Cofilin phosphatases and regulation of actin dynamics
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Cofilin is a ubiquitous actin-binding factor required for the reorganization of actin filaments in eukaryotes. The dephosphorylation of cofilin enables its actin severing and depolymerizing activity and drives directional cell motility, thus providing a simple phosphoregulatory mechanism for actin reorganization. To date, two cofilin-specific phosphatases have been identified: Slingshot and Chronophin. These cofilin phosphatases are unrelated in sequence and regulatory properties, each potentially providing a unique mechanism for cofilin activation under varying biological circumstances.

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Introduction
The reorganization of actin at the leading edge of eukaryotic cells is a fundamental aspect of cell motility, and requires the coordinated function of both actin-polymerizing and actin-depolymerizing/severing factors. To drive the cell front forward, branched actin filaments are generated at the leading edge through the action of the Arp2/3 complex and/or filamin A [1,2]. Actin depolymerizing components, including proteins of the cofilin/ADF (actin depolymerizing factor) family, disassemble F-actin from the rear of the actin network to recycle actin monomers to the leading edge for further rounds of polymerization. There is also growing evidence that cofilin-dependent F-actin severing activity is important for actin polymerization, as cofilin-severed F-actin fragments act as preferred substrates from which Arp2/3 builds actin networks [3]. Precise spatial regulation of cofilin-dependent actin-depolymerizing/severing activity appears to be crucial to cell motility, since site-specific activation of caged cofilin can determine the direction of cell movement [4**].

We discuss here current knowledge of the regulation and biological roles of the cofilin phosphatases in actin dynamics.

Single-site phosphoregulation of cofilin
Cofilin and related ADF family proteins (henceforth generically referred to as cofilin) are phosphorylated at a conserved N-terminal serine, Ser3 [5]. Early studies revealed that in its phosphorylated form cofilin is unable to bind actin, and that dephosphorylation of this site reactivated the actin-depolymerizing activity of cofilin [6]. From these studies, it became clear that cofilin phosphorylation/dephosphorylation at Ser3 acts as a simple switch for actin assembly and disassembly/severing (Figure 1).

Two kinase families have been shown to phosphorylate and deactivate cofilin: the LIM (Lin-11/Isl-1/Mec-3) kinases and the TES (testicular protein) kinases. Interestingly, no other substrates have been identified for these kinases. The LIM kinases, LIMK1 and LIMK2, are ubiquitous in their tissue distribution [7,8], whereas TESK1 is expressed most abundantly in testicular tissue [9,10]. LIM kinases are activated by phosphorylation of Thr508/505 (LIMK1/2) within the kinase activation loop through divergent Rho GTPase pathways: Rac/Cdc42 acting through p21-activated kinase (PAK)1 and PAK4 [11,12], Cdc42 acting through MRCK (myotonic-dystrophy-related cdc42-binding kinase) [13], and RhoA through ROCK (Rho-associated coiled-coil-containing kinase) [14]. By contrast, TESK activation is dependent on integrin engagement upon cell attachment, and occurs independently of PAK1/ROCK activation [15]. Functionally, the LIM and TES kinases promote F-actin stability through cofilin dephosphorylation and deactivation, as their overexpression in cell lines promotes F-actin accumulation [15].

Clearly, LIMK regulation is a key factor in the cofilin phospho-regulatory switch mechanism, but it was not certain whether lack of LIM kinase activity is sufficient to cause the decreases in phosphorylation that result in cofilin activation, or whether phosphatase activation is also necessary. In some cell types, cell stimulation induced changes in net phosphocofilin levels, while in other cells (e.g. 3T3 and A431) a significant increase in phosphocofilin turnover was observed with no significant change in the total phosphocofilin pool [16]. The latter observations suggested that the activity of both cofilin kinases and cofilin phosphatases was being regulated by common upstream stimulatory signals. As LIMK and TESK evolved the specific task of phosphorylating cofi-
lin, the possibility was raised that cofilin-specific phosphatases might also exist.

Although earlier studies implicated the involvement of phosphatases with broad substrate specificities, such as PP1, PP2A and PP2B, inhibitors active against these phosphatases largely fail to block cofilin dephosphorylation [5]. Such observations suggested that these general phosphatases may not account for the majority of cofilin phosphatase activity observed in response to cellular stimuli. Recent observations also indicate that cofilin dephosphorylation can be spatially restricted within specific cellular subcompartments [17,18] and that directed cofilin phosphatase activity within these locations may be sufficient to drive processes such as cell protrusion and motility, neurodegenerative stress responses, and even apoptosis.

Thus far, two types of cofilin-selective phosphatases have been identified: the Slingshot family of phosphatases, and Chronophin.

**Slingshot, a conserved family of cofilin phosphatases**

Slingshot was initially identified as a dedicated cofilin phosphatase through genetic studies in *Drosophila*, where its dysfunction was noted to cause disorganized epidermal cell morphogenesis, including splintered hair bristles (hence the name Slingshot) [19]. A single gene (*D-ssh*) in *Drosophila* codes for the 125-kDa Slingshot (dSSH)
protein. In both human and mouse, the Slingshot phosphatases are represented by three genes (SSH-1, -2, and -3), each with long and short variants with distinct tissue expression patterns (Table 1). SSH seems to be widely expressed in various organisms, but is notably absent in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Arabidopsis thaliana*. In mammalian cells SSH-1L, along with SSH-2L and SSH-3L, dephosphorylates both phospho-ADF and phospho-cofilin at the critical Ser3 residue, thereby suppressing actin filament assembly induced by LIMK1 or TESK1 [19]. Notably, SSH-3L was less effective in dephosphorylating these substrates in comparison with the two other isoforms. SSH phosphatases contain a protein phosphatase domain (PTP) with a canonical catalytic HCxxGxxR sequence found within other dual-specificity and protein tyrosine phosphatases. Although SSH-1L and SSH-2L both co-localize with actin filaments in mammalian cells through a C-terminal F-actin binding region, the three SSH family phosphatases differ in their subcellular localization, F-actin binding activity, specific activity and tissue expression patterns (Table 1) [20].

**Biological activities**

Localized cofilin function was previously shown to be of importance during cell division, which suggested that cofilin regulators may also be involved in the cell division process. Indeed, SSH-1L was found to localize at the cleavage furrow and midbody during cytokinesis [21]. Localization to these sites correlated with elevated SSH-1L activity during telophase and cytokinesis, and coincided with the temporal pattern of cofilin dephosphorylation during these stages [21]. Overexpression of phosphatase-inactive SSH1 led to aberrant accumulation of F-actin and phosphocofilin during late cytokinesis, accompanied by frequent regression of the cleavage furrow and formation of multinucleated cells, further supporting a role for SSH in cell division.

SSH has also been implicated in the control of the motility and morphology of the growth cone and in neurite extension through cofilin regulation. SSH induced highly motile growth cones and enhanced neurite extension rates, while antagonizing the repressive effects on growth cone behavior of LIMK1 [22]. SSH may regulate actin dynamics during membrane protrusion, as SSH-1L is activated in insulin-stimulated MCF7 cells and accumulates in protrusions where active cofilin, but not inactive phosphocofilin, is concentrated [23,24]. These studies reflect multiple roles for SSH in various cell types.

**Localization**

As site-specific cofilin activation was previously shown to be critical for the regulation of various biological processes,
the localization of SSH family phosphatases to specific subcellular sites is also likely to be required for their role in regulating actin dynamics. During cell motility, coflin is an important catalyst of actin filament disassembly required to maintain lamellipodial protrusion. To facilitate actin reorganization at the leading edge, it has been reported that SSH becomes activated through its release from 14-3-3 in the cytoplasm, consequently translocating to the F-actin-rich lamellipodia during cellular stimulation with neuregulin-1 [24]. The subcellular distribution of SSH-1L varies in mitotic HeLa cells, ranging from a uniform colocalization with cortical F-actin throughout prophase and metaphase to a distribution coincident with F-actin staining at the cleavage furrow during telophase [21].

The extent to which SSH localization is determined by its F-actin binding activity or through its association with other cellular components is still not entirely clear. For example, SSH localization to insulin-induced membrane protrusions was found to be PtdIns-3-kinase-dependent [23]. Whether this was due to a direct regulatory effect of PtdIns-3-kinase activity on SSH localization as opposed to an effect on SSH binding to F-actin remains unknown. Moreover, recent evidence indicates that 14-3-3 proteins associate with coflin and SSH-1L in a phosphorylation-dependent manner, thereby preventing coflin dephosphorylation and translocation of SSH to F-actin-rich cortical regions, respectively [24,25,26]. 14-3-3 proteins may therefore play a role in the formation of localized regulatory complexes, which include coflin, LIMK and F-actin as well as SSH [25,26,27].

Regulation
Multiple signaling pathways, including those involving Ca²⁺, cAMP and PtdIns 3-kinase, have previously been proposed to modulate stimulus-induced coflin dephosphorylation in many different cell types. Accordingly, several of these coflin-dephosphorylating pathways have now been linked to SSH. Recent studies have shown that the Ca²⁺ ionophore A23187 and Ca²⁺-mobilizing agonists, such as ATP and histamine, induced SSH-1L activation, correlating with coflin dephosphorylation in vivo [28]. Calcineurin, a calcium-regulated protein phosphatase, was observed to dephosphorylate and activate SSH-1L, suggesting the idea that SSH-1L is negatively regulated by phosphorylation. Indeed, SSH activity was inhibited by PAK4-mediated phosphorylation, and dephosphorylation of SSH by λ-phosphatase was shown to boost its phosphatase activity [26]. In most systems, there is also evidence for calcium-independent regulatory pathways. The PtdIns 3-kinase inhibitor wortmannin has been shown to antagonize coflin dephosphorylation induced by several receptor stimuli [5]. Nishita et al. (2004) reported that insulin-dependent actin reorganization occurs through PtdIns 3-kinase and SSH, since insulin-stimulated MCF7 cells exhibit SSH activation and coflin dephosphorylation that is abrogated by PtdIns 3-kinase inhibition [23]. Additionally, SSH phosphatase activity appears to be directly stimulated up to 10-fold by F-actin binding, indicating that existing actin filaments may participate in promoting additional actin reorganization through SSH/cofilin activation [24,26].

Interestingly, recent evidence indicates that SSH phosphatase activity may not be limited to coflin, as SSH appears to dephosphorylate and inactivate LIMK1 [26]. SSH-1L and LIMK1 form a complex, resulting in dephosphorylation of both autophosphorylated LIMK1 sites and the critical Thr508 residue in the activation loop of LIMK1, thereby inactivating its ability to phosphorylate coflin. This finding probably explains the robust coflin dephosphorylation observed in vivo upon overexpression of SSH-1L. These observations complicate our understanding of coflin regulation, as it is difficult to demonstrate whether changes in phosphocofilin levels are due to direct SSH-mediated coflin phosphatase activity, SSH-mediated LIMK inactivation, or a combination of both. Furthermore, it is unclear whether certain signals upstream of SSH can affect its specificity towards LIMK or coflin, and whether different signals can affect the association of SSH with LIMK. These issues will have to be addressed in any future studies involving the biological effects of SSH-1L.

Chronophin, a unique HAD-family coflin phosphatase
Chronophin (CIN) has been identified as a second potential coflin phosphatase through a biochemical screen based on in vitro dephosphorylation of coflin [29]. CIN belongs to the haloacid dehalogenase (HAD) family of phosphatases, whose involvement in mammalian signal transduction pathways is poorly characterized. CIN contains a highly conserved catalytic domain and three conserved sequence motifs characteristic of the HAD hydrolases. HAD phosphatases use an unconventional catalytic mechanism whereby a transition state phospho-aspartate intermediate forms as a result of nucleophilic attack on the substrate phosphate group [30]. Mutation of the nucleophilic aspartate (Asp25 in CIN) markedly compromises CIN activity in vitro. Interestingly, CIN has also been shown to exhibit pyridoxal (vitamin B6) phosphatase activity [31,32].

In addition to its biological activity (described below), CIN exhibits several unique characteristics that suggest it may represent a novel means of regulating coflin-dependent actin dynamics. First, HAD family members bearing similarity to CIN can be found in many organisms, including several that lack SSH orthologs (e.g. yeast and bacteria), and is widely distributed in human tissues, being particularly abundant in brain. Second, CIN coflin phosphatase activity is insensitive to classical thiol-based serine/threonine phosphatase inhibitors, as reported for


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physiological cofilin phosphatase activity [5,33]. Third, CIN exhibits several predicted interaction motifs potentially linking CIN to regulation by both PtdIns3-kinase and PLCγ, both of which have been suggested to be involved in signaling to cofilin dephosphorylation in vivo [5].

**Biological activities**

CIN dephosphorylates a limited number of protein substrates in vivo and in vitro (see supplementary data to [29**]), and is active against both cofilin and ADF in vivo [29**]. As opposed to SSH, CIN fails to exhibit significant activity towards LIMK, perhaps accounting for its less dramatic effect on phosphocofilin levels when overexpressed in cells. However, CIN overexpression does decrease basal phospho-cofilin levels, and effectively antagonizes the enhanced cofilin phosphorylation stimulated by LIMK. By contrast, catalytically inactive CIN or CIN depletion by siRNA induces enhanced cellular phospho-cofilin pools. High CIN activity is consistently associated with decreased cellular F-actin content, while decreased CIN activity promotes the accumulation of polymerized actin. CIN localizes to actin-rich ruffles and membrane protrusions, and our preliminary studies indicate that CIN function is directly linked to actin dynamics at the leading edge of motile cells (C DerMardirossian, V Delorme and GM Bokoch, unpublished).

Manipulation of CIN activity produces pronounced effects on cell division. Specifically, the overexpression of dominant negative forms of CIN (or downregulation by CIN siRNA treatment) causes aberrant actin assembly, frequent cleavage furrow regression, and a significant population of multinucleated cells, correlating with changes in phosphocofilin levels. Similar defects have been reported when cofilin (Twinstar in Drosophila) function is disrupted genetically in the fruit fly, and upon the injection of cofilin antibodies in dividing Xenopus blastomeres, suggesting that CIN and cofilin function are linked during cell division [34,35]. The colocalization of CIN with cofilin to the ingressing cleavage furrow and to the actomyosin contractile ring at later mitotic stages supports the observation that CIN function is critical during cell division. Interestingly, CIN appeared to localize to membranous regions at the cell poles during telophase, and seemed to have a more cytoplasmic distribution with limited localization to the cleavage furrow at earlier stages of mitosis. This differs from SSH, which strongly localizes to the ingressing furrow at early telophase, suggesting that these two cofilin phosphatases may play distinct regulatory roles during cell division (Table 1).

**Conclusions**

The identification of two unrelated phosphatases, SSH and CIN, that selectively dephosphorylate cofilin brings into question whether these phosphatases work redundantly or whether they activate cofilin in specific functional contexts. Superficially, both phosphatases associate with actin (CIN through its colocalization with cellular F-actin, and SSH through direct binding), and the overexpression or dysfunction of both phosphatases leads to qualitatively similar cellular phenotypes. However, the two phosphatases are not functionally redundant in the context of mitosis, as siRNA-mediated depletion of either protein in the same cell type caused distinct temporal changes in phospho-cofilin levels in synchronized mitotic cells, and depletion of both proteins caused enhanced increases in phospho-cofilin levels [29**]. Viewed from a reductionist perspective, the lack of any sequence similarity between SSH and CIN suggests that the phosphatases may fundamentally differ in their biochemical nature (for example, how they are activated and regulated), and in their regulatory function. Furthermore, associated signaling proteins have been identified that may form localized regulatory complexes and hence modify the functional properties of these cofilin phosphatases. A challenge for future studies will be to investigate the activities of cofilin phosphatases in the context of the normal biological, regulatory, spatial and temporal constraints that control their function in actin dynamics.

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**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


Using a photoactivatable form of cofilin, the authors show that spatially localized activation of cofilin will drive cell protrusion and determine the direction of cell migration.


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neuregulin-induced activation of cofilin-phosphatase

F-actin is found to stimulate slingshot (SSH) phosphatase activity in vitro, whereas 14-3-3 is found to inhibit SSH activity. Neuregulin stimulation induces SSH accumulation in lamellipodia and F-actin-enriched detergent fractions, whereas overexpressed 14-3-3 inhibits this translocation, localizing SSH to the cytoplasm.


29. Goila A, Birkenfeld J, Bokoch GM: Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics. Nat Cell Biol 2005, 7:21-29. This article describes the biochemical isolation using a novel in-gel phosphatase assay of chronophin (CIN), an HAD family phosphatase from bovine brain, and its characterization as a functional cofilin phosphatase. CIN overproduction and siRNA downregulation are shown to affect the cellular phosphocofilin pool, particularly in mitotic cells, thereby inducing abnormal cell division and aneuploidy.


