Ins and outs of ADF/cofilin activity and regulation

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Abstract

The actin-binding proteins of the actin-depolymerisation factor (ADF)/cofilin family were first described more than three decades ago, but research on these proteins still occupies a front role in the actin and cell migration field. Moreover, cofilin activity is implicated in the malignant, invasive properties of cancer cells. The effects of ADF/cofilins on actin dynamics are diverse and their regulation is complex. In stimulated cells, multiple signalling pathways can be initiated resulting in different activation/deactivation switches that control ADF/cofilin activity. The output of this entire regulatory system, in combination with spatial and temporal segregation of the activation mechanisms, underlies the contribution of ADF/cofilins to various cell migration/invasion phenotypes. In this framework, we describe current views on how ADF/cofilins function in migrating and invading cells.

Introduction

More than 30 years ago a novel actin-binding protein (ABP) was discovered in chicken and porcine brain based on its capacity to form 'cofilamentous' structures with actin filaments and to depolymerise them (Bamburg et al., 1980; Nishida et al., 1984). These proteins and their many homologues constitute the actin-depolymerisation factor (ADF)/cofilin family that is ubiquitously present throughout the eukaryotic kingdom. In different organisms, it has been demonstrated that ADF/cofilin activity is essential for life (e.g. Gurniak et al., 2005; Moon et al., 1993) and a factor contributing to various human diseases (Bamburg and Wiggan, 2002). The characteristic three-dimensional fold of these small (15–21 kDa), single-domain ABPs is termed the ADF-homology domain (ADF-H, InterPro entry IPR002108, Pfam family PF00241). In the closely related twinfilins, two ADF-H domains are present (Paavilainen et al., 2007), and in several proteins (e.g. drebrins) the ADF-H domain is part of a more complex protein architecture (Lappalainen et al., 1998). ADF-H domains also bear structural and functional similarity to the domains characteristically present in the gelsolin family of ABPs (PF00626) (Hatanaka et al., 1996; Van Troys et al., 1997, 2000) (see Pfam clan ADF CL0092).

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In this review, we focus on the ADF/cofilin family in mammalian systems, on their effects on actin dynamics and on the multiple mechanisms by which these proteins are regulated. We discuss recent research that reveals how the complex regulation of this ABP activity may allow controlled initiation of cell protrusive activity and chemotactic response and how this is crucial for migrating and invading cells.

The mammalian ADF/cofilin family

The ADF/cofilin family in mammalian systems consists of three highly similar paralogs: cofilin-1 (Cfl1, non-muscle cofilin, n-cofilin), cofilin-2 (Cfl2, muscle cofilin, m-cofilin (Ono et al., 1994)) and ADF (actin-depolymerising factor or destrin). The relative expression levels of these three isoforms vary in a cell/tissue-specific manner, as documented in mouse (Gurniak et al., 2005; Vartiainen et al., 2002). During development, Cfl1 is the predominant isoform and it remains ubiquitously expressed in most adult tissues. ADF becomes post-natally upregulated mainly in epithelial and endothelial tissues, albeit usually at concentrations lower than Cfl1. In late embryogenesis and after birth, Cfl2 replaces Cfl1 in striated muscle and forms the only isoform expressed in differentiated skeletal muscle and the main one in cardiac muscle (Nakashima et al., 2005).

It is presently unclear why mammals have different ADF/cofilin isoforms that are often co-expressed in one cell type. Obviously, multiple non-mutually exclusive answers to this question are possible. ADF/cofilin genes display different transcriptional regulation, e.g. during development (see above) or in response to specific cellular actin levels (Minamide et al., 1997) or cellular conditions (Estornes et al., 2007). In addition, they may be subject to differential posttranscriptional regulation by which their mRNA is site-specifically enriched (Lee and Hollenbeck, 2003; Willis et al., 2005), or they may display differential activation downstream of signalling or (subtle) functional differences (discussed below).
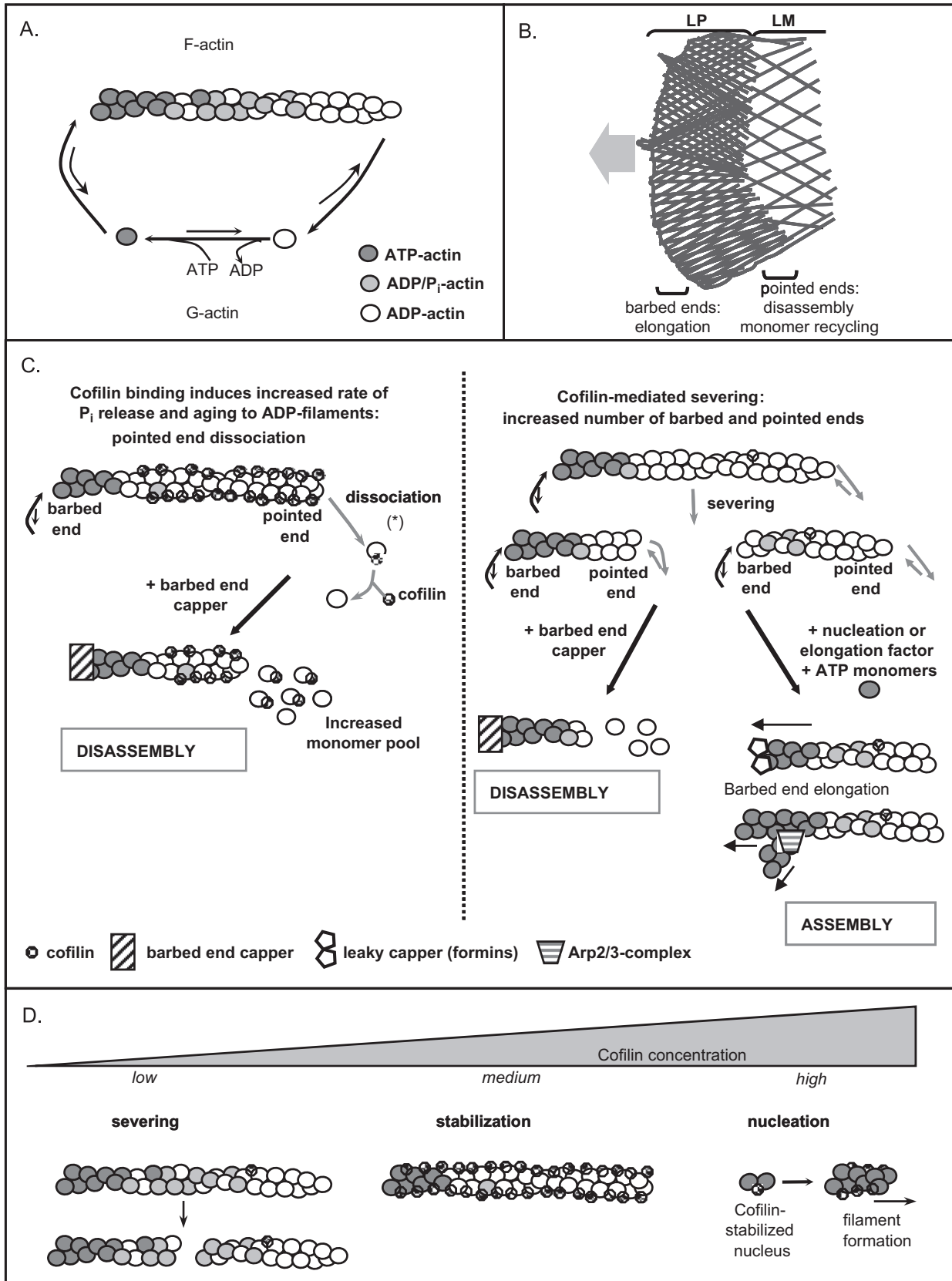
In cultured cells, Cfl1 downregulation can be rescued by ADF expression and vice versa (Hotulainen et al., 2005). In contrast, in more complex conditions, e.g. during specific developmental or physiological processes, different cofilin/ADF isoforms display distinct effects. This is underscored by the fact that Cfl1$^{-/-}$ mice are not viable (Gurniak et al., 2005) but ADF$^{-/-}$ mice are (Ikeda et al., 2003). Even though Cfl1$^{-/-}$ mice display an upregulation of ADF, likely allowing their survival past gastrulation, they die after E9.5. In this phase of development, Cfl1 appears crucial for cell migration events in specific cell lineages derived from the neural ectoderm and the paraxial mesoderm. Brain-specific knockout recently revealed that Cfl1 crucially controls both cell migration and cell cycle progression in the cerebral cortex (Bellenchi et al., 2007). In contrast, ADF$^{-/-}$ mice display normal embryonic development (Gurniak et al., 2005), suggesting that the lack of ADF is sufficiently rescued by Cfl1. However, shortly after birth ADF$^{-/-}$ mice develop abnormal thickening of the cornea by hyperproliferation of the corneal epithelial cells and become blind (Ikeda et al., 2003). Despite an upregulation of Cfl, the ADF-deficient corneal cells have abnormally high levels of F-actin.

These studies thus revealed that the different ADF/cofilin isoforms are not completely redundant. This is in line with biochemical studies demonstrating that different cofilin/ADF isoforms have qualitatively similar but quantitatively different effects on actin dynamics as discussed below.

Biochemical characterisation of ADF/cofilins: actin-dynamising proteins

ADF/cofilins have been termed actin-dynamising proteins based on their capacity to enhance the turnover of actin filaments in vitro (Carlier et al., 1997). Fig. 1A shows the actin polymerisation cycle. At steady state, actin filaments (F-actin) preferentially grow at one end (called the fast growing or barbed end) by association of ATP-loaded monomeric actin molecules (ATP-G-actin,

Fig. 1. Actin treadmilling and effects of ADF/cofilins. (A) Steady-state actin polymerisation cycle. Thick arrows indicate the preferred direction of this cyclic process. Barbed (fast growing) and pointed (slow growing) ends of the actin filament are indicated; a grey-scale code is used to represent the nucleotide status of the actin monomers (G-actin) or subunits in the filament (F-actin). (B) Scheme of a cellular protrusion (actin filaments in grey). The direction of growing protrusion is indicated as well as the separation of the protrusion in lamellipodium (LP) and lamellum (LM). The LP contains the treadmilling actin network and is a tropomyosin-free zone. The LM is rich in high-molecular-weight tropomyosins and in actomyosin activity. (C) Schematic representation of the proposed roles of ADF/cofilins in pointed end depolymerisation (left) and severing (right) of actin filaments leading to filament disassembly and/or assembly. A positive effect of cofilin on the dissociation rate of actin subunits from the pointed end (*) is a matter of debate (Andrianantoandro and Pollard, 2006; Carlier et al., 1999). Monomers released from the pointed end can be recycled for actin polymerisation. Cofilin is shown to sever distant from the binding site as described in Bobkov et al. (2006). Barbed end-capping proteins and actin-binding proteins promoting filament nucleation (e.g. Arp2/3 complex) or barbed end elongation (e.g. formins) are proposed to act in synergy with cofilin. (D) A new paradigm of ADF/cofilin activity (based on Andrianantoandro and Pollard (2006)). In a dose-dependent manner, ADF/cofilin can sever, stabilise or nucleate actin filaments.

A. F-actin

ATP ADP

ATP-actin
ADP/P$_i$-actin
ADP-actin

G-actin

B. LP LM

barbed ends: elongation

pointed ends: disassembly monomer recycling

C.

Cofilin binding induces increased rate of P$_i$ release and aging to ADP-filaments: pointed end dissociation

barbed end   pointed end

dissociation (*)

cofilin

+ barbed end capper

DISASSEMBLY

Increased monomer pool

Cofilin-mediated severing: increased number of barbed and pointed ends

severing

barbed end   pointed end

barbed end   pointed end

+ barbed end capper

+ nucleation or elongation factor + ATP monomers

DISASSEMBLY

Barbed end elongation

ASSEMBLY

cofilin   barbed end capper   leaky capper (formins)   Arp2/3-complex

D.

Cofilin concentration

low   medium   high

severing   stabilization   nucleation

Cofilin-stabilized nucleus

filament formation

globular actin) whereas monomers dissociate at the other (slow growing or pointed end). Incorporated monomers undergo hydrolysis of the bound ATP to ADP, with ADP/Pi-loaded actin as important intermediates. ADP-loaded actin is more prone to dissociation and recycles back to the monomer pool. Dissociated ADP-loaded actin monomers need to exchange their nucleotide before entering a new polymerisation cycle. The polarised growing and shrinking, resulting in dynamic turnover of actin filaments (also called treadmilling), forms the fundament of protrusive forces in cells. It occurs in lamellipodia and filopodia in migrating cells and it is also the basis of propulsion of specific vesicles in the cytosol (Chang et al., 2003). In these cellular nano-scaled force machineries, actin filaments are organised in polarised arrays (i.e. (branched) networks or bundles) that display array treadmilling (Pollard and Borisy, 2003) (Fig. 1B). The dynamic turnover of such an actin array can be enhanced either by an increase in the number of filament ends (resulting from de novo nucleation of filaments or from severing existing filaments) or by an increase in the extent or rate of monomer association at barbed filament ends and/or dissociation from pointed filament ends.

A wealth of data has been generated on ADF/cofilins in vitro, demonstrating that the effects of these proteins on actin dynamics are multiple and complex (see Andrianantoandro and Pollard, 2006; Bamburg and Wiggan, 2002; Bobkov et al., 2006; Carlier et al., 1999; McGough et al., 2001; Wang et al., 2007a). ADF/cofilins bind actin monomers and filaments. Based on images of cofilin-decorated actin filaments obtained using cryo-electronmicroscopy, it is suggested that each cofilin molecule is contacting two actin subunits in an actin filament by binding in a cleft between them. Cofilins have a higher affinity for ADP-loaded than for ATP-loaded actin; they decrease nucleotide exchange on ADP-loaded monomers and promote Pi release from ADP/Pi subunits in the filament. They accelerate spontaneous polymerisation of monomers (nucleation) and are hypothesised to increase the rate of actin subunit dissociation from the pointed end. Cofilin binding to F-actin induces a conformational twist in the actin filament structure that propagates over a long range from the actual cofilin-binding site (i.e. hundreds of subunits), and this is suggested to underlie their fragmenting/severing activity.

These activities of ADF/cofilins crucially contribute to actin dynamics. Cofilin is part of the machinery that is minimally required for actin-based propulsion used by intracellularly moving pathogens like *Listeria* (Lambrechts et al., 2008; Loisel et al., 1999). How exactly ADF/cofilins dynamise the actin polymerisation process in vitro has been strongly debated for several decades

and resulted in two leading models: either ADF/cofilins increase the dissociation rate of actin subunits from pointed ends or they sever actin filaments (Bamburg and Wiggan, 2002; Carlier et al., 1999; Ichetovkin et al., 2002; Maciver et al., 1991; Pavlov et al., 2007). Whereas both models imply a direct role of ADF/cofilins in F-actin disassembly, only the latter supports a direct function in assembly through the rapid generation of new barbed ends (Fig. 1C).

Important mechanistic insight into ADF/cofilin activity was recently provided by Andrianantoandro and Pollard (2006) favouring and extending the severing model and countering the enhanced dissociation model. Using real-time microscopic assays analysing single actin filaments, they were able to study the various effects of cofilins (severing, end-kinetics, nucleation) in isolation and observed switches in cofilin activity depending on its concentration. A new paradigm was proposed illustrating how the fine-tuning of ABP activity could occur (Fig. 1D). In line with previous studies (Ichetovkin et al., 2002; Orlova et al., 2004; Pavlov et al., 2007), F-actin filament-severing activity was found to be highest at low cofilin concentrations. When only a few cofilin molecules are bound to an actin filament, the number of torsionally strained interfaces between twisted filament regions (by long-range effects of cofilin binding) and non-twisted regions may be maximal and this is hypothesised to result in frequent breakage (Bobkov et al., 2006). At higher cofilin concentrations, when filaments are largely cofilin-decorated, severing is no longer observed (the high degree of decoration eliminates strained interfaces). Under these conditions, dissociation from pointed ends is observed but, importantly and in contrast with previous reports, not with rates beyond that of undecorated ADP-pointed ends. The observed disassembly is in line with the capacity of cofilin to enhance Pi release and thus promote aging of filaments to their ADP-loaded form. Finally, at very high concentrations of cofilin versus actin monomers, monomer binding and de novo nucleation appear strongly enhanced and assembly is promoted. Such de novo nucleation activity of ADF/cofilin (also observed by Carlier et al. (1997), Kudryashov et al. (2006) and Yeoh et al. (2002)) had been largely ignored as a potential mechanism to explain the assembly of new filaments in cells.

Taken together, these observations suggest that in a cell region where a gradient of high to low cofilin activity is present, the activity of ADF/cofilin could shift from (i) nucleating new filaments (that can initiate the formation of a branched network by the Arp2/3 complex (DesMarais et al., 2004, 2005; Ichetovkin et al., 2002)) to (ii) actin filament stabilisation and aging by cofilin decoration, and finally (iii) filament severing, which creates barbed ends capable of elongation or will, in the presence of barbed end-capping

proteins, results in net disassembly from pointed ends (Andrianantoandro and Pollard, 2006) (Fig. 1C,D).

In addition to this concentration dependence of the cofilin activity mode, an additional layer of complexity is added by the differences between the Cfl1, 2 and ADF cofilin isoforms. ADF appears most efficient at turning over actin filaments; it has weaker nucleating activity and promotes a stronger pH-dependency of actin filament disassembly than Cfl1 or Cfl2. Cfl2 has weaker F-actin depolymerisation activity than the other two isoforms and promotes filament assembly, rather than disassembly, in steady-state assays (Chen et al., 2004; Nakashima et al., 2005; Vartiainen et al., 2002; Yeoh et al., 2002).

The actin-modulating activities of ADF/cofilins are important for multiple cellular functions. Where and when cofilins are active in cells is, as outlined below, the resultant of a complex regulatory network that researchers are steadily disentangling.

Regulatory mechanisms of ADF/cofilin activity

Multiple mechanisms have been identified that regulate ADF/cofilins, including ADF/cofilin inactivation via phosphorylation and by polyphosphoinositide interaction, the effects of pH and the synergistic or competitive interactions of ADF/cofilins with other ABPs.

An inactivation/activation switch mediated by ADF/cofilin phoshorylation/dephosphorylation

ADF/cofilins are inactivated by phosphorylation on Ser3. This posttranslational modification results in inhibition of G- and F-actin binding and of F-actin severing (references in Bamburg and Wiggan (2002)). Mutants carrying S3D/E and S3A substitutions have been successfully used in vivo to mimic inactive phosphoADF/cofilin (P-ADF, P-CFL) and active ADF/cofilin, respectively. Two families of ubiquitous kinases, with related catalytic domains, are responsible for the inactivation of cofilins by phosphorylation: the LIM (Lin-11, Isl1, and Mec-3) kinases (LIMK) and testicular kinases (TESK) (Scott and Olson, 2007; Toshima et al., 2001b). The phosphatases (PPases) of the Slingshot (SSH) family and the haloacid dehalogenase phosphatase chronophin (CIN) are the enzymes that reactivate phosphorylated ADF/cofilin (Huang et al., 2006). Table 1 provides an overview of known characteristics of each of these enzymes.

Several studies in diverse cell types document that altering the expression levels or activities of the kinases and PPases that regulate ADF/cofilin disturbs actin reorganisation. Similarly cell motility and migration are affected, albeit contradictory results have sometimes been obtained (references in Huang et al. (2006), Ono (2007), Scott and Olson (2007) and Wang et al. (2007a)). Three aspects need to be considered in trying to understand the relative contributions of the ADF/cofilin kinases and PPases: first, their expression levels and tissue distributions, second, their activation pathways and activity levels and, finally, their subcellular localisation via potential scaffolding factors and alterations therein upon specific cell stimulation. Many of the available data on functionality have, however, been derived by studying only one isoform within the regulatory families of kinases and PPases. Studies that perform isoform comparisons are still scarce, but reveal that isoforms mediate specific effects, and insight herein will be important to understand the true complexity of the regulation of ADF/cofilin at the cellular level.

Widespread tissue distribution of multiple regulators of ADF/cofilin phosphorylation: potential for cooperative or parallel action

ADF/cofilin kinases and PPases all appear widely expressed and display overlapping tissue distributions as revealed by Northern blot, in situ hybridisation and in situ histochemistry on embryonic and adult tissues (Table 1). With some exceptions, multiple isoforms of each of these enzymes are present in cells albeit isoform-specific differences in expression levels have been observed in some tissues, for example for LIMK1 and 2 (Acevedo et al., 2006; Foletta et al., 2004) and for SSH1-3 (Ohta et al., 2003) (Table 1). Occasionally, a particular tissue or cell line expresses only a single isoform, for example in different cell populations of testis only TESK 1 or 2 are present (Toshima et al., 2001b). For LIMKs, the simultaneous expression of the two isoforms is in line with the defects observed in knockout mice. These defects are mainly limited to hippocampal dendritic spine structures for the LIMK1 knockout and to spermatogenesis in testis for the LIMK2 knockout (Meng et al., 2002; Takahashi et al., 2002). This suggests that only for these specialised processes a particular LIMK isoform is essential or the other isoform is not present and/or cannot provide rescue. Taken together, the broad tissue distribution of the multiple direct ADF/cofilin regulators indicates that in most cell types the mutual expression and activation of all these regulators needs to be considered. The picture is likely even more complex, since until now little is known about differences for reported splice variants of the different isoforms of LIMKs and SSHs (Table 1) (Ott et al., 2007).

Expression levels are mainly regulated at the transcriptional level, but in recent years mechanisms of translational control, e.g. via micro-RNAs, and mechanisms regulating protein lifetime via proteasome-mediated degradation have been outlined. Two E3 ubiquitin ligases

Table 1. Cofilin kinases[a] and phosphatases

Enzyme	LIM kinases	TES kinases	Slingshot phosphatases	Chronophin phosphatase
Isoform/splice variants[b]	LIMK1 (in humans: full length and variant lacking kinase domain) LIMK2 (in humans: variants 1a, 2a, 2b)	TESK1 TESK2	SSH1L (long) SSH2L SSH3 (in humans also: SSH1S (short), SSH2, SSH3L)	CIN (pyridoxal phosphatase)
Protein structural features[b]	2 LIM domains, PDZ domain, Pro/Ser-rich region, C-terminal kinase domain	N-terminal kinase domain, three conserved regions in C-terminal domain	Conserved domains: A and B, PPase domain In L- and S-isoforms: C-terminal domain of variable length with F-actin- and/or 14-3-3-binding capacity	Catalytic domain and three conserved motifs characteristic of haloacid dehalogenases
Tissue distribution[c]	LIMK1: widespread (abundant in brain, kidney, lung, stomach, and testis) LIMK2: widespread in all tissues (not in glial cells, kidney glomeruli) LIMK1 and 2: expressed in different testis cell populations	Present in several tissues, enriched in testis TESK1 in germ cells TESK2 in somatic Sertoli cells	Widespread, extensive (but incomplete) overlap in tissue distribution for SSH1, 2 and 3	Widespread, abundant in brain, heart, skeletal muscle, liver, and kidney
Substrate(s)[d] Activation (+) Inactivation (−)	(−) Cofilin, ADF (+) CREB (−) Nurr1 (−) p25α (promotes tubulin polymerisation)	(−) Cofilin, ADF	(+) Cofilin, ADF (−) LIMK1 and 2	(+) Cofilin, ADF (+) Pyroxidal 5′-phosphate
Posttranslational activation[d]	Phosphorylation	Unknown	Dephosphorylation	Unknown
Upstream cellular activators (+) and inhibitors (−)[e,f]	(+) Rho GTPases via: PAKs, MRCKα, ROCKs, CIB1-PAK1, MAPKAPK2 Dimerisation (Hsp-90 mediated) (−) Autoinhibition, SSH1L, PAR2-β-arrestin, LATS-1, LIMK2: Par-3	(+) Rac1 (−) α-parvin, sprouty-4, 14-3-3β	(+) Calcineurin (PPase2B), PI-3 kinase (−) PAK4, 14-3-3 (different isoforms)	β-Arrestin

[a]Additional cofilin kinase: Nck-interacting kinase (NIK)-related kinase (NRK)/NIK-like embryo-specific kinase (NESK) (Nakano et al., 2003), predominantly expressed in skeletal muscle during the late stages of mouse embryogenesis.

[b]LIMKs: References in Scott and Olson (2007), Ott et al. (2007), TESKs: Toshima et al. (2001a, b), SSHs: Ohta et al. (2003), CIN: Gohla et al. (2005).

[c]LIMKs: References in Scott and Olson (2007), Acevedo et al. (2006), Foletta et al. (2004), TESKs: Rosok et al. (1999), Toshima et al. (2001b), SSHs: Huang et al. (2006), Ohta et al. (2003), CIN: Huang et al. (2006).

[d]LIMKs: References in Scott and Olson (2007), SSHs: Niwa et al. (2002), Soosairajah et al. (2005), CIN: Huang et al. (2006).

[e]LIMKs: References in Scott and Olson (2007), Kobayashi et al. (2006), Leisner et al. (2005), Li et al. (2006), Zoudilova et al. (2007), TESKs: LaLonde et al. (2005), Raymond et al. (2004), Toshima et al. (2001c), Tsumura et al. (2005), SSHs: References in Huang et al. (2006), Soosairajah et al. (2005), Wang et al. (2005), CIN: Zoudilova et al. (2007).

[f]Additional regulation of LIMKs: protein cleavage and stability of LIMK1: via caspase-3 cleavage, E3-ubiquitin ligases RFn6 and parkin; translational inhibition of LIMK1 by microRNA miR134; cytoplasm/nuclear shuttling of LIMK1 by p57[KIP2]; of LIMK2 by PKC (references in Scott and Olson (2007) and Lim et al. (2007)).

and an inhibitory micro-RNA have been identified in neuronal systems as regulatory partners of LIMK1 protein and its RNA, respectively (footnote (e), Table 1). For the other players in ADF/cofilin phosphorylation, these types of regulation remain to be identified.

Complex regulation of the phosphorylation/dephosphorylation switch

Fig. 2 illustrates the complexity of the main signalling pathways that regulate the ADF/cofilin kinase and PPase activities. LIMKs are activated by phosphorylation on a threonine residue in the catalytic domain by various kinases that are themselves activated downstream of small Rho GTPases (references in Scott and Olson (2007)). LIMKs have been shown to be essential regulators of actin cytoskeletal reorganisation downstream of these GTPases and implicated in Rac-dependent lamellipodia formation and Rho-dependent stress fibre and focal adhesion formation (Sumi et al., 1999). Based on current knowledge, the isoforms LIMK1 and LIMK2 are partially under distinct control (Fig. 2). Rho and Cdc42 signal to both LIMK1 and 2 via the Rho kinases ROCK I and II and via the myotonic dystrophy kinase-related Cdc42-binding kinase MRCKα, respectively. PAK1 and 4, downstream of Rac activation, also activate LIMK1, but not LIMK2. A Rac-PAK2 activating pathway has also been described but, at present, it is not yet analysed whether PAK2 displays LIMK isoform specificity (Misra et al., 2005).

In addition, GTPase-independent LIMK activation also occurs. Examples are CIB1 (calcium- and integrin-binding protein 1)-PAK1 activation of LIMK1 induced by adhesion to fibronectin (FN) (Leisner et al., 2005) (Fig. 2) or LIMK1 phosphorylation and activation downstream of VEGF in endothelial cells by the MAPKAPK kinase. In contrast to Rho GTPase-dependent kinases, the MAPKAPK kinase phosphorylates LIMK1 on a Ser residue in the PDZ domain, and this phosphorylation is suggested to release an auto-inhibitory interaction between the amino terminal domain of LIMK and its kinase domain (Kobayashi et al., 2006).

Today the only PPase known to dephosphorylate and inactivate LIMK 1 and 2 is a PPase that also dephosphorylates cofilin, namely SSH1L (Soosairajah et al., 2005), suggesting a positive feedback loop via simultaneous cofilin activation and LIMK inhibition (Fig. 2).

As shown in Fig. 2, the known pathways affecting TESK activity are very different and mainly integrin mediated and adhesion dependent. TESK1 activity is inhibited by sequestration by α-parvin (or actopaxin), a focal adhesion protein, and 14-3-3β, a member of a large family of scaffold proteins that binds phosphoserine/threonine motifs, and this inhibition is relieved upon fibronectin–integrin engagement (LaLonde et al., 2005; Toshima et al., 2001c). Sprouty-4 (Spry-4, an inhibitor of receptor tyrosine kinase (RTK)-MAP kinase signalling) inhibits cell spreading of C2C12 cells by binding TESK1 and suppressing its activity (Tsumura et al., 2005). Since TESK1/2 activation was shown to be independent of ROCK or PAK in HeLa cells (Toshima et al., 2001b), TESKs and LIMK appear to relay different extracellular signals to cofilin phosphorylation.
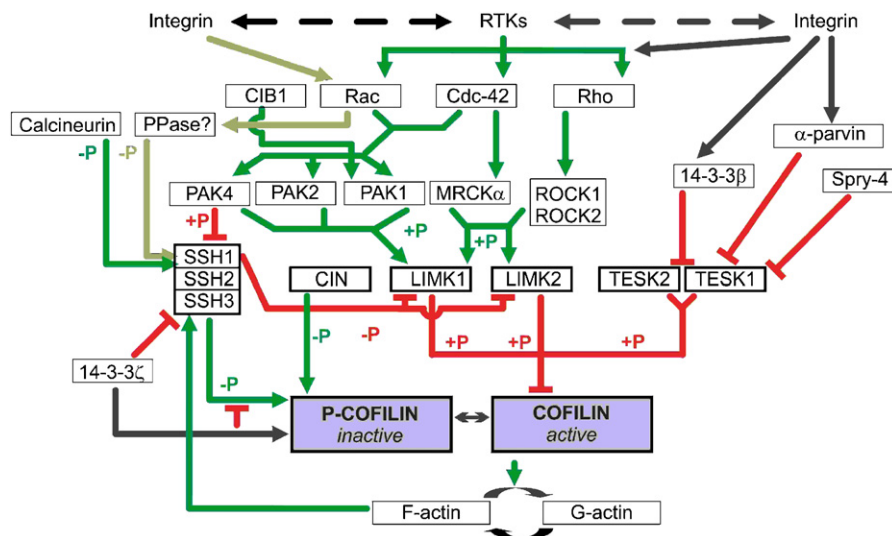

Fig. 2. The cofilin regulatory system: signals controlling cofilin phosphorylation and dephosphorylation. Schematic overview of the ADF/cofilin kinases and PPases and their currently known upstream regulators. Phosphorylated ADF/cofilin (P-ADF/P-CFL) is inactive and no longer binds or severs F-actin. Rho GTPases have a prominent role in control of kinases and PPases. Red, green (light or dark) and black lines or arrows point at an inhibitory interaction, an activation or an interaction with no (or unknown) effect on activity, respectively; +P and −P point to phosphorylation and dephosphorylation. The striped arrows connecting integrins and RTKs indicate the cross-talk between these receptor systems; see main text for details and acronym explanations.

This demonstrates that ADF/cofilins are integrators of multiple upstream cellular signals.

The regulatory pathways that modulate the ADF/cofilin PPases (Fig. 2) are less clear. A number of consensus motifs, e.g. for PKC phosphorylation, or binding sites, e.g. for p85 SH3, are present in CIN, but their involvement in its regulation remains to be demonstrated (Huang et al., 2006). Only a few direct modulators of SSH activity have presently been identified (Fig. 2). SSH1L activity is negatively regulated via direct phosphorylation by PAK4 in different cell types. This provides an additional reciprocal control of LIMK1 and SSH1L activity, in addition to the direct SSH1L–LIMK1 interaction (described above), and suggests a negative role of Rac1 activation on SSH activity and cofilin activity (Soosairajah et al., 2005). In other cell types, however, Rac1 signalling leads to SSH activation. This has been demonstrated in keratinocytes migrating on laminin and involves integrin $\alpha6\beta4$ activation; which SSH isoform is involved downstream of integrin-Rac1 activity was not identified (Kligys et al., 2007). Increased intracellular Ca^{2+} also activates SSH in HeLa cells by activating PPase2B (calcineurin) that in turn directly dephosphorylates SSH1L (Wang et al., 2005).

SSH1L contains three F-actin-binding sites, and F-actin interaction is a feature of SSH1L and SSH2L. The shorter SSH3, which also appears to be a weaker ADF/cofilin PPase, does not interact with F-actin. Actin filament binding by long SSHs strongly enhances their capacity to dephosphorylate cofilin in vitro and in vivo (Kiuchi et al., 2007; Nagata-Ohashi et al., 2004; Ohta et al., 2003; Soosairajah et al., 2005; Yamamoto et al., 2006). This may have a role in directing cofilin activation to specific cellular regions of high F-actin density.

Subcellular localisation of ADF/cofilin kinase and PPase isoforms

The subcellular localisation of ADF/cofilins and the regulators of their phosphorylation status are of great importance in the context of a dynamic cellular environment. Simultaneous recruitment of kinases and PPases to a particular region would allow for a spatially confined and highly dynamic regulation of ADF/cofilin activity (local phosphocycling). This could be further fine-tuned by the temporal regulation of the activities of the respective enzymes or by differences in their activation mechanisms. Conversely, specific types or isoforms of ADF/cofilin kinases or PPases may be exclusively recruited to a specific subcellular location. Evidence is emerging that cells exploit both strategies to control the impact of ADF/cofilin on diverse actin-dependent cellular processes.

A striking example of the relationship between subcellular localisation and temporal activation of ADF/cofilin regulators comes from studies on mitotic and dividing cells. In fact, these cells undergo extensive actin and microtubule reorganisation events that are well defined in time and space (Glotzer, 2005). ADF/cofilins and their regulators localise to the cytokinesis apparatus, and cell division is impaired by disturbing ADF/cofilin activity (directly or via targeting LIMK, SSH or CIN activity) (Gohla et al., 2005; Hotulainen et al., 2005; Kaji et al., 2003; Nagaoka et al., 1995; Sumi et al., 2006). During mitotic progression P-LIMK1 (but not P-LIMK2) and P-cofilin levels vary in a strict temporal pattern that is inverse to the timing of SSH1 activation (Gohla et al., 2005; Kaji et al., 2003; Sumi et al., 2006). This suggests that cofilin is maximally activated during the late mitotic steps in which assembly and disassembly of the contractile ring occurs and the cleavage furrow ingresses to complete cytokinesis. This temporal control is matched by a very striking isoform-specific localisation of LIMKs throughout the mitotic cycle (Sumi et al., 2006). During telophase, LIMK1 is colocalised with both actin and cofilin at the cleavage furrow/contractile ring whereas LIMK2 is found at the mid-zone microtubules but not associated with actin and cofilin. The molecular basis of this difference still needs to be determined, and clues may come from the recent finding that the microtubule polymerisation-promoting factor p25α is a LIMK1 substrate (Acevedo et al., 2007). Differences in isoform specific localisation during cell division for TESKs or SSHs have not yet been explored.

In interphase cells and in cells with protrusive membrane activity, localisation of ADF/cofilin kinases and PPases has also been studied, although not yet in the same detail for all of these enzymes. Endogenous CIN is present in membrane ruffles and at the leading edge of lamellipodial protrusions in HeLa cells (Gohla et al., 2005). In this cell line, overexpressed mouse SSH1L and 2L co-localise with F-actin and accumulate on thin and thick actin fibres in the cytoplasm as well as on actin bundles in the cell periphery. SSH2L also accumulated in focal adhesions. The SSH3 isoform was diffusely distributed in the cytoplasm and in the nucleus, in line with its inability to interact with actin.

Two studies report a relatively more restricted subcellular localisation pattern for LIMK1 than for LIMK2. In several cell types, including HeLa, LIMK1 is enriched in focal adhesions (Acevedo et al., 2006), although another study using HeLa cells showed it to be enriched at cell–cell adhesion sites (Sumi et al., 2006). Conversely, LIMK2 displayed either a punctate or diffuse pattern in the cytosol. The localisation of LIMK1 at focal adhesions appears in line with the negative effects on cell adhesion on fibronectin observed in an invasive hepatoma cell line by specifically down-regulating LIMK1 (Horita et al., 2008). In contrast, in MTLn3 mammary carcinoma cells, in which LIMK1 is upregulated and reported to be the dominating cofilin

kinase, LIMK1 and active P-LIMK1 do not appear enriched in a specific structure or in the cellular periphery, not even after epidermal growth factor (EGF) stimulation (Song et al., 2006).

An emerging role for scaffolding mechanisms?

A number of molecules have been suggested as possible scaffolding molecules for enzymes controlling the ADF/cofilin phosphorylation status (Fig. 3). Different 14-3-3 isoforms (β, γ, ζ) interact with inactive, phosphorylated SSH isoforms 1, 2 and 3 (Kligys et al., 2007; Nagata-Ohashi et al., 2004; Soosairajah et al., 2005), and this interaction may inhibit SSH activity (Nagata-Ohashi et al., 2004). Moreover, this interaction appears to compete with the F-actin-dependent SSH1 activation (Soosairajah et al., 2005) (Fig. 2). In cells stimulated with neuregulin, expression of 14-3-3γ prevents translocation of both SSH1L and cofilin to lamellipodia and inhibits cofilin dephosphorylation (Nagata-Ohashi et al., 2004). Furthermore, interactions between 14-3-3ζ and P-cofilin and LIMK have also been reported (Birkenfeld et al., 2003; Gohla and Bokoch, 2002), and SSH1L, Cfl and LIMK1 co-immunoprecipitate with several 14-3-3 isoforms (Soosairajah et al., 2005). This suggests that the various 14-3-3 isoforms can scaffold different components required to locally control cofilin activity (Fig. 3A) in a dynamic, probably signal-responsive manner.

Analogously, 14-3-3β was also identified as a modulator of integrin $\alpha4\beta6$-Rac1 activation of SSH1L in keratinocytes migrating on laminin (Kligys et al., 2007). These authors propose 14-3-3β as a controlling shuttle, promoting or inhibiting keratinocyte migration depending on its interaction with phospho-SSH1L in the cytosol or with integrin $\alpha4\beta6$ at the membrane. Finally as mentioned above, 14-3-3β was reported to bind and inhibit TESK1, an inhibition that is abrogated by integrin activation (LaLonde et al., 2005; Toshima et al., 2001c) (Figs. 2 and 3A).

A comparable multi-component complex, which potentially brings together both an ADF/cofilin inhibitor and activator and has a β-arrestin protein as scaffold, has also been proposed (Fig. 3B). β-Arrestins are recruited to several G-protein-coupled receptors, including activated protease-activated receptor PAR2 (Ge et al., 2003). β-Arrestins associate with cofilin, CIN and LIMK and redistribute together to membrane protrusions upon PAR2 activation. The net result, in breast cancer cell lines, is maximal cofilin dephosphorylation, 5 min after receptor activation (Zoudilova et al., 2007). Using β-arrestin knockout cells, it was demonstrated that the β-arrestin scaffolding proteins are required for this effect on cofilin activity. Whether CIN, LIMK and cofilin are all present together in a single macromolecular complex remains to be determined (Zoudilova et al., 2007).

Other examples of interactions that may scaffold ADF/cofilin-modulating enzymes either by sequestering them in the cytosol or by recruitment to specific cellular structures have been reported. In cortical neurons stimulated with bone morphogenic protein (BMP), LIMK1 is recruited to the tips of neurites by directly binding to the tail of the BMP receptor II and this interaction is implicated in dendritogenesis (references in Wen et al. (2007)) (Fig. 3C). In epithelial cells, LIMK2 (but not LIMK1) binds the polarity protein Par-3 that is important for proper assembly of tight junctions (Chen and Macara, 2006). Following growth factor-dependent Src kinase phosphorylation of Par-3, LIMK2 is released from this complex and locally affects actin dynamics (Wang, Y., et al., 2006) (Fig. 3D).
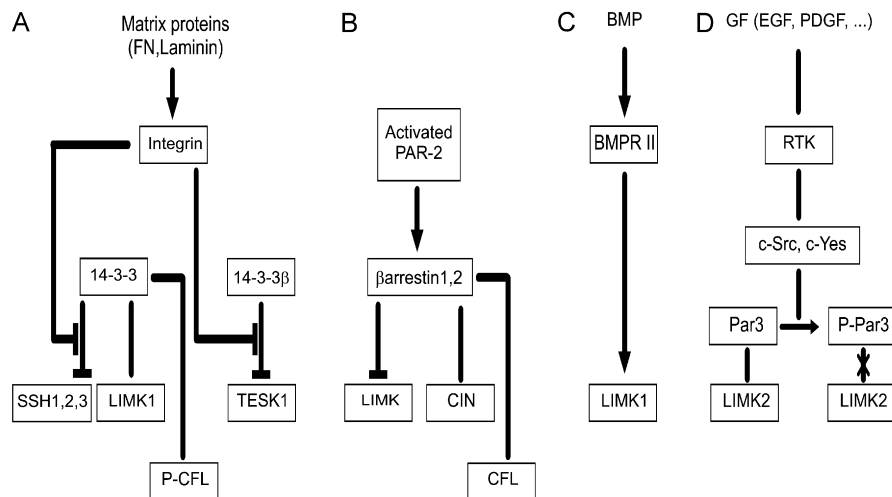

Fig. 3. Scaffolding mechanisms for cofilin regulators. Schematic representation of reported mechanisms, which scaffold one or more regulators of ADF/cofilin allowing localisation of cofilin activity and/or sequestration of cofilin to prevent activation. For details, see main text. Connecting lines, '⊥' and arrows indicate interactions, inhibiting interactions and activating interactions, respectively.

Given the diversity of cellular processes in which cofilin-dependent actin dynamics have been implicated, it is likely that many more of these scaffolding mechanisms are active in cells to recruit cofilin-activating and -regulating proteins to specific actin-rich regions or structures.

An inactivation/activation switch mediated by ADF/cofilin-polyphoshoinositide binding and release

Membrane polyphosphoinositides (PPI), in particular phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and phosphatidylinositol-4,5-bisphosphate (PIP_2), as well as the enzymes producing or hydrolysing these lipids upon cell stimulation (Fig. 4), are well-recognised signalling molecules controlling the dynamic turnover of the actin cytoskeleton and focal adhesions, and ultimately cell migration (reviewed in Ling et al. (2006)). PPI interaction affects the activity of several ABPs (reviewed in Niggli (2005)).

Since their discovery, ADF/cofilins have been known to interact with PPI in vitro (Yonezawa et al., 1990). Most studies focused on the cofilin–PIP_2 interaction although cofilin also binds with similar affinity to PIP_3 in vitro (Gorbatyuk et al., 2006; Ojala et al., 2001). The actin-binding capacity of ADF and Cfl is lost upon interaction with PIP_2. This inhibition is due to competitive binding since F-actin and PIP_2 target overlapping binding sites on cofilin (Van Troys et al., 2000). Actin turnover is needed at the cell periphery to form the membrane protrusions required for migration or at vesicles for their intracellular motility. In many cell types (Dawe et al., 2003; Nishita et al., 2005; Song et al., 2006; van Rheenen et al., 2007) cofilin, but not P-cofilin, is present at the membrane, strongly suggesting that PIP_2 acts to sequester unphosphorylated cofilin. Stimulus-induced PIP_2 hydrolysis and subsequent cofilin release would allow mobilisation of relatively high local concentrations of active cofilin near the membrane, i.e. exactly where its activity is needed (Fig. 4). The PIP_2-hydrolysing enzyme phospholipase C (PLC) has been shown to act upstream of cofilin activation in various stimulated cells (Matsui et al., 2001; Mouneimne et al., 2004; Zhan et al., 2003; Zhou et al., 2007). This provided indirect evidence for a PIP_2–cofilin interaction in cells. The release kinetics of cofilin from the plasma membrane upon PIP_2 hydrolysis downstream of EGF stimulation were recently studied in carcinoma MTLn3 cells (van Rheenen et al., 2007). In this cell line, a first transient of EGF-induced actin barbed end formation and polymerisation is crucially dependent on both cofilin and PLC activity (Mouneimne et al., 2004; Wang et al., 2007a). During this time span cofilin dephosphorylation does not occur, suggesting that the PIP_2-mediated pathway is important for initial cofilin activation, at least in cellular protrusions (Song et al., 2006; Wang et al., 2007a). By using fluorescence resonance energy transfer (FRET) and fluorescence loss in photobleaching (FLIP) techniques, it was shown that cofilin is released from the membrane following PIP_2 hydrolysis, and also that it is released in its active form and subsequently translocates to the actin filaments close to the membrane to generate new barbed ends (van Rheenen et al., 2007). This cofilin translocation may still be an indirect effect of PIP_2 hydrolysis mediated by an unidentified PIP_2-binding partner. Nevertheless, this work underscores that the PLC–PIP_2–cofilin pathway is a physiologically relevant activation/deactivation switch for ADF/cofilins, in addition to regulation by (de)phosphorylation.

Enzymes, other than PLC, also modulate PIP_2 levels in a stimulus-responsive manner. Several PI-5 kinases and PI-5 PPases (e.g. synaptojanins) have been shown to play a role in actin cytoskeleton dynamics and in cell migration and invasion (reviewed in Chuang et al. (2004), Heck et al. (2007)) (Fig. 4). It remains to be shown whether these enzymes are also actively involved in cofilin regulation via PIP_2.

Modulation of ADF/cofilin activity by pH

Variation in intracellular pH is an additional mechanism that can modulate ADF/cofilin activity in cells. Changes in pH over the physiological range (6.8–7.4) alter the severing capacity of active ADF/cofilin in vitro. Interestingly, a much more potent pH-dependency was observed for ADF than for Cfl1 both in vitro and in vivo (references in Bamburg and Wiggan (2002), and Bernstein et al. (2000)) suggesting differential cellular mechanisms for ADF/cofilin isoforms. Regulators of intracellular pH such as Na^+/H^+ exchanger, and more indirectly Na^+/K^+ pumps, are shown to enhance cell migration and to interact with components of the actin cytoskeleton, including cofilin (Chiang et al., 2008; Kaplan, 2005; Lee et al., 2001).

Modulation of ADF/cofilin activity by other ABPs

Actin-interacting protein 1 (Aip1) is a conserved WD-repeat protein that specifically enhances ADF/cofilin-induced actin dynamics (Iida and Yahara, 1999; Rodal et al., 1999). Aip1 itself has only small effects on actin filament dynamics in vitro, but by binding to cofilin it actively promotes severing by ADF/cofilins (references in Ono (2003, 2007)). The relative local levels of Aip1 and Cfl in cells will consequently be a determining factor in regulation of dynamic actin processes including cell migration efficiency, as demonstrated in T-lymphocytes and Drosophila S2 cells (Li et al., 2007; Rogers et al., 2003). In T-lymphocytes, Aip1 depletion indeed leads to
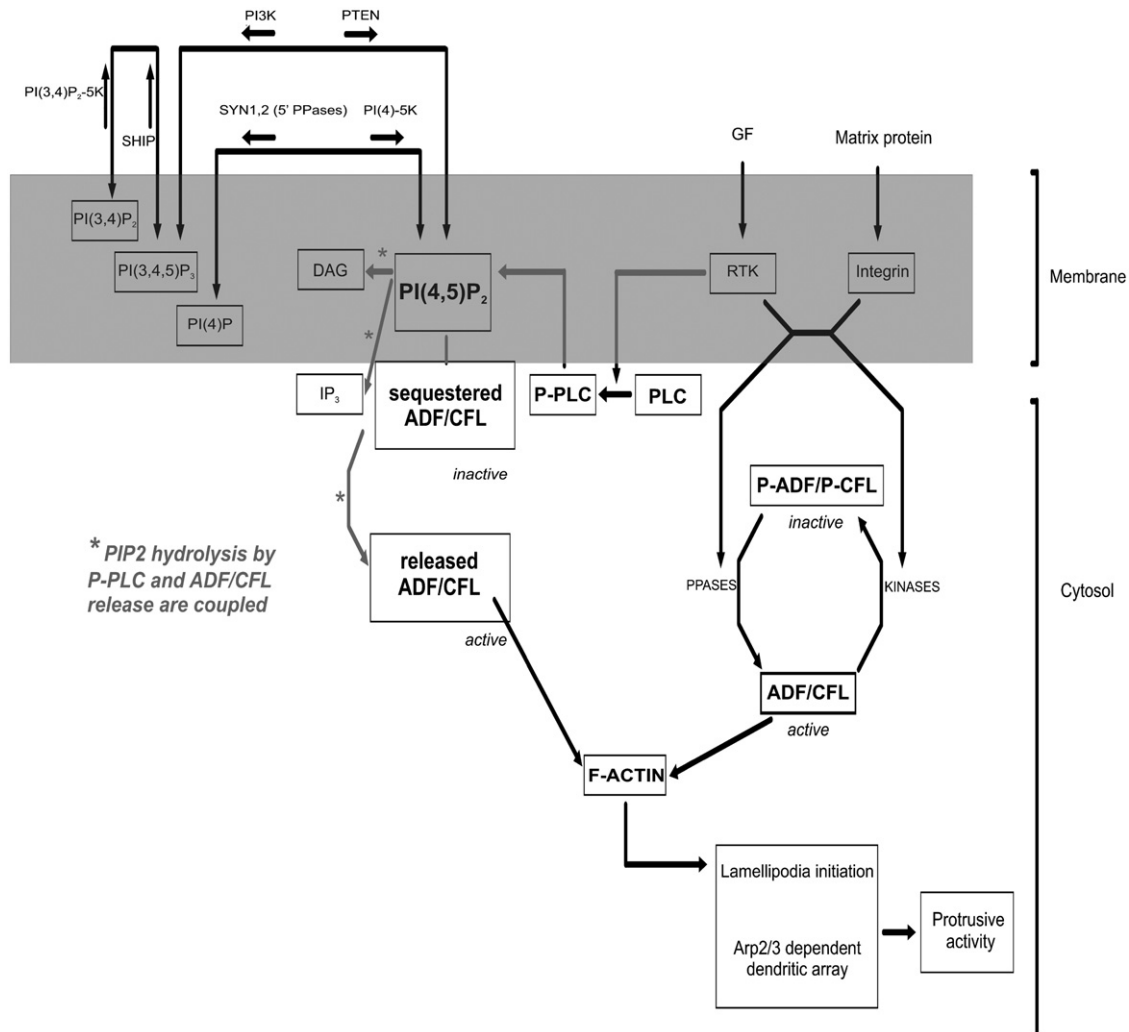
Fig. 4. The cofilin regulatory system: focus on regulation via PIP_2 interaction. This scheme shows the different enzymes that can directly or indirectly modulate PIP_2 levels (upper left), but focuses in particular on the phospholipaseC (PLC)-PIP_2 pathway downstream of growth factor (GF) stimulation. ADF/cofilin, which is sequestered by PIP_2, is inactive and is released as active protein upon hydrolysis of PIP_2 by activated, i.e. phosphorylated PLC (P-PLC). The released cofilin is translocated to F-actin (van Rheenen et al., 2007). This PLC-PIP_2 activation switch is acting in coordination with activation/deactivation by cofilin-dephosporylation/phosphorylation. The latter mechanism is shown on the right in a condensed manner (for details see Fig. 2). Acronyms: lipids, e.g. $PI(3,4)P_2$, polyphosphoinositide-3,4-bisphosphate; DAG, diacylglycerol; lipid kinases, e.g. $PI(3,4)P_2$-5K, polyphosphoinositide-3,4-phosphate-5-kinase; lipid phosphatases (PPases), SYN, synaptojanin; PTEN, phosphatase and tensin homolog; SHIP, Src homology-2 domain-containing inositol-5-PPase; IP_3, inositoltrisphosphate; (P-)PLC, (phospho)phospholipase C; (P-)ADF, (phosphorylated) actin depolymerisation factor; (P-)CFL, (phosphorylated) cofilin; GF, growth factor; RTK, receptor tyrosine kinase.

a strong migration defect, and intriguingly Aip1-cofilin activity is further enhanced via caspase-11 binding to both Aip1 and F-actin (Li et al., 2007).

ADF/cofilins cannot sever actin filaments that are decorated with high-molecular-weight tropomyosins. Tropomyosins are rod-shaped molecules that span several actin subunits along the actin filament long axis and have a strong stabilising effect. Tropomyosins also prevent the filament-branching activity of the Arp2/3 complex ((DesMarais et al., 2002; Ono, 2007) and references therein). The action of tropomyosin isoforms

results in different actin filament populations in discrete cellular regions. A growing protrusion consists of a tropomyosin-free, branched actin network (termed lamellipodium) at the extreme peripheral edge of the protrusion and a second actin network (termed lamellum) that contains tropomyosin-decorated filaments and depends on actomyosin contractility (Danuser, 2005) (Fig. 1B). Given the competitive binding between tropomyosins and ADF/cofilins, cofilin is mainly present in the tropomyosin-free lamellipodial region (DesMarais et al., 2002).

ADF/cofilins in cellular protrusions: a complex regulation system at work

ADF/cofilins are implicated in several cellular processes including neuronal outgrowth, T-cell activation, phagocytosis, endocytosis, receptor recycling, regulation of ion channels, and maybe, via the formation of actin–cofilin rods, in cellular ATP-energy management (Bamburg, 1999; Bamburg and Wiggan, 2002; Ono, 2007). In the following paragraphs, mainly the role of ADF/cofilins in migrating and invading cells is highlighted. Cell migration is needed for proper morphogenesis during development, for wound healing and for lymphocyte-mediated immune responses. It is also a determining feature of cancer malignancy as it is required for dissemination of tumour cells throughout the metastatic process (Van Troys et al., 2007). A common and early requirement for different forms of cell motility is the formation of membrane protrusions (lamellipodia, filopodia, pseudopods and also growth cones) via pushing forces mainly generated by actin polymerisation against the membrane. Chemotactic cells need to form these protrusions along extracellular gradients whereas neuronal growth cones display attraction or repulsion toward a guidance signal. This underscores the importance of spatio-temporal control of this actin-based protrusive activity.

The role of ADF/cofilins during protrusive activity in cells

Manipulation of ADF/cofilin activity – or that of its regulators – has been shown to affect formation of protrusions (growth cones, lamellipodia) and cell migration. The different biochemical activities of ADF/cofilins are probably important for obtaining either local assembly or local disassembly of actin filaments in cells. These two cofilin-mediated outcomes are not necessarily mutually exclusive and may depend on the local presence of other ABPs (Fig. 1C). We here discuss selected observations supporting either a role of ADF/cofilin in filament assembly or in disassembly in lamellipodial protrusions.

In mammary carcinoma cells, the activation of caged cofilin leads to the generation of new actin filament barbed ends and to actin filament assembly. Local photo-release resulted in local lamellipodia formation (Ghosh et al., 2004). This supports the fact that active cofilin can initiate actin filament assembly in cells albeit in combination with other ABPs. A model has been proposed by which new barbed ends (or nuclei), generated by cofilin, subsequently elongate and serve as basis for the Arp2/3 complex to initiate a dendritic array and lamellipodia formation (Andrianantoandro and Pollard, 2006; DesMarais et al., 2004; Ichetovkin

et al., 2002). Cofilin is from this perspective an early effector of the formation of lamellipodia since its local activation sets the site where new lamellipodia appear as well as the direction that a migrating cell takes (Ghosh et al., 2004; Mouneimne et al., 2006; Sidani et al., 2007). To result in filament disassembly, however, filament barbed ends that are locally generated by cofilin-mediated severing likely need to be barbed-end capped with high efficiency. This can lead to local depolymerisation of filaments from their pointed ends and an increase in actin monomers available for new polymerisation or assembly. In support hereof, global inactivation of cofilin in Cos and MCF7 cells reduces the amount of actin monomers in the cytosol and the latter had a negative effect on growth factor-stimulated lamellipodial outgrowth (Kiuchi et al., 2007).

The localisation of cofilin in lamellipodia is also indicative of its function in this cellular structure. In rapidly moving keratinocytes, ADF/cofilins were shown to localise to the middle and rear of the lamellipodium (which is the small treadmilling zone of the protrusion (Fig. 1B)) (Svitkina and Borisy, 1999). This observation is potentially consistent with both the assembly and disassembly activities of cofilins. In a rapidly moving keratinocyte, spacing may be created between the fast protruding edge and the zone where barbed ends are generated by cofilin. Conversely, the localisation in the lamellipodial rear fits the disassembly/monomer recycling scenario. In respect to the latter localisation, cofilin activity has recently also been suggested to be important in the segregation of a protrusion in a lamellipodium and a lamellum (Fig. 1B) and in the spatial interaction of these zones that appears important in efficient protrusion (Delorme et al., 2007). Recently, FRAP-based recovery kinetics in protruding lamellipodia have been reported for different ABPs in several cell types. This revealed that cofilin recovery was very different from that of the Arp2/3 complex, since it did not first reappear at the leading edge but simultaneously throughout the lamellipodium (Lai et al., 2008). This appears to suggest that cofilin may not have a prominent role in nucleation of actin filaments, at least not to maintain turnover rates in an existing and extending lamellipodium.

Despite a wealth of data indicating that ADF/cofilins are players in the cellular protrusive machinery, many apparently conflicting results have arisen from studies addressing their cellular function. The questions whether ADF/cofilins contribute to actin cytoskeleton assembly or disassembly in cells and in lamellipodia are still a matter of controversy, as well as whether they are positive or negative effectors of protrusive activity in cells. These contradictory results are, however, partly due to the inherent complexity of the cofilin-activating system (described above) of which

in many previous studies only one aspect (usually the phosphorylation status) was (or could be) assessed. Protrusive activity in cells has thus been linked to both high and low cofilin phosphorylation status and consequently either to cofilin inactivation or activation. As elaborated below, this likely is an oversimplification since in most cells several activation/deactivation systems will cooperate to regulate ADF/cofilin activity and, consequently, relying only on cofilin phosphorylation status to address cofilin activation may be misleading.

Taking into account the complexity of the ADF/cofilin regulatory system

Further understanding of the cellular impact of ADF/cofilins will benefit from comprehensive approaches. First, ADF/cofilin activity should be treated as output of the entire regulatory system, i.e. the different activation/deactivation switches. Cell stimulation may indeed simultaneously or sequentially activate multiple inhibitors and activators of cofilin activity within these different signaling pathways and ultimately it is their combined (antagonistic or synergistic) effect that is of interest. For example, several cell stimuli are known to signal both via small GTPases – that are upstream of ADF/cofilin kinases and PPases – and via enzymes modulating levels of PIP_2, a second cofilin regulator (see also Figs. 2 and 4).

Second, differences in timing and subcellular location of ADF/cofilin activity also need to be carefully considered, i.e. when, for how long and where exactly in the cell are ADF/cofilins activated? Separation of phosphorylation and dephosphorylation in time or subcellular space has indeed been demonstrated, for example during platelet activation (Pandey et al., 2007), during neuronal outgrowth (Endo et al., 2003, 2007; Meyer et al., 2005) and in T-lymphocytes (Nishita et al., 2005). A recent striking example is the asymmetric spatial distribution of P-cofilin levels observed across a single growth cone of a *Xenopus* spinal neuron that was stimulated from one side with BMP-7 (Wen et al., 2007). Intriguingly, with increasing time of stimulation a switch occurred in the spatial asymmetry. Concomitantly the growth cone switches from attractive to repulsive movements towards the signal. This points to a delicate balance between kinase and PPase activities during BMP 7-induced growth cone turning (Wen et al., 2007). Another study addressing the complexity of cellular ADF/cofilin activation was performed in breast cancer MTLn3 cells. This clearly illustrates the requirement to tackle cofilin activation in a comprehensive manner, i.e. by addressing the different activation mechanisms and their specific spatial activities (van Rheenen et al., 2007). As described above, active cofilin in MTLn3 cells is transiently released from the membrane after EGF stimulation and PLC-mediated PIP_2 hydrolysis and this is followed by translocation to the peripheral F-actin where new barbed ends are generated (Wang et al., 2007a). Nearly simultaneously, cofilin inactivation by phosphorylation is also occurring and this is mediated by LIMK. Importantly, LIMK is active throughout the cells. Based on these observations, a local excitation-global inhibition (LEGI) model was proposed for cofilin control in these mammary carcinoma cells (Mouneimne et al., 2006; van Rheenen et al., 2007; Wang et al., 2007a). Following this model, the global inactivation of cofilin is only compensated at the membrane facing the EGF gradient where PLC is slightly more active. This results in a sharp zone of cofilin activity near the membrane where lamellipodia are being formed to drive directional migration. The transient PLC-dependent peripheral activation is followed by a second phase of actin polymerisation, mediated by other ABPs, during which lamellipodial outgrowth in the direction of the signal can occur (Song et al., 2006). The transient character of the PLC-dependent activation of cofilin at the membrane may allow MTLn3 cells to swiftly respond to changes in the orientation of the external gradient. In line with this LEGI model, the phenotype of cofilin suppression in migrating MTLn3 cells is decreased turning frequency and highly directional migration (Sidani et al., 2007).

It remains to be verified whether the LEGI model, which strongly depends on PLC-mediated activation, also applies to other chemotactic cells or whether other combinations of cofilin regulatory mechanisms are used to control and restrict cofilin activation. Given the complexity and versatility of the cofilin regulatory system, it is likely that, depending on cell type, on mode of migration, on cell stimulus etc., the various activation/deactivation switches may indeed cooperate in a different manner. Current findings indicate that in some cellular systems, activation by dephosphorylation in the peripheral zone is more prominent than for example observed in MTLn3 cells (Dawe et al., 2003; Nishita et al., 2005; Soosairajah et al., 2005). It remains to be determined whether PIP_2-mediated cofilin release is occurring. It is also not unlikely that cofilin control may be very different in polarised cells that – in contrast to amoeboid moving cells (like MTLn3 cells) – inherently move in a directional fashion. Indeed, Rho-ROCK signalling, which controls cofilin activity, is very different in cells moving as amoeboids or in a mesenchymal fashion (Sahai and Marshall, 2003). In line with this argument, downregulating cofilin has a different effect in fibroblast-like cells. These cells lose their inherent polarity and form protrusions in multiple directions, which is in contrast to MTLn3 cells that become more polarised after cofilin depletion (Dawe et al., 2003; Sidani et al., 2007). Consequently,

further studies may reveal that cofilin activation during chemotaxis follows not just one universal mechanism.

ADF/cofilins and cancer cell invasion

In a malignant primary tumour, cancer cells invade surrounding tissues and reach blood or lymphatic vessels. This and subsequent steps of the metastatic process, that ultimately lead to the formation of secondary tumours at distant sites, require active migration in a complex microenvironment. The actin machinery is of central importance in the regulation of cell migration. Hence, the role of its various components, including ADF/cofilins, in cancer cell invasion is under intense investigation (reviewed in Van Troys et al. (2007), Wang et al. (2007a)).

ADF/cofilins have been implicated in invadopodia formation (Yamaguchi et al., 2005). Invadopodia are actin-rich membrane extensions that adhere to and protrude into the matrix. They are transiently formed by aggressive tumour cells and are hot spots of proteolytic degradation of the extracellular matrix (Gimona, 2008; Linder, 2007). Suppression of cofilin expression in MTLn3 cells resulted in formation of short-lived invadopodia with less matrix degradation activity that did not fully mature (Yamaguchi et al., 2005). In addition, expression of constitutively active cofilin upregulates the expression of the matrix-degrading enzymes matrix metalloproteinase 2 (MMP2) and 14 in melanoma cells (Dang et al., 2006). Similarly, expression of both the serine protease urokinase type plasminogen activator (uPA) and its receptor uPAR are higher in the breast cancer cell line MDA-MB-435 upon LIMK1 overexpression (Bagheri-Yarmand et al., 2006). uPa is a also matrix-degrading protease and an activator of multiple other matrix proteases, including MMPs. In breast tumours, a coordinated up regulation of cofilin and uPAR is observed (Bagheri-Yarmand et al., 2006). These data suggest a correlation, but do not directly link between cofilin activity and invasive potential.

Other studies have reported that ADF/cofilins or its regulators, especially LIMK1, display altered expression levels in invasive or metastatic cancer cells (reviewed in Wang et al. (2007a)). In two mouse models of mammary cancer, high cofilin activity is correlated with invasion, intravasation and metastasis (Wang, W., et al., 2006). This is apparently achieved by changes in the cofilin regulatory system triggered by the tumour microenvironment. This was analysed in an MTLn3 xenograft in mammary fat pad of rats (Wang et al., 2004) and in the polyoma-middle-T oncogene (PyMT)-derived mammary tumour mouse model (Wang et al., 2007b). The migrating population of these tumour cells was collected using an in vivo invasion assay and compared to the bulk tumour via micro-array analysis. Transcript changes were found that are in agreement with low proliferation, high cell survival and upregulated motility for the invasive cell population (Wang et al., 2004). The transcripts within the cofilin system that are simultaneously changed in these studies intriguingly include both inhibitors and activators of cofilins. Indeed, in the xenograft model the level of the cofilin transcript is increased in combination with that of its inhibitor LIMK1 (Wang et al., 2004). In the PyMT mouse model, LIMK1 and SSH1 transcript levels are simultaneously increased. These simultaneous deregulations within the cofilin system, observed in correlation with invasion, appear in accord with in vitro findings of cofilin regulation during protrusive activity. This suggests that also during growth factor-induced invasion in a three-dimensional matrix, strict regulation of cofilin activity may be required, possibly to obtain activation in one cellular region combined with inactivation in a different region. The data from different tumour cells in addition indicate that different strategies of transcript level changes can result in the necessary control of cofilin activity for increased migration/invasion. This may also explain why in some invasive cell lines or tumours upregulation of LIMK1 was reported whereas in others this protein appeared downregulated. Information on the entire (de)phosphorylation pathway is clearly required to make valid statements on the correlation of cofilin activity with invasive capacity. The situation may even be more complex since in the invasive subpopulation of the primary tumours analysed by Wang et al. (2004, 2007b) several LIMK activators as well as modulators of PIP$_2$ levels (a PI4P-5 kinase and PLC) are also upregulated. This indicates that changes within the cofilin system occur at different hierarchical levels of regulation as well as in different activation switches. Finally, little is known about possible isoform-specific changes in invasive cancer cells at the different levels of cofilin regulation. A recent report describing a specific role for ADF in invasion of human colon cancer cells (Estornes et al., 2007) indicates that it is warranted to analyse in more detail isoform usage in the altered cofilin activation/deactivation system in invasive cancer cells.

Conclusions

The actin-binding proteins of the ADF/cofilin family play a key role in cell migration and cancer cell invasion. Not surprisingly, their activity is strictly regulated. Many regulatory cascades have been described, with (de)phosphorylation and PIP$_2$ binding and release as the currently best-documented activation/deactivation switches. Extracellular stimuli appear to initiate a combination of these activating/deactivating switches to create spatially, as well as temporally, segregated

pools of active and inactive cofilin. For mammary carcinoma cells in a chemotactic gradient, the interplay between the different regulatory mechanisms results in local excitation at the front of the cells and global inactivation in the rest of the cell. Future research will require a comprehensive approach in which cofilin activity is studied as the output of all regulators, each with a spatially and temporally finely tuned activity. Current data in cancer cell lines and rodent tumour models suggest that the ADF/cofilin system is altered in correlation with invasive potential. This system consequently holds promise as a potential target in invasion-inhibiting strategies. Successful strategies will, however, need to focus on the entire regulatory system and not on single components.

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