of the two proteins in cells [30,31], and the high Ca^{2+} concentrations (~ 2 mM) required for S100B to bind to the isolated α-subunit peptides *in vitro* [26,28].

For the β subunit of CP, no experiments have tested the prediction of mobility and flexibility; however, its C terminus is extended and surrounded by solvent in the crystal structure [21] (Figure 3), suggesting that it is probably mobile and flexible in solution. Structure–function analyses also suggest that the two C termini have different roles in functionally capping the barbed end [22,23,26]. The capping on-rates for α-subunit C-terminal deletion mutants and a chicken CP α1-subunit Arg259Ala mutant (Arg239Ala in budding yeast) were decreased by 10–20-fold as compared with those of wild-type CP [22,23]. Arg259 is highly conserved and its side chain protrudes inward to make apparent ionic and hydrogen bond contacts with residues in the body of the protein on the β subunit [21,22] (Figure 3a,b). Arg259 might therefore influence the structure and/or the orientation of the actin-binding C terminus of the α subunit.

By contrast, the CP β-subunit C-terminal deletion mutants had normal capping on-rates [22,23]. Furthermore, the activity of a chicken CP variant carrying a Arg244Ala mutation in the β1 subunit (Arg244 is highly conserved in the C terminus of the β subunit and is the structural analog of Arg259 in the α subunit; Figure 3a,b), was indistinguishable from that of wild-type CP in functional assays *in vitro* [23]. Thus, the apparently more constrained C terminus of the α subunit might provide some form of specificity for the initial interaction of CP

with the barbed end, whereas the apparently more mobile and flexible C terminus of the β subunit might provide cap stability.

The observation that a CP mutant containing only a single actin-binding region and the isolated C-terminal 28 or 34 amino acids of the β subunit are both able to cap the barbed end suggests that each C-terminal region might bind to the filament at an interface between the actin subunits (Box 1 and Figure 4). The precise molecular details of this interaction and the exact binding sites on the barbed end remain, however, to be addressed experimentally.

Box 1. How might CP cap the barbed end of an actin filament?

The actin filament can be viewed as a two-stranded long-pitch helix, in which the ends of the two protofilaments are staggered. Functionally capping a filament end requires a decrease in both subunit addition and subunit loss. To inhibit subunit addition, a protein could bind anywhere near the end and sterically block the access of free monomer to the end. Alternatively, the protein might change the conformation of the actin subunits at the filament end, such that free actin monomers are unable to add.

Capping also requires the inhibition of actin subunit loss from the end. Here, the key factor is a decrease in the off-rate constant for the terminal actin subunit at the end, which requires an increase in the number and/or strength of the binding interactions between the terminal subunit and the other subunits of the filament. A protein that caps could bind to two actin subunits – the ones at each end of the protofilaments – thereby increasing the binding energy between the terminal subunit and the filament. Dissociation of a capping molecule bound to these two subunits from the barbed end of a filament would involve breaking more bonds than would dissociation of a single actin subunit.

A model (termed here the 'dimer-binding model') has been proposed for barbed end capping by the gelsolin family of proteins, in which domain 1 and domain 4 from gelsolin each bind a separate actin subunit – related to each other across the short-pitch helix of the filament – at a site between subdomains I and III on the actin monomer [24]. If CP were to bind in a similar fashion, with each C terminus contacting only one actin subunit at the barbed end, both C-terminal regions would probably need to be extended.

Recent structure–function studies [22,23,26] lend credence, however, to an alternative mechanism in which CP binds at the interface between the terminal actin subunit and the adjacent actin subunit at the end of the other protofilament, thereby increasing the number of bonds that connect the terminal actin subunit to the filament. CP lacking either one of its two C-terminal actin-binding regions can still cap the end with an affinity that is significantly higher than that of an actin subunit for the barbed end, owing to a decrease in the off-rate constant [22,23].

It seems unlikely that capping by CP could occur by the interaction of one actin-binding region with only a single terminal actin subunit, as predicted by the dimer-binding model. In addition, the C terminus of the α subunit of CP might remain immobile and constrained to the upper surface of the CP protein body [26], even on interaction with the filament, and such an orientation is difficult to reconcile with the dimer-binding model. Finally, 28- or 34-residue peptides derived from the C terminus of the chicken CP β1 subunit can cap the barbed end [23], suggesting that the binding site for these peptides is an actin–actin subunit interface. These small peptides would seem to be too short to bind simultaneously to the two terminal actin monomers that are related to each other across the short pitch helix.

But both models might turn out to have some relevance. The C-terminal region of one CP subunit might bind at a subunit interface, whereas the other might not. Furthermore, the binding interaction at an actin–actin subunit interface might comprise more interactions from one actin subunit than from the other.

Physiological significance of CPs actin binding activity

The significance of the actin-binding activity of CP has been tested *in vivo* in budding yeast by determining the ability of a set of CP mutants to rescue CP-null mutant phenotypes, such as decreased actin polarization [22]. The ability of these mutants to rescue *in vivo* was found to correlate well with their ability to cap *in vitro* [22]. In addition, localization of the mutant CPs to cortical actin patches (motile cortical actin structures that are involved in polarized growth in yeast) *in vivo* correlated well with their ability to cap *in vitro*. Thus, actin capping seems to be necessary for CP to function and to localize *in vivo* [22]. A likely scheme is that actin filaments in patches are nucleated by the Arp2/3 complex, grow for a while, and then become capped by CP.

In addition, CP might have other important functions *in vivo*, such as those mediated by a direct interaction with twinfilin [18,32], a ubiquitous conserved protein that binds and sequesters actin monomers [18]. Twinfilin also binds directly to CP [32], and in budding yeast the physiological function of twinfilin seems to require twinfilin binding to both actin and CP [22,32]. Twinfilin localizes at the actin patch in wild-type yeast, but not when CP is absent or does not itself localize to the actin patch [22,32].

In *Drosophila*, CP is important for development and morphogenesis. Loss-of-function mutations in the β subunit are lethal at an early larval stage [33]. When CP function was reduced in the *Drosophila* bristles, which depend on bundles of actin filaments for their morphology, the actin became disorganized and bristles developed with abnormal shapes [33]. Mutations in profilin suppressed the bristle morphology phenotypes of CP mutants [34], and overexpression of profilin in the bristle had effects similar to those observed in CP mutants [34]. These results suggest that profilin and CP have antagonistic functions in actin assembly in the bristle [34], although the biochemical nature of these functions is not yet clear.

The role of CP in dendritic nucleation

Capping protein is an important component of the dendritic nucleation model that has been proposed to account for actin polymerization and the generation of protrusive force at the leading edge of cells [5]. In this model, nucleation of actin is driven by activation of the Arp2/3 complex [35,36], which creates filament branches and free barbed ends. Actin subunits add to the free barbed ends, which grow and push the plasma membrane outward. Over time, the barbed ends become capped by CP (Figure 5). Filament aging leads to breakdown of the actin filament network and depolymerization of the filaments. *In vitro*, the addition of CP to actin polymerization reactions along with active Arp2/3 complex increases the degree of branching and shortens the filaments [37].

The dendritic nucleation model is also proposed to account for the reconstitution of actin-based motility in synthetic reconstitution systems *in vitro*. Actin polymerization can drive the movement of objects in solutions containing only CP, active Arp2/3 complex and ADF/cofilin [38]. The reason why CP should be required in this system might be found in the 'funneling' hypothesis of Carlier and
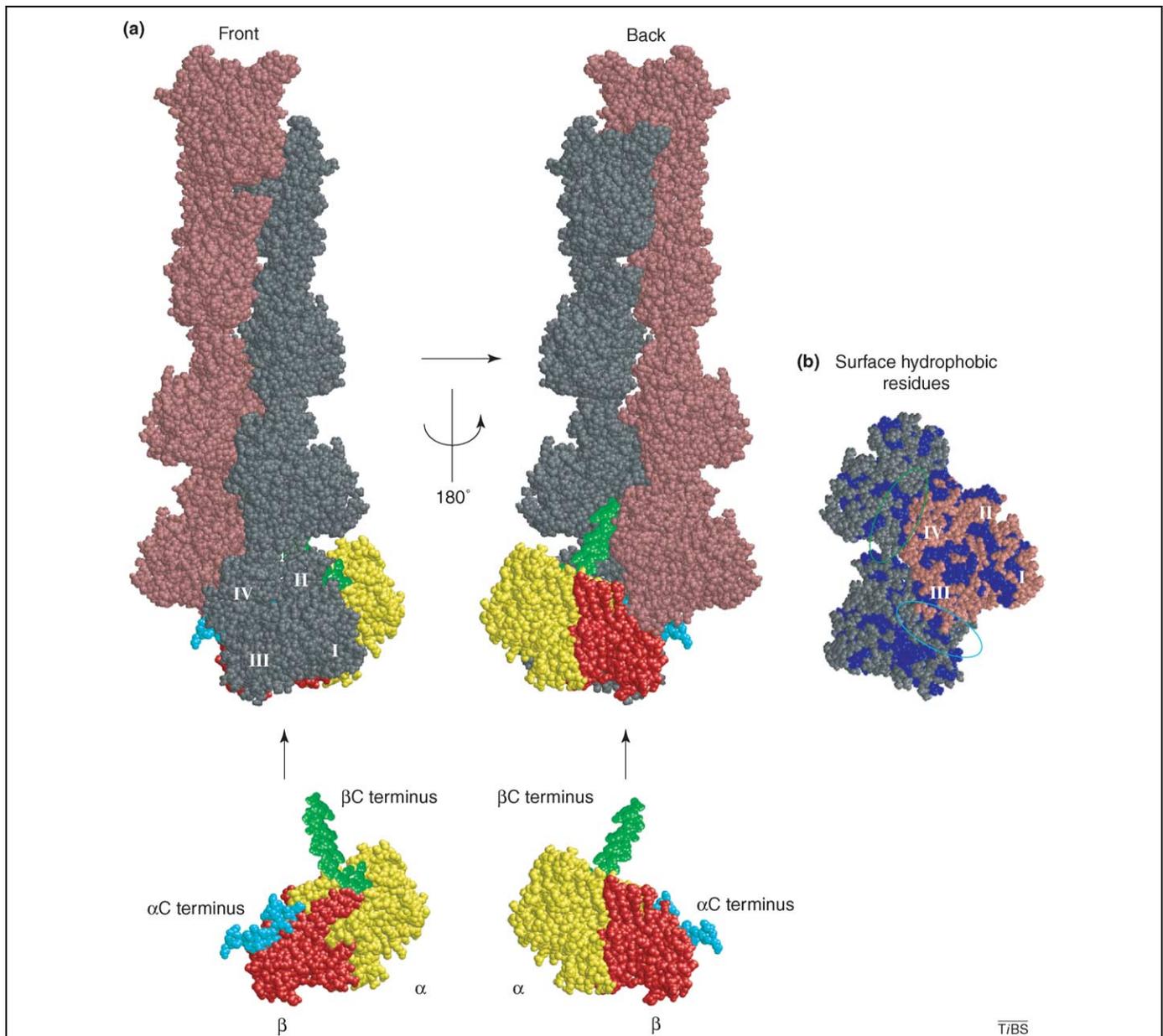
Figure 4. Subunit interface binding mechanism showing how capping protein (CP) might cap the barbed end. **(a)** This speculative model, in which each C-terminal region of CP makes contact with two or three actin subunits, shows how CP might cap the filament barbed end by binding at an interface between the two protofilaments. An actin filament with eight subunits is viewed from the front and, by a rotation of 180° about the filament long axis, from the back. The filament is illustrated in space-filling representation with each protofilament colored differently (gray and rose). Subdomains I–IV of actin are indicated on the terminal subunit in the front view orientation. The X-ray structure of CP is shown in space-filling representation at the same scale as the actin filament and from the same two relative orientations. The C terminus (green) of the β subunit (red) has been manually 'flipped up' from its orientation in the X-ray structure, to illustrate its apparent mobility and flexibility. The C terminus (cyan) of the α subunit (yellow) remains folded down on top of the body of the heterodimer. The whole CP molecule has then been manually docked onto the barbed end. Because the CP structure is a rigid body, the assignment of these binding sites is essentially arbitrary; however, the binding sites on actin for both of the CP C-terminal regions are assigned such that each C terminus makes contact with a surface at a subunit interface that has hydrophobic character. With the C terminus of the α subunit folded down on the top of the body of the protein (as shown), the hydrophilic residues on the surface of this region would be in contact with actin. On binding to the filament, however, it is conceivable that the conformation of the C terminus of the α subunit could change to allow occluded hydrophobic residues, such as Trp271, to interact directly with actin. **(b)** Surface hydrophobic residues (blue) on the three terminal actin subunits at the barbed end, viewed from the same perspective as the back orientation of the filament in (a). The essentially arbitrarily assigned binding sites of the α (cyan oval) and β (green oval) C-terminal regions of CP and subdomains I–IV on the foremost actin subunit are indicated. There are other regions of significant hydrophobic character at the barbed end that might also be candidates for interaction with CP.

Pantaloni [39]. In this hypothesis, the actin cytoskeleton is in a steady state, far from equilibrium, and actin subunits continuously flux onto free barbed ends. To create movement of the object, the addition of actin subunits must be confined to the newly created barbed ends near the object. In addition, the growing filaments need to be short and branched to sustain the pushing force, because long unbranched filaments bend easily [40]. To accomplish this, barbed ends are created near the membrane by activated Arp2/3 complex and then CP caps these ends in a stochastic manner. Thus, older barbed ends are capped, newer ones are free, the filaments are kept short, and subunits are available for nucleation to create new branches with activated Arp2/3 complex. The flux of polymerizing actin subunits is thereby 'funneled' to the region near the object or membrane [39] (Figure 5).
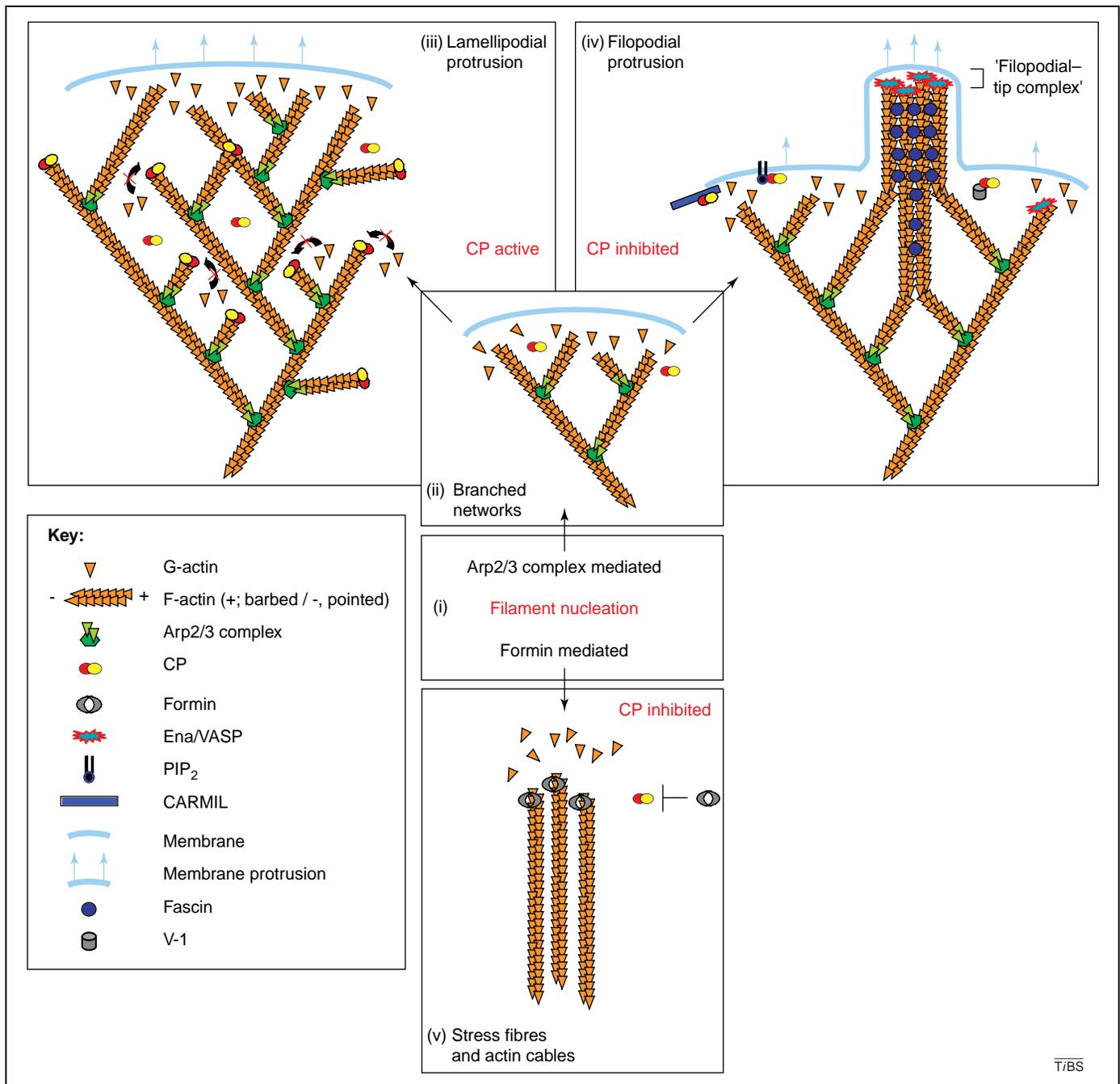
Figure 5. How modulating the barbed end capping activity of capping protein (CP) might influence the generation of different actin filament architectures. (i) Cells can nucleate new actin filaments by two main mechanisms mediated either by the Arp2/3 complex or by formins. (ii) Activation of the Arp2/3 complex leads to the generation of branched actin filament networks, and modulation of CP activity could lead to the generation of structurally and functionally distinct actin filament architectures from the same underlying filament network. (iii) In lamellipodial protrusion, capping results in the generation of a highly branched network of short filaments. New barbed ends nucleated by the Arp2/3 complex polymerize and push the membrane forwards. Older ends further back in the network are capped rapidly by CP, resulting in the 'funneling' of subunit flux from disassembling filaments further back in the cell to the free ends near the membrane. (iv) In filopodial protrusion, filopodial extensions are generated from the underlying Arp2/3-complex-mediated network. The inhibition or antagonism of CP by molecules such as phosphatidylinositol (4,5)-bisphosphate (PIP$_2$), V-1, CARMIL or Ena/VASP (as a component of a 'filopodial tip complex') results in free barbed ends that persistently elongate. This generates longer filaments that can be crosslinked and bundled (by proteins such as fascin) into parallel arrays. (v) In stress fiber and actin cable formation, formins nucleate actin filaments and antagonize the interaction of CP with the barbed end. This results in more persistent elongation and the generation of parallel unbranched bundles of filaments. This actin architecture is probably important for generating the cytokinetic actin ring, actin cables and stress fibers, all of which lack CP or have CP distributed very sparsely among the filament arrays.

The dendritic nucleation model and the funneling hypothesis are beginning to be tested *in vivo*. Kinetic rate constants for CP capping measured *in vitro* are consistent with the proposed *in vivo* functions. In vertebrate cells, the cytoplasmic concentration of CP is about 1 μM [11] and the on-rate for CP binding the barbed end is 2–7 μM^{-1} s^{-1} [23,41], suggesting that free barbed ends would become capped with a half-time of about 1 s.

Allowing a barbed end to grow for roughly 1 s at a rate of around 0.3–3 μm s^{-1} (an estimate that assumes an actin monomer concentration of 10–100 μM) could account for the lengths of filaments seen in branched networks at the leading edge of cells [42,43].

Actin dynamics might be faster in yeast than in vertebrates. Yeast CP caps barbed ends roughly 10-fold less tightly than does chicken CP, owing to a higher

off-rate constant [22]. Therefore, the half-life for uncapping a capped actin filament in yeast should be about 20–60 s, which is much less than the value of 30 min estimated for vertebrates *in vitro* [23,41]. *In vivo*, however, there are regulated mechanisms for the active disassembly of filaments (e.g. filament severing and enhancement of subunit dissociation from the pointed ends mediated by the ADF/cofilin family of proteins [17]) that indicate that filament turnover in non-muscle cells is likely to be much more rapid – possibly on the order of 30–60 s [44].

In studies in *Dictyostelium,* changes in the levels of CP resulted in changes in resting and chemoattractant-induced actin assembly, consistent with the capping of barbed ends by CP [45]. Decreased expression of CP caused actin filaments to be longer and more bundled. During cell migration, cells overexpressing CP moved faster, whereas those underexpressing CP moved slower, than control cells [45]. These changes are consistent with the proposed funneling role of CP in the dendritic nucleation model.

In yeast, the dendritic nucleation model seems to be valid for cortical actin patches in some respects but not others. The Arp2/3 complex is important for actin patch assembly and motility [46], and actin polymerization is important for patch movement [47], as predicted by the model. By contrast, some results with CP [22] and cofilin [48] run counter to the model, and cofilin might not be required for patch movement in budding yeast [48]. The funneling hypothesis of the role of CP in dendritic nucleation predicts that an absence of CP should cause a decrease in actin assembly at patches and a decrease in patch movement, owing to the increase in actin assembly at barbed ends at other cellular locations. Complete loss of the actin-capping activity of CP caused an increase in both the numbers of free barbed ends and the amount of F-actin at patches [22]; however, actin patches moved at normal speeds in both CP actin-binding and CP-null mutants [22].

A potential explanation for this discrepancy is that yeast cells might not have a substantial pool of barbed ends, other than those in patches and perhaps at cable ends, in the cytoplasm in general and thus might not need CP to cap those ends. Alternatively, another factor or factors might cap barbed ends in yeast. Aip1 has been reported to cap barbed ends in complex with cofilin in *Xenopus* and yeast [49,50]. However, an *aip1 cap2* double null mutant in yeast shows only a minimal synthetic effect in terms of growth [51]. No other types of barbed end capping protein have been found in yeast by sequence or biochemical analyses.

A role for CP in stable arrays of actin filaments *in vivo*

Actin filaments are far more stable in some situations than they are at the cortex of metazoan or yeast cells. In the sarcomere of striated muscle [6], for example, the actin-based thin filaments remain in place even though their subunits exchange and turn over [52]. CP seems to cap the barbed end of every thin filament of the sarcomere, possibly helping to anchor the filament end to the Z-line and to prevent the growth of that filament into the neighboring sarcomere [53,54]. Expression of a mutant CP with decreased actin-binding ability (β1 L262R) was found to disrupt the sarcomere in hearts of transgenic mice [55], as predicted. Furthermore, expression of the non-sarcomeric CP isoform, β2, had similar effects on sarcomere assembly, indicating that the β2 non-sarcomeric isoform cannot substitute for the β1 sarcomeric isoform [55].

On the basis of some intriguing circumstantial evidence, CP might also help to attach barbed ends to membranes. Actin filament barbed ends seem to be attached to the plasma membrane in the microvilli of epithelial cells, and CP might mediate this attachment [56]. In plasma membranes in general, some barbed ends might be stably attached to the membrane, on the basis of the observation that some actin filament ends remain attached to purified *Dictyostelium* membranes after treatment with the myosin fragment S-1 [57]. CP binds tightly to the membrane-associated protein CD2AP, which binds the integral membrane protein CD2 [58]. CD2AP colocalizes with CP, the Arp2/3 complex and cortactin at dynamic foci of actin assembly in the lamella of fibroblasts [59]. Expression of a truncated variant of CD2AP was found to inhibit T-cell polarization, a process that involves actin assembly [60], and the CD2AP knockout mouse has a defect in the podocytes of the kidney glomerulus [61,62], which have actin-rich foot processes.

Potential mechanisms for regulating CP
Direct regulation of CP

Several molecules influence the actin-binding ability of CP, either by binding directly to CP or by binding to filament barbed ends and thereby preventing CP from binding (Figure 5). Phosphatidylinositol (4,5)-bisphosphate (PtdIns$[4,5]P_2$) [19] and the proteins V-1 [63] and CARMIL [64,65] bind directly to CP and inhibit its ability to bind actin. PtdIns$(4,5)P_2$ rapidly and reversibly inhibits CP and uncaps barbed ends *in vitro* [19]. During platelet activation, PtdIns$(4,5)P_2$ is implicated in the removal of CP from capped actin filaments at the onset of actin polymerization [66].

V-1, also known as myotrophin, is a small protein of 12 kDa consisting of two complete and two incomplete ankyrin repeat motifs that has potential roles in neural development and cardiac hypertrophy [67]. *In vitro*, purified V-1 binds to CP with moderately high affinity ($K_d \approx 0.12$ μM) and a 1:1 stoichiometry [63]. In addition, V-1 has been found to inhibit the interaction of CP with actin filaments *in vitro* in a dose-dependent manner [63]. V-1 is expressed in the cytoplasm at relatively high levels in wide range of cells and tissues [68]. Expression of V-1 in skeletal muscle cells decreases during differentiation in culture and *in vivo*, and increases on differentiation in both individuals affected with Duchenne muscular dystrophy and the *mdx* mouse model [69]. The hypothesis that V-1 inhibits CP *in vivo*, with consequences for actin assembly, remains, however, untested.

Coactosin, a protein of about 16 kDa that is associated with the *Dictyostelium* and metazoan actin cytoskeleton, also seems to inhibit the capping activity of CP *in vitro* [70]. Whether coactosin has a direct effect on CP or an indirect effect on actin is not known.

CARMIL is a large (∼1050–1450 amino acids) scaffold protein found in all metazoans [64]. *Dictyostelium* CARMIL binds CP, the Arp2/3 complex and a class I myosin with a Src homology SH3 domain [64]. In *Dictyostelium*, CARMIL localizes to both actin-rich cellular extensions in chemotaxing cells and sites of macropinocytosis [64]. *Dictyostelium* mutants lacking CARMIL have severely abnormal chemotactic responses and reduced rates of fluid-phase pinocytosis [64]. CP binds to a region of about 200 amino acids in the proline-rich C terminus of *Acanthamoeba* CARMIL with submicromolar affinity [65]. CARMIL purified from *Acanthamoeba* is accompanied by CP in a 1:1 molar stoichiometry [65]. The affinity of binding and the cellular concentrations, about 1 μM for both CP and CARMIL, suggests that a substantial amount of CP–CARMIL complex might exist *in vivo*. CARMIL is particularly interesting because, in addition to binding to the major barbed end capper, it can bind a barbed end nucleator, the Arp2/3 complex, and a barbed end directed motor, myosin I [64]. The physiological significance of these various interactions remains to be determined.

Indirect regulation of CP

Capping protein can be also regulated indirectly by proteins that bind the barbed end of the actin filament. In one of the first studies to suggest such a mechanism, the addition of GTPγS-activated Cdc42 to neutrophil cell extracts induced the polymerization of actin filaments with barbed ends that were protected from CP [71]. The physiological significance and mechanism of this effect remain to be determined; however, recent work on formins has provided great insight into an indirect mechanism. Formins are a conserved superfamily of large auto-inhibited [72], multidomain proteins that are characterized by the formin homology domains FH1 and FH2 [12–14]. Formins nucleate actin filaments from monomers *in vitro*, creating single unbranched filaments that can grow at their barbed and pointed ends [73,74]. Kinetic analysis suggests that formins stabilize actin dimers and trimers during the nucleation process [75].

Formins bind the barbed ends of filaments and their presence functionally caps the barbed end; however, the extent of this inhibition is often only partial [75–77]. Actin subunits can still add at reduced rates, even when ends are completely saturated with formin, which has inspired the term 'leaky' or 'processive' cap [76–79]. Leaky cappers are different from weak cappers, which inhibit actin addition completely but bind with low affinity. Notably, kinetic analysis argues that formin molecules might remain bound to a barbed end as the end adds actin and grows [76].

Some formins also prevent the addition of CP to the barbed end. The FH1 and FH2 domains of budding yeast Bni1p [76] and mouse FRLα-C [77], and the FH1 domain of mouse mDia1 [77], allow barbed ends to grow even in the presence of CP. The combined properties of leaky capping, surfing with the barbed end, and inhibiting capping by CP predicts that *in vivo* formins will allow barbed ends to grow and become long, unbranched filaments. Thus, the effect of formins is in marked contrast to that of the Arp2/3 complex, which creates branched networks of short filaments (Figure 5).

In budding and fission yeast, formins are required for *in vivo* assembly of actin cables in growing cells and actin rings in dividing cells [73,80,81]. These structures presumably contain unbranched filaments. As predicted, cables and rings do not depend on the Arp2/3 complex [82] and do not seem to contain CP [83]. The fine structure of actin cables and the manner in which they disassemble support a model in which cables are composed of several both short and long overlapping actin filaments [84]. How formins might actually regulate and generate such filament arrays is not clear. In addition, the observation that CP-null mutants in budding yeast show a loss of actin cables implies that CP has a role in promoting cable formation rather than in antagonizing it [85]. Even less is known about the function of formins *in vivo* in vertebrates, although the mouse formin mDia1 localizes to structures lacking CP, including stress fibers and cytokinetic rings [86] (Figure 5). The roles of both formins and CP in the formation of actin structures such as cables and stress fibers are currently not well understood.

Members of the Ena or vasodilator-stimulated phosphoprotein (Ena/VASP) family [2] also seem to antagonize the capping of barbed ends by CP. VASP has been found to associate with free barbed ends *in vivo* [87]. Targeting VASP to the plasma membrane in fibroblasts resulted in a decrease in cell migration and lamellipodial extension, and the actin filaments in these lamellipodia were relatively long and ran parallel to the membrane [87]. Conversely, depletion of VASP from lamellipodia resulted in an increase in cell movement, and the actin filaments were shorter and more branched [87]. When purified proteins were studied *in vitro*, VASP inhibited CP in barbed end actin assembly assays [87]. Thus, Ena/VASP proteins might interact with the barbed end of filaments to prevent or to delay capping by CP. In cells, VASP also localizes to the tips of filopodia, which contain a bundle of unbranched filaments [88]. Filopodia seem to form from the lamellipodial network of branched filaments [89] (Figure 5). Where this happens, a 'filopodial tip complex' has been proposed to prevent capping and to enable filaments to grow long [89] (Figure 5).

Concluding remarks

Recent research has increased our understanding of how CP interacts with actin filaments at the molecular level, and new potential mechanisms for regulating CP have been suggested. The role of CP in actin assembly and cell motility *in vivo* is beginning to be elucidated, especially in connection with the leading edge of motile cells, the cortical actin patches of yeast and the actin bundles of *Drosophila* bristles. But many issues remain unresolved regarding the regulation and function of CP in actin assembly and motility in cells.

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