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Regulating actin-filament dynamics *in vivo*

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The assembly and disassembly (i.e. turnover) of actin filaments in response to extracellular signals underlie a wide variety of basic cellular processes such as cell division, endocytosis and motility. The bulk turnover of subunits is 100–200 times faster in cells than with pure actin, suggesting a complex regulation *in vivo*. Significant progress has been made recently in identifying and clarifying the roles of several cellular proteins that coordinately regulate actin-filament turnover.

THE DYNAMIC NATURE of actin filaments allows cells to respond to extracellular signals by moving, changing shape and translocating intracellular organelles. Such processes are critical to the development and function of a multicellular organism, but are also important in the disease state, for example the ability of a tumor cell to become metastatic. A number of proteins are involved in modulating actin dynamics, among which are proteins in the actin-depolymerizing factor (ADF)/cofilin (AC) family. The regulation and function of AC proteins must be integrated with the activity of actin-interacting protein 1 (Aip1), profilin, β-thymosins, gelsolin, tropomyosin and capping proteins (including the actin-related protein complex Arp2/3) to control processes such as lamellipodial extension, which will be discussed.

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Actin dynamics

Actin is a major constituent of the cytoskeleton of almost all eukaryotic cells. Actin exists either in a monomeric form, G-actin, or in a filamentous form, F-actin, and each actin subunit binds to either ATP or ADP. The minimal concentration of actin required for assembly [i.e. the critical concentration (Cc)] is lower for ATP–actin than for ADP–actin[1]. At the Cc of the filament end, the rate of subunit addition to the end of filament equals the rate of subunit dissociation from the same end. Net polymerization occurs when the G-actin concentration is higher than Cc, and net depolymerization occurs when the G-actin concentration is lower than Cc. The major difference between ATP–actin and ADP–actin concerns the behavior of the polymer. In the absence of nucleotide hydrolysis, polymers behave as equilibrium polymers with identical Ccs for both ends. However, in reality, actin filaments are non-equilibrium polymers because nucleotide hydrolysis occurs in the filament. Furthermore, this hydrolysis lags behind the assembly process.

Filaments have a pointed end (an arrowhead structure seen in filaments decorated with myosin fragments), which is the slow-growing (minus) end with higher Cc (Cc^{-}). The barbed-end of the filament is the fast-growing (plus) end with lower Cc (Cc^{+}). At steady state (Fig. 1a), the G-actin concentration (also called the steady-state concentration, Cs) is such that a net assembly of subunits at the plus end equals the net disassembly at the minus end, a process called treadmilling[2]. Filament length and number are relatively constant.

The filament-turnover cycle is thought to consist, subsequently, of the addition of ATP–monomer to the barbed end, hydrolysis of ATP within the incorporated subunit, release of P_i into solution, dissociation of the ADP–monomer from the pointed end, and exchange of ATP for ADP on the monomer (Fig. 1b). *In vivo*, ATP–actin is the predominant form in the monomer pool[3], whereas ADP–actin and, under conditions of rapid assembly, perhaps ADP-P_i–actin[4] are the major internal subunits of F-actin.

The motile events of non-muscle cells involve active, precisely controlled reorganization of the actin-filament network. Filament turnover is 100–200-fold faster in cells than for pure actin *in vitro*[5]. Because actin filaments gain and lose their subunits only at their ends, this enhanced turnover could arise from an alteration in subunit on and off rates, an increase in number of free filament ends, or both. The rapid turnover cannot be accomplished without proteins that regulate actin assembly through a variety of activities. Some proteins sequester actin monomers to prevent

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spontaneous nucleation of filaments (β-thymosins) or interact with actin monomers to enhance nucleotide exchange (profilin). Some sever F-actin to generate more filament ends for assembly or disassembly (AC proteins, gelsolin). Other proteins cap filament ends to regulate addition or loss of actin subunits (capping protein, gelsolin, Arp2/3 complex), to nucleate filament growth (Arp2/3 complex), or to enhance subunit dissociation by AC proteins.

How AC proteins modulate actin-filament dynamics

AC proteins are an essential group of actin-binding proteins ubiquitous among eukaryotes. Their highly complex regulation allows them to modulate with spatial and temporal precision the filament turnover needed for many actin-based processes in non-muscle cells. A growing body of evidence supports the importance of AC in actin turnover in cells: (1) AC proteins are localized to cell regions with highly dynamic actin filaments[6,7]; (2) AC proteins increase the turnover rate of actin comet tail of *Listeria monocytogenes*[8,9]; and (3) unlike higher eukaryotes that express two or three different AC proteins, yeast express only one, and mutations that reduce its activity cause defects in filament disassembly[10].

Many AC proteins bind to ADP–G-actin with ~30–80-fold higher affinity than for ATP–G-actin at physiological ionic strength[8,11,12]. AC proteins bind monomeric actin in a 1:1 complex and inhibit nucleotide exchange[13,14]. With the exception of AC from yeast and *Dictyostelium*, the activity of AC is inhibited by phosphorylation at Ser3 (Ref. 15) or its equivalent Ser6 in plants. In metazoans, ACs are the only substrates for LIM kinases[16,17], two forms of which are regulated differentially by members of the Rho family of GTPases[18]. Both ubiquitous and specific phosphatases are involved in dephosphorylation of AC (Ref. 19). AC proteins are also inhibited by PtdIns(4,5)P_2 binding, pH <7.0 and tropomyosin-saturation of F-actin[20].

With the exception of actophorin (i.e. AC protein in amoeba) binding to amoeba F-actin[21], AC proteins bind to F-actin in a cooperative manner[13,22]. AC binds to ADP–actin with higher affinity than to ADP-Pi–actin or ATP–actin[8,23], suggesting that the change in conformation of F-actin that follows P_i release[24] enhances AC binding. Normal F-actin has a helical twist that results in a crossover every 35 nm. Actin filaments decorated with AC have shorter actin crossovers (~27 nm), although the rise per subunit remains the same[25,26]. The consequence of this AC-induced helical twist of approximately −5° per subunit is the weakening of the lateral actin–actin contacts in the filament, which could cause filament fraying and severing[26]. The AC-induced twist also eliminates the phalloidin-binding site on F-actin so that AC-saturated F-actin does not stain with fluorescent phalloidin.

The mechanism by which AC proteins enhance filament dynamics has been somewhat controversial. In early studies of AC action, a weak (non-stoichiometric) filament-severing activity was detected[4,13,27], suggesting that one mechanism for enhanced depolymerization was the generation of additional filament ends. The concentration of filament ends reached a plateau and the number of filaments eventually declined[13]. However, Carlier *et al* reported that recombinant AC proteins enhanced both the association rate (up to 12-fold) at the barbed ends of filaments and the dissociation rate (up to 22-fold) at the pointed ends in the apparent absence of any filament-severing activity[8]. Other scientists reported less of an effect on subunit off-rates and more severing activity with different AC proteins[21,23,28]. Du and Frieden[29] interpreted their actin-assembly kinetics in the presence of yeast cofilin completely in terms of severing, although other models cannot be excluded. Some of these differences in AC activity could be due to a weaker severing activity of recombinant proteins compared with tissue-derived proteins[20]. Furthermore, upon binding to F-actin, different AC proteins induce a different extent of twist[25], and the degree of twist might determine the ability of the AC protein to sever the filament. Moriyama and Yahara[28] developed an assay that looked at both filament numbers (by trapping with a barbed-end capping complex) and rates of subunit loss. They found that porcine cofilin increased filament numbers by severing filaments with a maximum of approximately one severing event per 290 actin subunits (~0.8 μm in length). Subunit off-rates were also enhanced to a maximum of ~6.4-fold. Mutant forms of cofilin were identified that differentially affected severing or the enhanced off-rate at the pointed end. Yeast cells expressing mutant cofilin with defects in severing are more impaired in their growth than those expressing mutant cofilins with defects in depolymerization, suggesting that the ability of AC to sever is an essential process, independent of depolymerization. Taken together, a

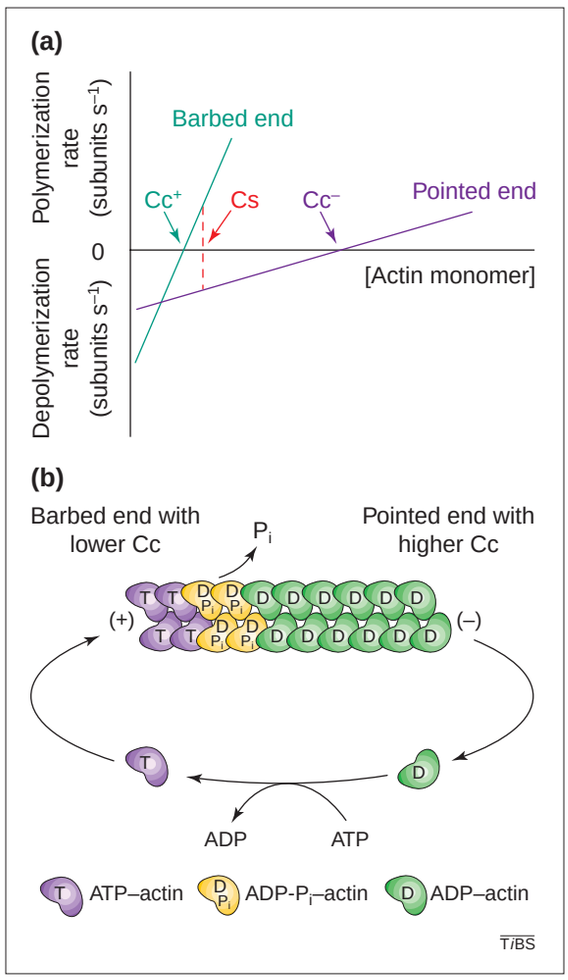

Figure 1

The turnover of an actin filament. **(a)** The actin filament is a polar structure with two different ends. The slow-growing pointed end has a higher critical concentration (Cc) than the fast-growing barbed end. At steady state, the net assembly at the barbed end equals the net disassembly at the pointed end (treadmilling). The G-actin concentration under these conditions is called steady-state concentration (Cs). **(b)** The filament turnover at steady state involves a sequence of actin assembly, ATP hydrolysis, P_i release, filament disassembly and nucleotide exchange. For more detailed discussion, see text, and for a more detailed kinetic analysis, see Ref. 1.

current model for generic AC activity (see Fig. 2) suggests that severing occurs, probably requiring saturation of relatively long regions of an actin filament with AC, and that subunit loss at the pointed end of the filament is also enhanced. Severing is most likely to occur at the junction between AC-saturated and naked F-actin, and possibly only on relatively long filaments (>1 μm) that might have the tendency to fragment as a result of thermal motion.

Another important consideration for the regulation of filament turnover in cells is the potential tight coupling of cycles of AC phosphorylation and dephosphorylation (phosphocycling) to filament turnover. Under *in vivo* conditions thought to enhance filament turnover, levels of phosphorylated and dephosphorylated AC often do not change, but the half-life of the phosphate group on AC decreases significantly[19]. Filament turnover could be linked to AC phosphocycling by the mechanism shown in Fig. 2. Nucleotide exchange can be a rate-limiting step in reassembly. AC inhibits nucleotide exchange on the ADP–actin that dissociates from pointed ends. Transient phosphorylation of AC that dissociates from this complex will allow the free ADP–actin to exchange nucleotides. Removal of the phosphate from AC then reactivates it for another round of subunit removal. Thus, phosphocycling of AC could help drive filament dynamics, especially if the kinase and phosphatase are spatially separated. Within cells, other proteins, in particular profilin, contribute to these regulatory activities.

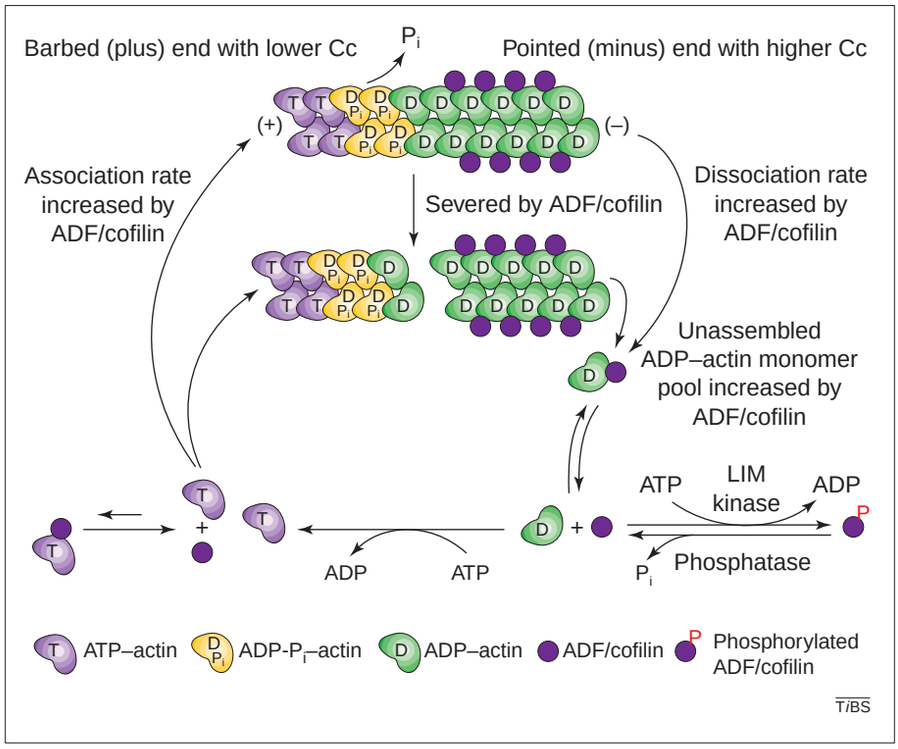

Figure 2

ADF/cofilin (AC) enhances the turnover of actin filaments. AC increases the dissociation rate at the pointed (minus) end and can enhance the association rate at the barbed (plus) end[8]. The dissociation of phosphate from ADP-P_i–actin filaments promotes AC binding to the filament[4,8,21], and the binding of AC to the filament can also promote P_i dissociation[21]. Filament severing by AC creates short filaments with additional free pointed and barbed ends that can contribute to enhanced turnover. The nucleotide exchange on the monomer can be a rate-limiting step. The binding of AC to ADP–actin monomer inhibits nucleotide exchange. The phosphorylation of AC prevents its association with ADP–actin, allowing the binding of profilin to actin to enhance the exchange of ATP for ADP on actin monomer. The dephosphorylation of AC reactivates it to bind and depolymerize actin filaments. Cc, critical concentration.

The complex regulation of actin dynamics in lamellipodial extension

Whether considering neurosecretion, lamellipodial extension or any other cellular process dependent upon actin filament turnover, eventually, the precise temporal and spatial coordination of assembly and disassembly needs to be understood. Currently, lamellipodial movement seems to be the best-understood process[30]. We suggest that the following three features can effectively integrate the activity of numerous actin-binding proteins required for coordinated cell movement: (1) protein localization, (2) signalling that modulates multiple proteins, and (3) a rate-limiting step that changes under different cellular conditions.

Molecules involved in actin-filament turnover are highly localized or spatially regulated in the leading edge. This allows actin to undergo cycles of assembly at the front and disassembly in the rear of the lamellipodium that generate force for extension[31]. Drugs that inhibit polymerization of actin block forward movement of the cell[32]. The barbed ends of the filaments are facing towards the leading edge where actin assembles predominately. Electron-microscopic examination of the lamellipodia of keratocytes and fibroblasts[33] showed an extensively branched array of actin filaments (called dendritic brush) at the leading edge. The pointed ends are associated through the Arp2/3 complex (comprising seven subunits) with the sides of other filaments[34], resulting in Y-junctions[33]. As an alternative to individual filament treadmilling, a treadmilling model for the entire actin-filament array has been proposed[35] (Fig. 3). The model involves steps of nucleation with pointed-end capping, elongation at free barbed ends at the leading edge, ATP-hydrolysis and P_i release, capping of barbed ends as the filament array moves away from the leading edge, pointed-end uncapping and disassembly, presumably from the pointed end. In Fig. 3 we incorporate other actin-regulating proteins into a model for array treadmilling that diagrams the spatial control of filament dynamics.

Actin assembles predominately at the leading edge because the concentration of uncapped barbed-ends is high in this region and can be rate-limiting for polymerization. The uncapped barbed ends can be generated by *de novo* nucleation, severing of existing filaments and removal of barbed-end capping proteins. The Arp2/3 complex might serve to generate new barbed ends through *de novo* nucleation[34,36] or it might capture and stabilize pointed ends of actin filaments generated through severing[37]. The availability of barbed ends is reduced by the barbed-end capping proteins that bind filaments with high affinity (K_d ~1 nM)[38]. These capping proteins are modulated by Ca^{2+} or PtdIns(4,5)P_2 levels, or both[38]. The effect of severing will greatly amplify barbed-end assembly in the presence of pointed-end capping proteins such as the Arp2/3 complex[39], simply because

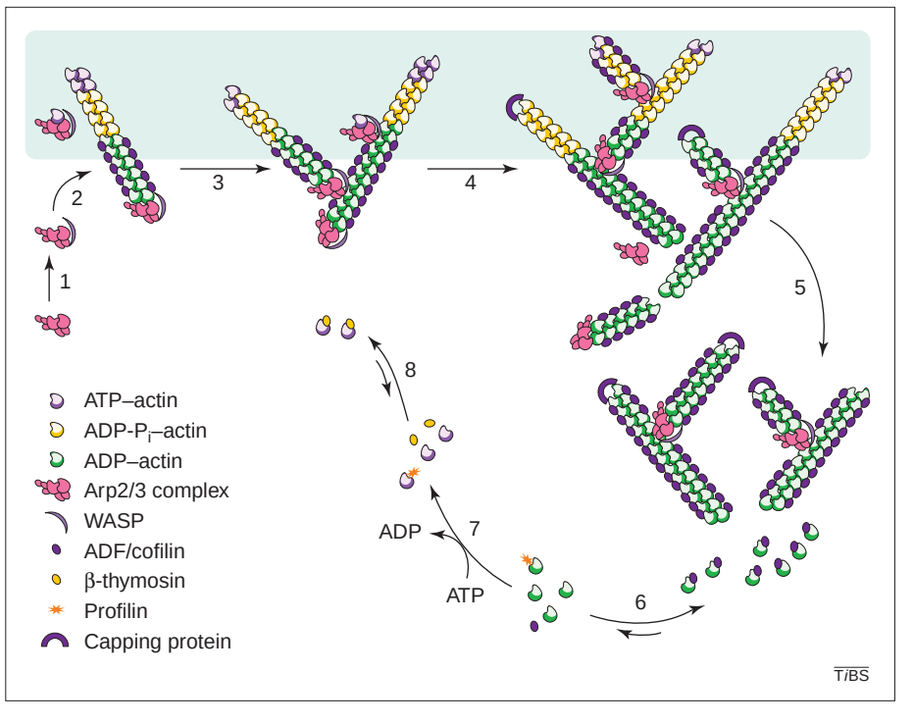

Figure 3

Model for the turnover of the actin-filament array at the leading edge of lamellipodia. The actin network at the leading edge of motile cells is characterized by an extensively branched array of actin filaments with barbed ends facing forwards and pointed ends making Y-junctions (at ~70° angles) with other filaments that are linked by the Arp2/3 complex[33]. The treadmilling of the dendritic array is regulated by several actin-binding proteins as described below. (1) The Arp2/3 complex is activated upon binding to WASP that is activated by the small GTPase cdc42. (2) The active Arp2/3 complex nucleates actin-filament assembly and caps the free pointed end of the filaments[34,36] or (3) it binds to the side of a filament and then nucleates filament growth or captures the pointed ends of a pre-existing filament[37]. Growth of filaments is rapid and the lag in P_i dissociation leads to filaments in the leading edge (green shading) that are composed predominantly of ATP– and ADP-P_i–actin and do not bind to ADF/cofilin (AC). (4) Capping of the barbed ends by capping proteins prevents their further elongation. At the rear of lamellipodia, two mechanisms, filament severing and uncapping of pointed ends by removal of Arp2/3 complex, could contribute to the rapid depolymerization. Severing by AC is likely to occur at junctions between regions of filaments that are saturated with AC and naked F-actin. The depolymerization is enhanced by Aip1 (not shown). (5) AC enhances depolymerization of ADP–actin from free filament ends in the rear of lamellipodia. (6) The complex of AC and ADP–actin that dissociates from the filament ends is in equilibrium with AC and ADP–actin monomer. (7) The nucleotide exchange on actin monomer is a slow process, further inhibited by AC, whereas profilin enhances this rate[11,49,50]. (8) ATP–actin monomers are sequestered by β-thymosins[47] to prevent spontaneous nucleation, but provide a pool of ATP–actin for assembly.

the number of free barbed ends relative to pointed ends will increase. The localization of Arp2/3 complex to the leading edge helps to provide the spatial organization necessary for locomotion. The Arp2/3 complex is activated for actin nucleation by binding to proteins in the Wiscott–Aldrich Syndrome protein (WASP) family, which are in turn activated by cdc42 (Refs 30,39). The implication of *in vitro* studies is that the WASP-activated Arp2/3 complex binds laterally along pre-existing filaments[36], thus promoting actin assembly and the formation of the Y-junctions in the dendritic brush. The filaments adjacent to the membrane are apparently resistant to AC depolymerization[33], possibly because they are composed predominantly of ATP–actin and ADP-P_i–actin. If actin-assembly rates are rapid and P_i release is slow, AC proteins would be restricted in binding to regions of filaments somewhat removed from the leading edge[4]. Another possible mode of AC regulation in the leading edge involves Arp2/3 complex capping of pointed ends. AC proteins can rapidly depolymerize filaments capped with Arp2/3 complex *in vitro*[40], but *in vivo*, filaments capped with Arp2/3 appear to be more stable[33], suggesting activation of Arp2/3 complex by WASP or some other factor inhibits its removal by AC.

The temporal coordination of assembly might be generated by signals such as PtdIns(4,5)P_2 that could modulate the activity of several proteins simultaneously. Extracellular signalling, which reduces phospholipase C activity or activates PtdIns-4 kinase and PtdIns-5 kinase, could increase PtdIns(4,5)P_2 and stimulate barbed-end assembly via several coordinated processes. These include PtdIns(4,5)P_2 inhibition of AC depolymerizing and severing activity[20], prevention of capping protein from binding to barbed ends, and uncapping of gelsolin-bound filaments[38,41]. However, other signals such as Ca^{2+} and pH might activate both assembly- and disassembly-enhancing proteins. The integrated effect will depend upon the relative concentrations and availability of each protein and signalling species in the modulated microdomain.

At the rear of lamellipodia, two likely mechanisms contribute to the fast depolymerization and supply of G-actin needed for assembly at the front. One mechanism might be the removal of the Arp2/3 complex, facilitating AC-dependent depolymerization from the pointed end. Another mechanism is a Ca^{2+}-independent filament severing by AC (Ref. 20), perhaps aided by active Aip1 (Refs 42,43), or a Ca^{2+}-dependent severing by gelsolin[38], or both. Aip1 is localized to dynamic regions of cell cortex including lamellipodia in *Dictyostelium*[44]. It binds with a maximum stoichiometry of 1:2:2 to AC and actin[42]. Aip1 itself has no significant F-actin severing or depolymerization activity, but it greatly enhances filament depolymerization promoted by yeast cofilin or *Xenopus* AC (Refs 42,43). In motile adenocarcinoma cells, a rapid increase in filament number at the leading edge occurs in a calcium-independent fashion[45], suggesting that the severing activity of AC proteins might be modulated rapidly.

Not all filaments lacking Arp2/3 complex can be depolymerized or severed by AC. The binding of AC and tropomyosin to actin filaments is mutually exclusive. Tropomyosin inhibition of AC activity[20] provides another level of control for coordinating both the spatial and temporal turnover of actin filaments. Specific isoforms of tropomyosin distribute differentially during the development of some cells[46]. How different filament populations arise remains an open question.

Although AC proteins are essential for enhanced actin dynamics *in vivo*[8–10], clearly they do not work alone. In addition to the multiple species controlling their behavior directly, their effectiveness in disassembly and severing is greatly augmented by other monomer-sequestering proteins. These proteins, profilin and

thymosin β4, might lack depolymerizing or severing activity themselves[47], but they are present in sufficiently high concentration to contribute significantly to the maintenance of the ATP–G-actin pool. AC can then rebind to filaments, severing and/or removing more monomers. In contrast to ACs, which bind with higher affinity to ADP–actin than to ATP–actin, β-thymosins have a 100-fold higher affinity for ATP–actin than for ADP–actin and sequester the ATP–actin monomers in a 1:1 complex[48]. Under normal intracellular conditions, profilin binding to G-actin accelerates nucleotide exchange[49], generating more of the preferred ATP–G-actin for filament addition. By enhancing nucleotide exchange, often the rate-limiting step in actin cycling, profilin increases the subunit turnover in the presence of AC up to 125-fold over actin alone[11,50].

Conclusions and outlook

Recent biochemical and structural studies have provided important insights into how AC proteins work with other actin-binding proteins to enhance the turnover of actin filaments, but a number of questions remain to be answered. Do different AC homologues vary qualitatively or quantitatively in their effects on filament turnover? What are the intracellular implications of these differences, especially in cells that contain more than one member of the AC family? How do the signal-transduction pathways controlling AC phosphocycling impact the activities of the other proteins discussed here? How are the bifurcating signaling pathways regulating AC phosphocycling kept in balance? Is the competitive binding of tropomyosin and AC used to distinguish different filament populations and if so how are these selected? A combination of molecular, biochemical and ultrastructural approaches will help answer these questions in the near future.

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