14-3-3 Regulates Actin Dynamics by Stabilizing Phosphorylated Cofilin

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Summary

The functionality of the actin cytoskeleton depends on a dynamic equilibrium between filamentous and monomeric actin. Proteins of the ADF/cofilin family are essential for the high rates of actin filament turnover observed in motile cells through regulation of actin polymerization/depolymerization cycles [1, 2]. Rho GTPases act through p21-activated kinase-1 (Pak-1) [3] and Rho kinase [4] to inhibit cofilin activity via the LIM kinase (LIMK)-mediated phosphorylation of cofilin on Ser3 [5, 6]. We report the identification of 14-3-3ζ as a novel phosphocofilin binding protein involved in the maintenance of the cellular phosphocofilin pool. A Ser3 phosphocofilin binding protein was purified from bovine brain and was identified as 14-3-3ζ by mass spectrometry. The phosphorylation-dependent interaction between cofilin and 14-3-3ζ was confirmed in pulldown and coimmunoprecipitation experiments. Both Ser3 phosphorylation and a 14-3-3 recognition motif in cofilin are necessary for 14-3-3 binding. The expression of 14-3-3ζ increases phosphocofilin levels, and the coexpression of 14-3-3ζ with LIMK further elevates phosphocofilin levels and potentiates LIMK-dependent effects on the actin cytoskeleton. This potentiation of cofilin action appears to be a result of the protection of phosphocofilin from phosphatase-mediated dephosphorylation at Ser3 by bound 14-3-3ζ . Taken together, these results suggest that 14-3-3ζ proteins may play a dynamic role in the regulation of cellular actin structures through the maintenance of phosphocofilin levels.

Results and Discussion

Purification of a Ser3 Phosphocofilin Binding Protein

Bovine brain cytosol was fractionated by DEAE anion exchange chromatography, and column fractions were tested for cofilin binding proteins by using an overlay assay with in vitro Ser3-phosphorylated *Xenopus* cofilin (XAC-P). *Xenopus* and human cofilin are highly homologous, and their actin binding and -depolymerizing activities are equally inhibited by LIMK-mediated Ser3 phosphorylation [1]. We observed that a 31 kDa protein (p31) bound to Ser3-phosphorylated cofilin but not to unphos-

phorylated cofilin (Figure 1A). A protein of 49 kDa bound cofilin regardless of its phosphorylation state. In order to exclude the possibility that the p31/XAC-P interaction resulted from cofilin phosphorylation on other sites in addition to Ser3 (catalyzed by unidentified kinases during the in vitro phosphorylation reaction), overlay assays were performed with the LIMK phosphorylation site mutant XAC-S3A, which was subjected to a typical in vitro kinase reaction. As shown in the bottom panel of Figure 1A, p31 did not detectably associate with XAC-S3A. These results suggest that p31 represents a protein that specifically binds to Ser3-phosphorylated *Xenopus* cofilin.

Identification of p31 as 14-3-3ζ by Mass Spectrometry

Employing the XAC-P overlay assay to detect p31, the protein was purified to near homogeneity (Figures 1B–1D) and was sequenced by MS/MS tandem mass spectrometry. The peptide sequences obtained corresponded exclusively to members of the 14-3-3 family of proteins. Notably, 14-3-3ζ was by far the most abundant 14-3-3 protein present in the fraction, and the generated peptides encompassed about 70% of the sequence of 14-3-3ζ. The 14-3-3 proteins are a family of ubiquitously expressed and evolutionarily conserved molecules, including seven highly homologous mammalian isoforms (β, ε, γ, η, σ, τ, and ζ) that primarily exist as homo- or heterodimers. 14-3-3 proteins play key regulatory roles in signal transduction, cell cycle control, and cell survival by binding to a multitude of functionally diverse ligands and affecting their enzymatic activities, directing their subcellular localization, or altering their association with other cellular components [7–10]. To our knowledge, a role for 14-3-3 proteins in cytoskeletal regulation has not been reported.

14-3-3ζ and Ser3 Phosphocofilin Associate In Vitro and In Vivo

The interaction between 14-3-3 and cofilin was confirmed in pulldown binding experiments with GST-14-3-3ζ. Cos-1 cells were either mock transfected or transfected with the constitutively active LIMK1 fragment Kd3 [3, 5, 6] in order to specifically increase the endogenous pool of Ser3 phosphocofilin. In lysates of control cells, only a small amount of cofilin was able to bind GST-14-3-3ζ. In contrast, the cofilin/14-3-3ζ interaction was strongly increased by expression of Kd3 (Figure 1E). Similar results were obtained with GST-14-3-3τ (not shown). Immunoblotting of precipitated proteins with α-phosphocofilin antibodies clearly confirmed that 14-3-3ζ specifically pulled down the phosphorylated cofilin in both control and Kd3-expressing cells. Furthermore, pretreatment of cell lysates with the serine/threonine phosphatases PP1 and PP2A (which dephosphorylate cofilin in vitro; [11] and data not shown) completely abolished the cofilin/14-3-3ζ interaction.

Figure 1F shows that endogenous cofilin coimmuno-

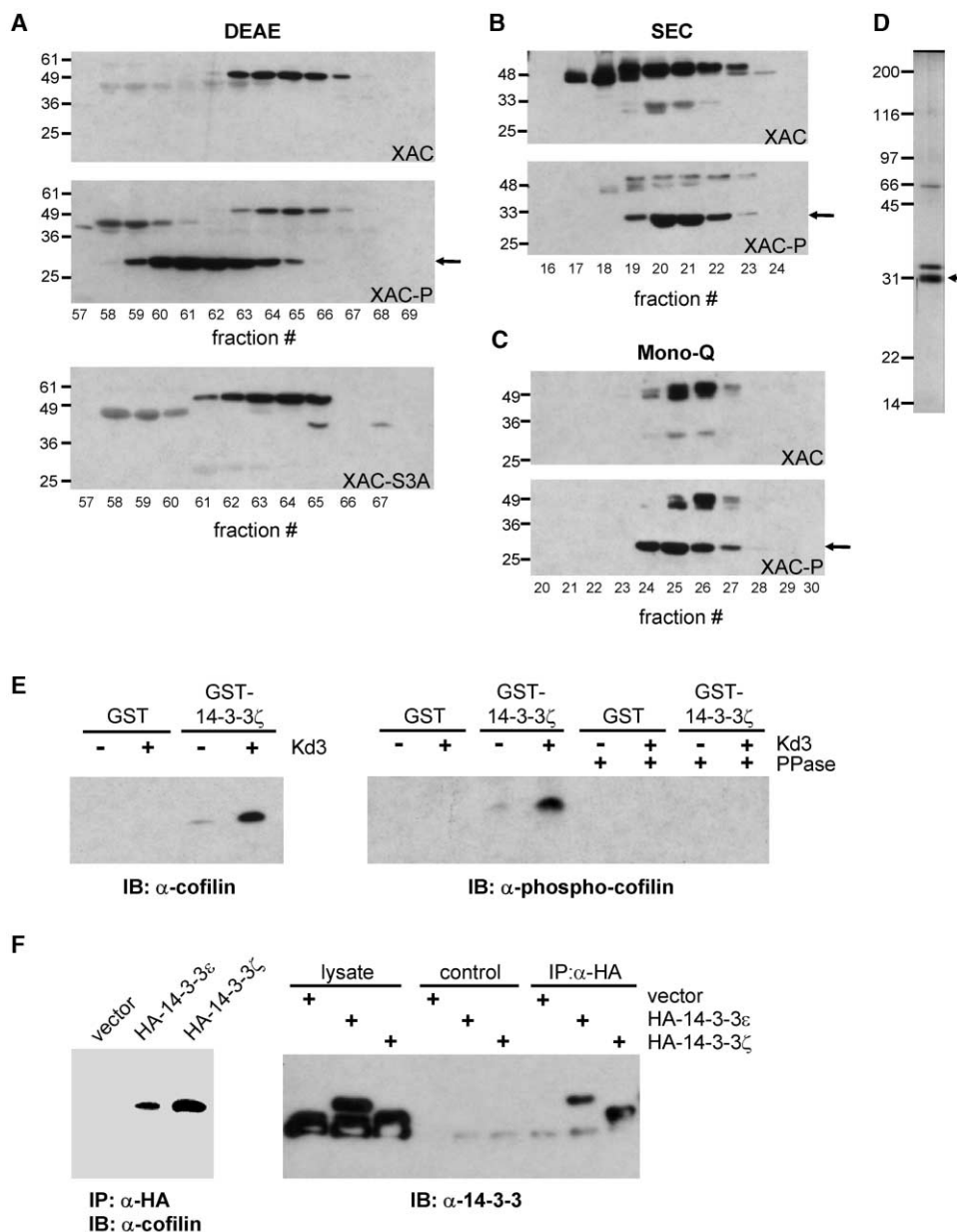[1]Correspondence: bokoch@scripps.edu

Figure 1. Purification and Identification of a Ser3 Phosphocofilin Binding Protein

(A–C) Bovine brain cytosol was fractionated as described in the Experimental Procedures by (A) DEAE, (B) SEC, and (C) Mono-Q column chromatography and was tested for cofilin binding proteins by using an overlay assay with purified XAC, XAC-P, or XAC-S3A. Far Western immunoblots detecting bound cofilin are shown. p31 is indicated by an arrow, and the position of molecular weight marker proteins is given on the left.

(D) A silver-stained gel of the Mono-Q fraction #24 after SDS-PAGE separation is shown. p31 is indicated by an arrowhead and was identified as 14-3-3 by tandem mass spectrometry.

(E) Cos-1 cells were transfected with empty vector or the LIMK1 fragment Kd3 to increase the endogenous pool of Ser3 phosphocofilin and were incubated with GST or GST-14-3-3ζ immobilized on beads. In some cases, cell lysates were treated with 0.1 U of PP1 and PP2A before incubation with glutathione beads. Left panel: GST-14-3-3 beads specifically associate with cofilin. The interaction is enhanced by expression of Kd3. Right panel: 14-3-3 preferentially binds to phosphocofilin, as pretreatment with PP1/PP2A abolishes the interaction.

(F) Left panel: HA-14-3-3ε and -ζ were expressed in Cos-1 cells, immunoprecipitated with anti-HA antibodies, and probed for coimmunoprecipitated cofilin. Both 14-3-3 isoforms associate with cofilin. The right panel shows the corresponding expression and immunoprecipitation of endogenous and transfected 14-3-3 proteins, as detected with the pan-14-3-3 antibody 14-3-3β. The control lanes represent immunoprecipitation experiments performed in the absence of anti-HA antibodies. All results shown are representative of at least two independent experiments.

precipitated with HA-14-3-3ζ, which is indicative of stable complex formation in cells. Cofilin also associated with HA-14-3-3ε, albeit to a lesser extent, suggesting that the interaction is not restricted to a single 14-3-3 isoform. Interestingly, 14-3-3 was able to immunoprecipitate cofilin even in the absence of increased LIMK
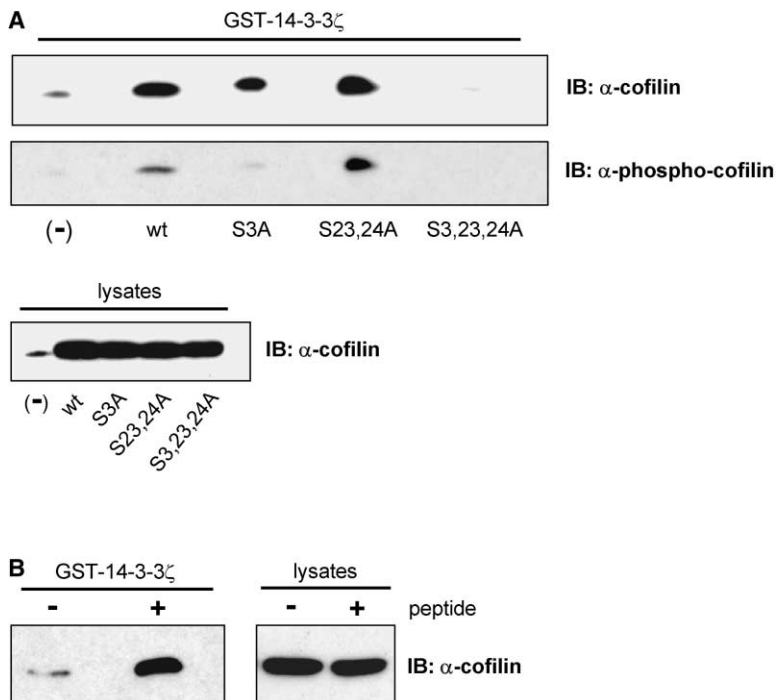
A

B

Figure 2. Mapping of the 14-3-3ζ Binding Site on Human Cofilin

(A) The indicated mutants of human cofilin or the empty vector control were transfected into Cos-1 cells, and cell lysates were incubated with purified GST-14-3-3ζ immobilized on glutathione beads. Precipitated proteins were probed with anti-cofilin antibody or anti-phosphocofilin antibody. The bottom panel demonstrates comparable expression of the cofilin mutants in cell lysates. The mutation of both the regulatory phosphorylation site and the predicted 14-3-3 binding motif (cofilin^{S3,23,24A}) abolished the cofilin/14-3-3 association.

(B) Pulldown experiments were performed with wild-type cofilin in the presence or absence of 0.1 μmol of a peptide comprising the eight amino-terminal residues of human cofilin. The cofilin/14-3-3 association is significantly enhanced in the presence of peptide. All results shown are representative of at least two independent experiments.

signaling. Results discussed below show that the expression of 14-3-3ζ itself can lead to an elevation of the basal phosphocofilin pool, which typically constitutes up to 20%–50% of the total cofilin in nontransformed cells [12].

The affinity of purified Ser3 phosphocofilin for 14-3-3ζ was determined by a solid-phase binding assay (see Figure S1). Binding was best fit to a one binding site model, with a Kd of $0.6–1 \times 10^{-7}$ M, suggesting equivalent and independent function of the two ligand binding sites on the 14-3-3 dimer. In summary, the in vitro and in vivo association of phosphocofilin and 14-3-3ζ is consistent with the phosphorylation-dependent interaction observed in overlay assays.

Mapping of the 14-3-3ζ Binding Site on Human Cofilin

Numerous 14-3-3 binding proteins associate with 14-3-3 through a phosphorylated serine contained within a prototypical 14-3-3-recognition motif, RSxpSxP (where x represents any amino acid and pS stands for phosphorylated serine) [7]. Human cofilin contains one putative 14-3-3 recognition sequence (RKS^{23}S^{24}TP). Although Ser24 in this motif exists within a conserved CaM kinase II consensus sequence, it is not a phospho-acceptor site in vivo [13]. It seems reasonable to assume that the extreme amino terminus of cofilin also plays a role in 14-3-3 binding, since Ser3 phosphocofilin specifically associates with 14-3-3ζ (Figure 1). However, the amino acid sequences surrounding Ser3 do not contain any of the described 14-3-3 interaction motifs. We note that some 14-3-3 binding partners interact with 14-3-3 in a phosphorylation-specific manner, but do not contain prototypical 14-3-3 recognition motifs [8].
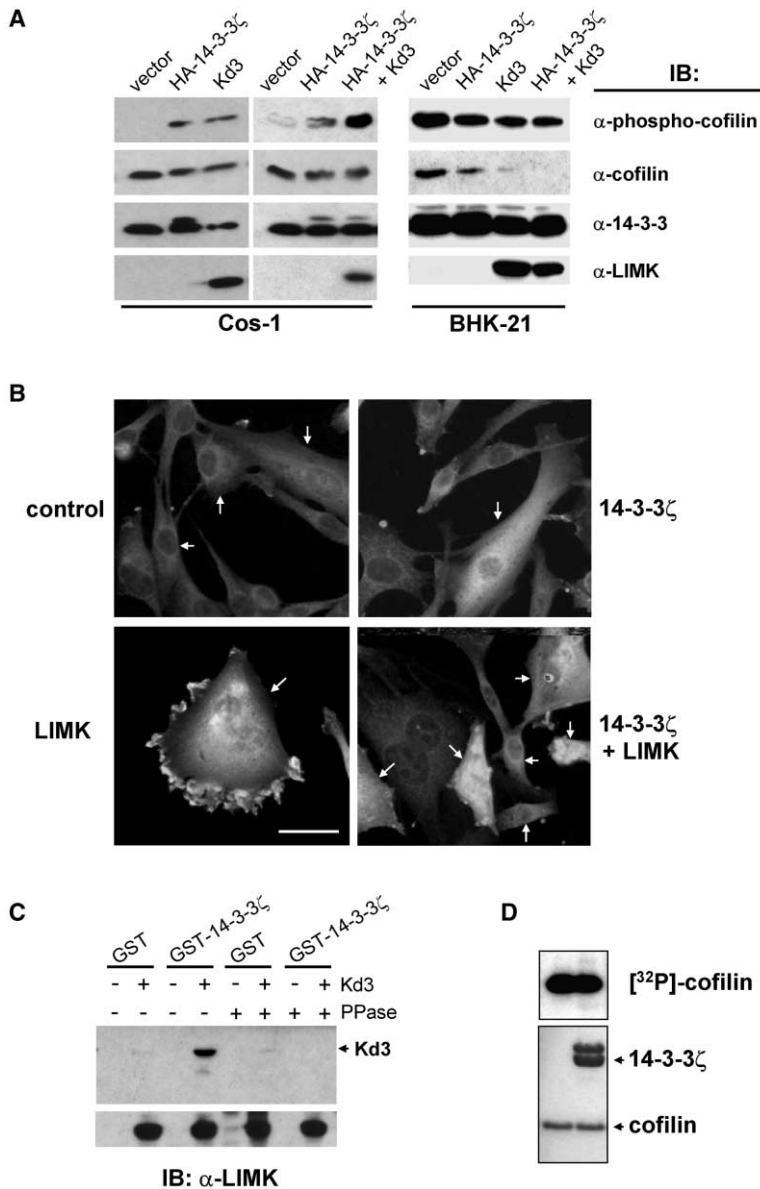
In order to map the 14-3-3 binding site(s) on cofilin, we expressed point mutants of human cofilin in Cos-1

cells and tested their binding to GST-14-3-3ζ. The employed mutants lack the regulatory phosphorylation site (cofilin^{S3A}) and/or contain a modification in the predicted 14-3-3 recognition motif (cofilin^{S3,23,24A} or cofilin^{S23,24A}). As shown in Figure 2A, mutation of Ser3 to Ala clearly reduced 14-3-3 binding, confirming that Ser3 phosphorylation of cofilin is important for the association with 14-3-3. Cofilin^{S23,24A}, which is Ser3 phosphorylated as efficiently as wild-type cofilin, also bound 14-3-3 comparable to wild-type cofilin. In contrast, the mutation of both the regulatory phosphorylation site and the predicted 14-3-3 binding motif (cofilin^{S3,23,24A}) abolished the cofilin/14-3-3 association. Similar results were obtained when GST-14-3-3τ was used (not shown). Thus, both the Ser3-phosphorylated amino terminus and the 14-3-3 consensus site of cofilin are functionally involved in 14-3-3 binding.

It is tempting to speculate that, when cofilin is active, the unphosphorylated amino terminus of cofilin folds over the putative 14-3-3 recognition motif, thus impeding 14-3-3 binding. Phosphorylation of Ser3 would then lead to a conformational change of the molecule, rendering the 14-3-3 recognition motif more accessible to 14-3-3. In support of this hypothesis, we observed that a peptide comprising the eight amino-terminal amino acids of human cofilin, which might compete with this putative intramolecular association, significantly enhanced cofilin binding to 14-3-3 (Figure 2B).

The Expression of 14-3-3ζ Increases Phosphocofilin Levels

To investigate the functional relevance of the cofilin/14-3-3-interaction, 14-3-3ζ was transiently transfected into Cos-1 or BHK-21 cells, and effects on steady-state phosphocofilin levels were studied biochemically and by immunofluorescence analysis. Remarkably, 14-3-3ζ

A

IB:

α-phospho-cofilin

α-cofilin

α-14-3-3

α-LIMK

Cos-1   BHK-21

B

control

14-3-3ζ

LIMK

14-3-3ζ
+ LIMK

C

- + - + - + - + Kd3
- - - - + + + + PPase

◄ Kd3

IB: α-LIMK

D

[³²P]-cofilin

◄ 14-3-3ζ

◄ cofilin

Figure 3. The Expression of 14-3-3ζ Increases Phosphocofilin Levels

(A) Cos-1 cells or BHK-21 cells were transfected with HA-14-3-3ζ and/or the LIMK1 fragment Kd3; then, the lysates were probed with the indicated antibodies. Blots were stripped and reprobed for cofilin with α-cofilin antibody to compare total cofilin in cell lysates.

(B) Immunofluorescence analysis of phosphocofilin levels and distribution in BHK-21 cells. Cells expressing the indicated transgenes are marked by arrows. The expression of 14-3-3ζ leads to a significant increase in the cytosolic phosphocofilin immunosignal, which is enhanced by coexpression with wt-LIMK. Representative cells from three independent transfections are shown. The scale bar represents 10 μm.

(C) Top panel: in vitro association of LIMK with immobilized GST-14-3-3ζ. The binding was markedly reduced after treatment of cell lysates with PP1/PP2A. Equal expression of LIMK was verified by probing cell lysates with anti-LIMK antibodies.

(D) 14-3-3 binding to LIMK does not affect the LIMK-dependent cofilin phosphorylation. A representative autoradiogram of *Xenopus* cofilin phosphorylated with LIMK in the presence or absence of 14-3-3ζ purified from bovine brain is shown. Similar results were obtained with purified GST-14-3-3ζ. The bottom panel shows a corresponding Coomassie blue-stained gel. The results shown are representative of at least two independent experiments.

elevated the phosphocofilin pool to a similar extent as the constitutively active LIMK1 fragment Kd3 in Cos-1 cells (Figure 3A). The coexpression of 14-3-3ζ and Kd3 further increased the amount of phosphocofilin compared to the effect of each protein alone. Densitometric analysis of the phosphocofilin/cofilin ratios showed that 14-3-3ζ or Kd3 alone increased relative phosphocofilin levels 10- to 13-fold compared to the vector control, whereas coexpression of both led to a 57-fold increase in phosphocofilin. In BHK-21 cells, we frequently observed a downregulation of total cofilin protein coincident with 14-3-3ζ and/or Kd3 expression. However, densitometric comparison of the phosphocofilin immunosignals with the amounts of endogenous cofilin revealed a 14-3-3ζ- and/or Kd3-dependent increase in the phosphocofilin pool similar to the levels obtained in Cos-1 cells (14-3-3ζ: 3-fold, Kd3: 10-fold, 14-3-3ζ + Kd3: 58-fold). Immunofluorescence analysis in BHK-21 cells (Figure

3B) could be performed after shorter expression times, and staining for endogenous cofilin under these conditions did not show any detectable decrease in protein levels (data not shown). The expression of 14-3-3ζ led to a diffuse increase in cytoplasmic phosphocofilin staining, consistent with the largely cytoplasmic distribution of 14-3-3 proteins [14]. Wild-type LIMK1 promoted an accumulation of phosphocofilin predominantly in membrane ruffles, where it colocalized with actin and 14-3-3 (see [5, 6], and data not shown). The coexpression of both 14-3-3ζ and LIMK1 led to a further increase in largely cytoplasmic phosphocofilin staining. In addition, these cells showed a strikingly altered morphology reminiscent of the phenotypical changes encountered in cells expressing high amounts of the constitutively active LIMK1 fragment Kd3 [3, 15].

Testicular protein kinase 1 (TESK1), an integrin-activated serine/threonine kinase with homology to LIMK1,

A

control

14-3-3ζ

LIMK

14-3-3ζ
+ LIMK

B
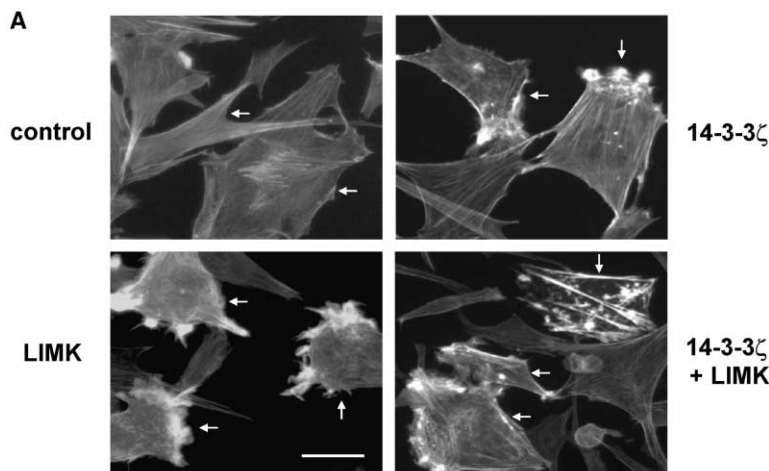
Figure 4. 14-3-3ζ Potentiates the LIMK-Induced Actin Phenotype

(A) BHK-21 cells were transfected with HA-14-3-3ζ and/or wild-type LIMK (arrows), and the actin cytoskeleton was visualized. The scale bar represents 10 μm.

(B) Distribution of actin cytoskeletal phenotypes in the population of cells expressing the indicated proteins. A minimum of 100 cells was scored in a blinded manner. Each transfection was repeated twice with similar results.
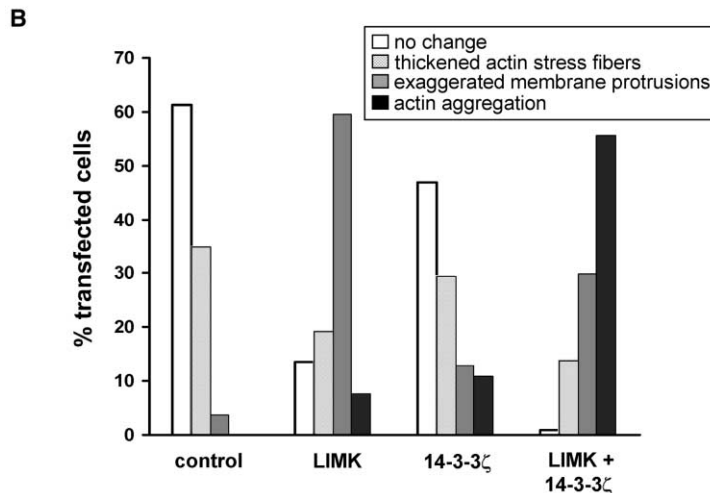
also induces actin cytoskeletal reorganization through phosphorylation of cofilin on Ser3 [16, 17]. Interestingly, TESK1 was recently shown to bind 14-3-3β, resulting in an inhibition of TESK1 kinase activity [18]. In in vitro kinase assays with immunoprecipitated, myc-tagged TESK1 and TESK2, we were, however, unable to detect any effect of up to 6 μg purified GST-14-3-3ζ or -τ on TESK-dependent cofilin phosphorylation (data not shown). Additionally, the expression of endogenous TESK1 or TESK2 proteins in Cos-1 or BHK-21 cells appeared to be very low, since neither isoform was detected in lysates of up to 5×10^6 cells when the original antibodies described in [16–18] were used (data not shown). We further investigated whether LIMK1 might be an interaction partner of 14-3-3ζ. As shown in Figure 3C, LIMK1 indeed associated with GST-14-3-3ζ in pull-down experiments. However, 14-3-3ζ (and 14-3-3τ; data not shown) had no effect on the LIMK1-mediated cofilin-phosphorylation in in vitro kinase assays (Figure 3D).

These results support the idea that the 14-3-3ζ-dependent increase in cellular phosphocofilin is a direct result of the phosphocofilin/14-3-3 interaction and impli-

cate 14-3-3 proteins in the control of the actin cytoskeleton. However, we cannot formally exclude the possibility that binding of 14-3-3 to LIMK regulates the phosphorylation of an unidentified LIMK substrate that also controls cofilin activity.

14-3-3ζ Potentiates the LIMK-Induced
Actin Phenotype

Expression analysis of wild-type and mutant LIMK in different cell types has revealed dose- and activity-dependent effects of LIMK on the actin cytoskeleton [5, 6, 15]. To obtain functional evidence that 14-3-3ζ is involved in cofilin-controlled actin dynamics, we expressed 14-3-3ζ and/or wild-type LIMK in BHK-21 cells. As shown in Figure 4A, 14-3-3ζ induced the formation of membrane protrusions and triggered actin aggregation in a subset of cells. The character of these actin-rich protrusions is at present unclear. Consistent with published observations, cells overexpressing wild-type LIMK were generally characterized by exaggerated membrane protrusions, and only a small subset of LIMK expressors contained actin aggregates. However, cells
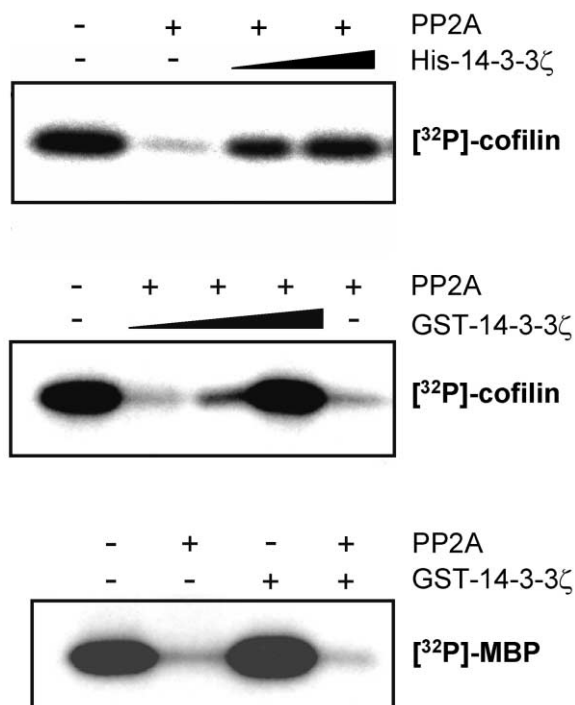
Figure 5. 14-3-3ζ Protects Cofilin from Dephosphorylation

Xenopus cofilin and myelin basic protein (MBP) were subjected to in vitro kinase reactions as detailed in the Experimental Procedures. A total of 1 μg XAC-P or 0.5 μg phospho-MBP per reaction was preincubated overnight at 4°C in phosphatase buffer in the absence or presence of 1 μg or 5 μg purified His-14-3-3ζ (UBI). The upper panel shows the result of a phosphatase assay using partially purified PP2A from bovine brain. Similar results were obtained with 0.02 U of purified PP2A (UBI) and purified, recombinant GST-14-3-3ζ (0.5 μg, 1 μg, or 5μg), as shown in the middle panel. A total of 5 μg GST-14-3-3ζ was used for the MBP experiments shown in the bottom panel. The results shown are representative of at least three independent experiments.

coexpressing 14-3-3ζ and wild-type LIMK displayed dramatic F-actin accumulation and clumping, reminiscent of the effects induced by Kd3. These results were quantified in Figure 4B and clearly demonstrate that 14-3-3ζ potentiates LIMK-mediated effects on the actin cytoskeleton through a mechanism synergistic with, but distinct from, effects on LIMK activity.

14-3-3ζ Protects Cofilin from Dephosphorylation
To address the issue of how 14-3-3 is able to increase the pool of inactive cofilin independently of regulating LIMK activity, we conducted in vitro phosphatase assays with purified proteins. Figure 5 shows that 14-3-3ζ inhibited the PP2A-mediated dephosphorylation of cofilin in a dose-dependent manner. The 14-3-3-mediated protection from dephosphorylation did not result from a general suppression of phosphatase activity, as 14-3-3 failed to antagonize the dephosphorylation of myelin basic protein (MBP) by PP2A. We have observed similar protection from dephosphorylation by a partially purified cofilin phosphatase from bovine brain (not shown). These results are biochemical evidence that 14-3-3ζ-bound phosphocofilin is specifically protected

against phosphatase attack and are in good agreement with published observations. For example, it has been reported that 14-3-3ζ blocked Raf [19, 20] and BAD [21] from PP1/PP2A-mediated dephosphorylation.

Conclusions
In summary, our results identify a previously uncharacterized role for 14-3-3 proteins in modulating the actin depolymerizing and/or severing functions of cofilin. Our data strongly indicate that LIMK and 14-3-3ζ act cooperatively to control actin cytoskeletal dynamics by increasing and stabilizing the levels of phosphocofilin. It will be of interest to evaluate whether the scaffolding function of 14-3-3 proteins plays any additional role in organizing and regulating the interplay between LIMK, cofilin, and various phosphocofilin regulatory phosphatases.

References

1. Bamburg, J.R. (1999). Proteins of the ADF/Cofilin family: essential regulators of actin dynamics. Annu. Rev. Cell Dev. Biol. *15*, 185–230.
2. Condeelis, J. (2001). How is actin polymerization nucleated *in vivo?* Trends Cell Biol. *11*, 288–293.
3. Edwards, D.C., Sanders, L.C., Bokoch, G.M., and Gill, G.N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat. Cell Biol. *1*, 253–259.
4. Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999). Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. Science *285*, 895–898.
5. Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature *393*, 805–809.
6. Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998). Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature *393*, 809–812.
7. Fu, H., Subramanian, R.R., and Masters, S.C. (2000). 14-3-3 proteins: structure, function, and regulation. Annu. Rev. Pharmacol. Toxicol. *40*, 617–647.
8. Tzivion, G., and Avruch, J. (2002). 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J. Biol. Chem. *277*, 3061–3064.
9. Yaffe, M.B. (2002). How do 14-3-3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett. *513*, 53–57.
10. Muslin, A.J., and Xing, H. (2000). 14-3-3 proteins: regulation of

subcellular localization by molecular interference. Cell. Signal. *12*, 703–709.

11. Ambach, A., Saunus, J., Konstandin, M., Wesselborg, S., Meuer, S., and Samstag, Y. (2000). The serine phosphatases PP1 and PP2A associate with and activate the actin-binding protein cofilin in human T-lymphocytes. Eur. J. Immunol. *30*, 3422–3431.

12. Meberg, P.J., Ono, S., Minamide, L., Takahashi, M., and Bamburg, J.R. (1998). Actin depolymerizing factor and cofilin phosphorylation dynamics: response to signals that regulate neurite extension. Cell Motil. Cytoskeleton *39*, 172–190.

13. Agnew, B.J., Minamide, L., and Bamburg, J.R. (1995). Reactivation of phosphorylated actin depolymerizing factor and identification of the regulatory site. J. Biol. Chem. *29*, 17582–17587.

14. Garcia-Guzman, M., Dolfi, F., Russello, M., and Vuori, K. (1999). Cell adhesion regulates the interaction between the docking protein p130[Cas] and the 14-3-3 proteins. J. Biol. Chem. *274*, 5762–5768.

15. Edwards, D.C., and Gill, G.N. (1999). Structural features of LIM kinase that control effects on the actin cytoskeleton. J. Biol. Chem. *274*, 11352–11361.

16. Toshima, J., Toshima, J.Y., Amano, T., Yang, N., Narumiya, S., and Mizuno, K. (2001). Cofilin phosphorylation by protein kinase testicular protein kinase 1 and its role in integrin-mediated actin reorganization and focal adhesion formation. Mol. Biol. Cell *12*, 1131–1145.

17. Toshima, J., Toshima, J.Y., Takeuchi, K., Mori, R., and Mizuno, K. (2001). Cofilin phosphorylation and actin reorganization activities of testicular protein kinase 2 and its predominant expression in testicular sertoli cells. J. Biol. Chem. *276*, 31449–31458.

18. Toshima, J., Toshima, J.Y., Watanabe, T., and Mizuno, K. (2001). Binding of 14-3-3β regulates the kinase activity and subcellular localization of testicular protein kinase 1. J. Biol. Chem. *276*, 43471–43478.

19. Dent, P., Jelinek, T., Morrison, D.K., Weber, M.J., and Sturgill, T.W. (1995). Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. Science *268*, 1902–1906.

20. Thorson, J.A., Yu, L.W., Hsu, A.L., Shih, N.-Y., Graves, P.R., Tanner, J.W., Allen, P.M., Piwnica-Worms, H., and Shaw, A.S. (1998). 14-3-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. Mol. Cell. Biol. *18*, 5229–5238.

21. Chiang, C.-H., Harris, G., Ellig, C., Masters, S.C., Subramanian, R., Shenolikar, S., Wadzinski, B.E., and Yang, E. (2001). Protein phosphatase 2A activates the proapoptotic function of BAD in interleukin-3-dependent lymphoid cells by a mechanism requiring 14-3-3 dissociation. Blood *97*, 1289–1297.