

## Phosphorylation of ADF/Cofilin Abolishes EGF-induced Actin Nucleation at the Leading Edge and Subsequent Lamellipod Extension

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**Abstract.** In metastatic rat mammary adenocarcinoma cells, cell motility can be induced by epidermal growth factor. One of the early events in this process is the massive generation of actin barbed ends, which elongate to form filaments immediately adjacent to the plasma membrane at the tip of the leading edge. As a result, the membrane moves outward and forms a protrusion. To test the involvement of ADF/cofilin in the stimulus-induced barbed end generation at the leading edge, we inhibited ADF/cofilin's activity *in vivo* by increasing its phosphorylation level using the kinase domain of LIM-kinase 1 (GFP-K). We report here that expression of GFP-K in rat cells results in the near total phosphorylation of ADF/cofilin, without changing either the G/F-actin ratio or signaling from the EGF re-

ceptor *in vivo*. Phosphorylation of ADF/cofilin is sufficient to completely inhibit the appearance of barbed ends and lamellipod protrusion, even in the continued presence of abundant G-actin. Coexpression of GFP-K, together with an active, nonphosphorylatable mutant of cofilin (S3A cofilin), rescues barbed end formation and lamellipod protrusion, indicating that the effects of kinase expression are caused by the phosphorylation of ADF/cofilin. These results indicate a direct role for ADF/cofilin in the generation of the barbed ends that are required for lamellipod extension in response to EGF stimulation.

**Key words:** ADF/cofilin • actin • LIM-kinase • EGF • cell motility

### Introduction

The early steps in actin reorganization after stimulation with chemoattractants have been well established in metastatic mammary adenocarcinoma (MTLn3)<sup>1</sup> cells. Upon stimulation with EGF, newly formed actin filament barbed ends accumulate at the leading edge, and a maximum level is achieved at one minute after stimulation (Chan et al., 1998; Bailly et al., 1999). This transient increase in the number of barbed ends constitutes a prerequisite for massive actin polymerization at the leading edge required for lamellipod extension, cell motility (Condeelis, 1993; Segall et al., 1996), and chemotaxis (Bailly et al., 1998). The accumulation of free barbed ends at the leading edge is accompanied by an increase in the number of pointed ends and a shortening filament length distribution suggesting that severing and/or de-

novo nucleation are involved, acting either separately or together (Bailly et al., 1998, 1999; Chan et al., 1998, 2000).

ADF/cofilin is an essential protein that is responsible for the enhanced turnover of actin filaments *in vivo* (Carlier et al., 1997; Lappalainen and Drubin, 1997; Rosenblatt et al., 1997). ADF/cofilin binds to monomeric actin (G-actin), as well as filamentous actin (F-actin), with greater affinity for the ADP forms (for review see Bamberg, 1999). It binds to F-actin in a cooperative manner and induces a twist in the double helix, without changing the length of the filament (McGough et al., 1997). ADF/cofilin has been shown to both depolymerize F-actin by increasing the off rate from the pointed ends of actin filaments (Carlier et al., 1997) and by directly severing filaments (Maciver et al., 1991; Du and Frieden, 1998; Chan et al., 2000; Ichevtovkin et al., 2000), but the relative importance of these two activities at the leading edge of the cell where barbed ends are formed remains unclear.

Cofilin's severing activity has been detected in lysates from EGF-stimulated metastatic cells using an *in vitro*

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<sup>1</sup>Abbreviations used in this paper: MAP, mitogen-activated protein; MTLn3, metastatic mammary adenocarcinoma cells.

light microscope–severing assay. The severing activity in these cell lysates is specifically inhibited by function blocking antibodies against cofilin. Furthermore, cofilin is recruited to the leading edge of cells at the time when free barbed ends appear after EGF stimulation. Finally, microinjection of function blocking antibodies against cofilin into resting cells inhibits the formation of barbed ends after EGF stimulation (Chan et al., 2000). These observations are consistent with an involvement of cofilin's severing and depolymerization activities in the EGF-stimulated generation of barbed ends. Cofilin's involvement in generating barbed ends at the leading edge could be either direct, contributing barbed ends by severing actin filaments, or indirect, supplying actin monomers for polymerization through its ability to enhance depolymerization of actin filaments. This study was performed to distinguish these possibilities.

The activity of ADF/cofilin can be regulated *in vitro* by pH, PIP<sub>2</sub>, and, in vertebrates, serine phosphorylation. Only phosphorylation has been shown to regulate its function *in vivo*. Both actin binding and depolymerizing activities of ADF/cofilin are inhibited by the phosphorylation on serine 3 (Moriyama et al., 1996; Nagaoka et al., 1996). Rapid dephosphorylation of ADF/cofilin has been observed in various cell types upon stimulation of cells with different hormones that induce changes in cytoskeleton organization and assembly (for reviews see Moon and Drubin, 1995; Bamburg, 1999). ADF/cofilin phosphorylation is under the control of an unconventional actin-binding serine/threonine kinase, LIM-kinase (Arber et al., 1998; Yang et al., 1998b). LIM-kinases contain two tandem LIM domains at the NH<sub>2</sub> terminus and a PDZ domain in the central region, which are presumably involved in the regulation of the kinase activity and its cellular distribution, respectively (Hiraoka et al., 1996; Yang et al., 1998a; Nagata et al., 1999).

ADF/cofilin coimmunoprecipitates with LIM-kinase 1. Only LIM-kinase and ADF/cofilin incorporate <sup>32</sup>P in these preparations, suggesting a high level of specificity of the kinase activity (Yang et al., 1998b). Expression of the kinase domain of LIM-kinase 1 in C2C12 cells is sufficient to cause phosphorylation of ADF/cofilin, and coexpression studies with mutants of cofilin indicate that the effects of LIM-kinase on the actin cytoskeleton are ADF/cofilin specific (Arber et al., 1998). The kinase domain of LIM-kinase optimally phosphorylates ADF/cofilin *in vivo* (Arber et al., 1998; Edwards and Gill, 1999). The K<sub>m</sub> of LIM-kinase for cofilin is ~7 μM, which is comparable to the intracellular concentration of cofilin (Edwards and Gill, 1999; Chan et al., 2000). Here, we took advantage of these observations and used the kinase domain of LIM-kinase 1 to quantitatively inhibit cofilin's activity *in vivo* by increasing its phosphorylation level. We analyzed the effects of inhibition of cofilin's activity on the appearance of barbed ends at the leading edge and lamellipod protrusion during EGF stimulation in the presence of known concentrations of G/F-actin.

## Materials and Methods

### Cells and Antibodies

Rat MTLn3 cells were maintained and stimulated with EGF, as described previously (Bailey et al., 1998, 1999). Ab287 was raised against a rat cofilin

peptide containing amino acids 57–71 present in both wild-type and S3A mutants of cofilin (Chan et al., 2000). Antiphospho-epitope antibody (provided by J.R. Bamburg, Colorado State University, Fort Collins, CO) was raised against a phosphorylated peptide corresponding to the NH<sub>2</sub> terminus of ADF and has been shown to recognize phospho-cofilin/ADF, but not the unphosphorylated form (Meberg et al., 1998). Cy5 goat anti-rabbit was from Accurate Chemical & Scientific Corp. Cy3 mouse anti-otitin (mAb, clone BN-34) was from Sigma-Aldrich. Antibodies against phospho-p44/p42 mitogen-activated protein (MAP) kinase were from New England Biolabs, Inc.

### Expression of Exogenous Proteins in MTLn3 Cells

GFP-F (GFP-tagged full-length LIM-kinase 1, active), GFP-FS (GFP-tagged LIM-kinase 1 Short, inactive), GFP-K (GFP-tagged kinase domain of LIM-kinase 1, active), and GFP-KS (GFP-tagged kinase domain of LIM-kinase 1 Short, inactive) were characterized previously (see Fig. 1 A) (Arber et al., 1998). The S3A cofilin mutant was in the pCDNA3 expression vector (Arber et al., 1998). MTLn3 cells were plated on acid washed glass bottom 35-mm MatTek dishes (2 × 10<sup>5</sup> cells) for 16–24 h before subsequent manipulation. The different constructs were introduced into cells either by transfection, using lipofectamine Plus Reagent as recommended by manufacturer (Life Technologies), or by direct microinjection in the perinuclear region, using an Eppendorf semiautomatic microinjection system. Cells were analyzed 16–24 h after transfection or 5–8 h after microinjection. The same results were obtained regardless of the method used to deliver DNA.

### In Situ Actin Nucleation Assay and Immunofluorescence Microscopy

The *in situ* actin nucleation assay was performed as described previously (Bailey et al., 1998, 1999; Chan et al., 1998) using 0.45 μM of either biotin- or rhodamine-labeled G-actin in saponin-containing permeabilization buffer. Under these conditions either biotin- or rhodamine-actin is incorporated only at the barbed ends of actin filaments (Bailey et al., 1998, 1999; Chan et al., 1998).

Immunofluorescence was performed as described previously (Bailey et al., 1998, 1999). Biotin-actin was detected using cy3 mouse antibodies.

### Lamellipod Extension Assay

Lamellipod extension results in a large increase in the footprint of the cell on a two-dimensional surface, and it can be quantified as an increase in the relative cell area (the ratio of cell area at a given time to cell area before treatment with EGF). The cell area was measured using DIAS software (Solltech) on the time lapse digital images taken during EGF treatment, as described previously (Segall et al., 1996; Bailey et al., 1998, 1999). The sensitivity of this technique allows a lamellipod as small as 1 μm across to be detected. Since MTLn3 cells extend lamellipods of more than 5 μm in response to EGF, the early stages of lamellipod extension can be detected by DIAS. Cells were analyzed and the mean relative cell area was plotted as a function of time to assess the kinetics of protrusive activity.

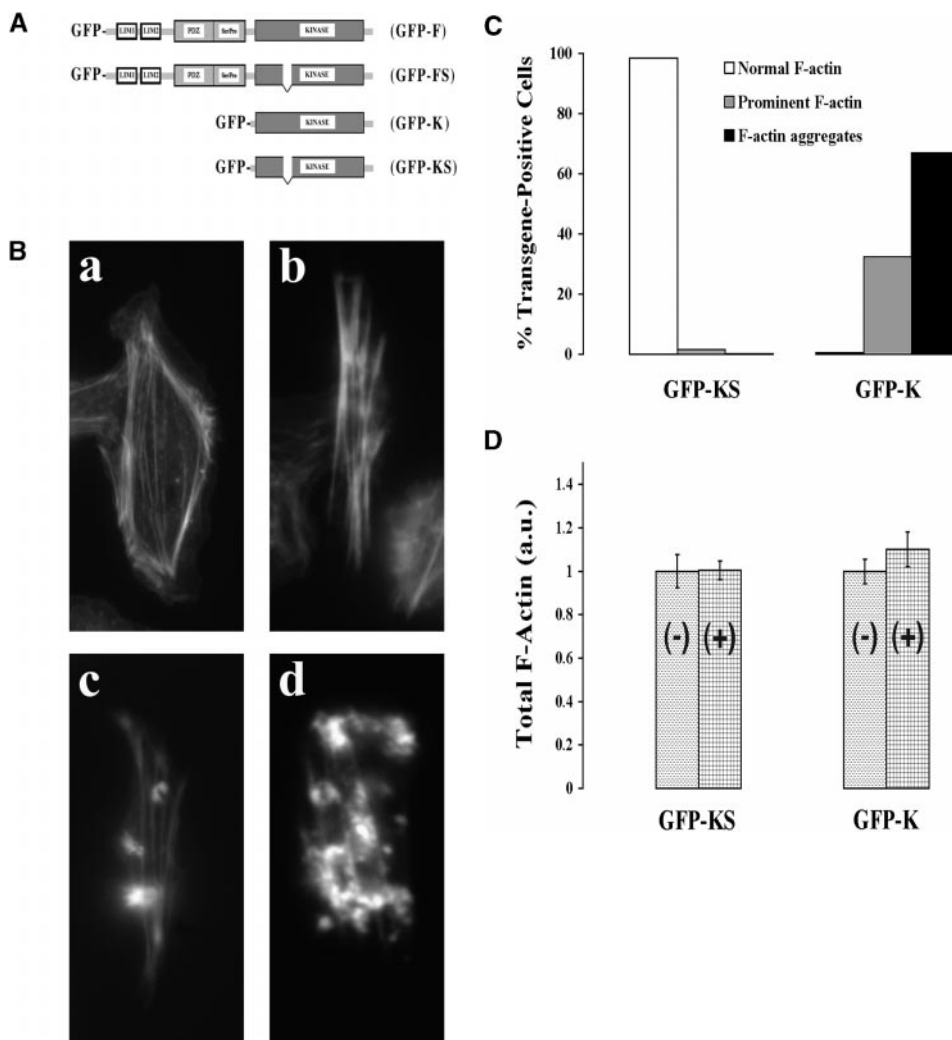
### Fluorescence Quantification

Fluorescence digital images of total cofilin, phosphocofilin, total F-actin, total actin, and barbed ends, captured as above, were converted linearly in NIH Image. The mean pixel intensity per cell and the cell area were determined, as described previously (Bailey et al., 1999), and the total pixel intensity per cell was calculated as their product. All measurements of total pixel intensity were done using double-blind scoring. Pixel intensities were normalized against the control cells that do not express any exogenous construct to overcome the variability between experiments and imaging fields. Measurements of relative pixel intensity were invariant with magnification and depth of field, indicating that 100% of the fluorescence from each cell was collected.

## Results

### Effect of the Kinase Domain of LIM-Kinase 1 on F-Actin Distribution

We first analyzed the effect of the expression of GFP-tagged kinase domain of LIM-kinase 1 (GFP-K) on the or-



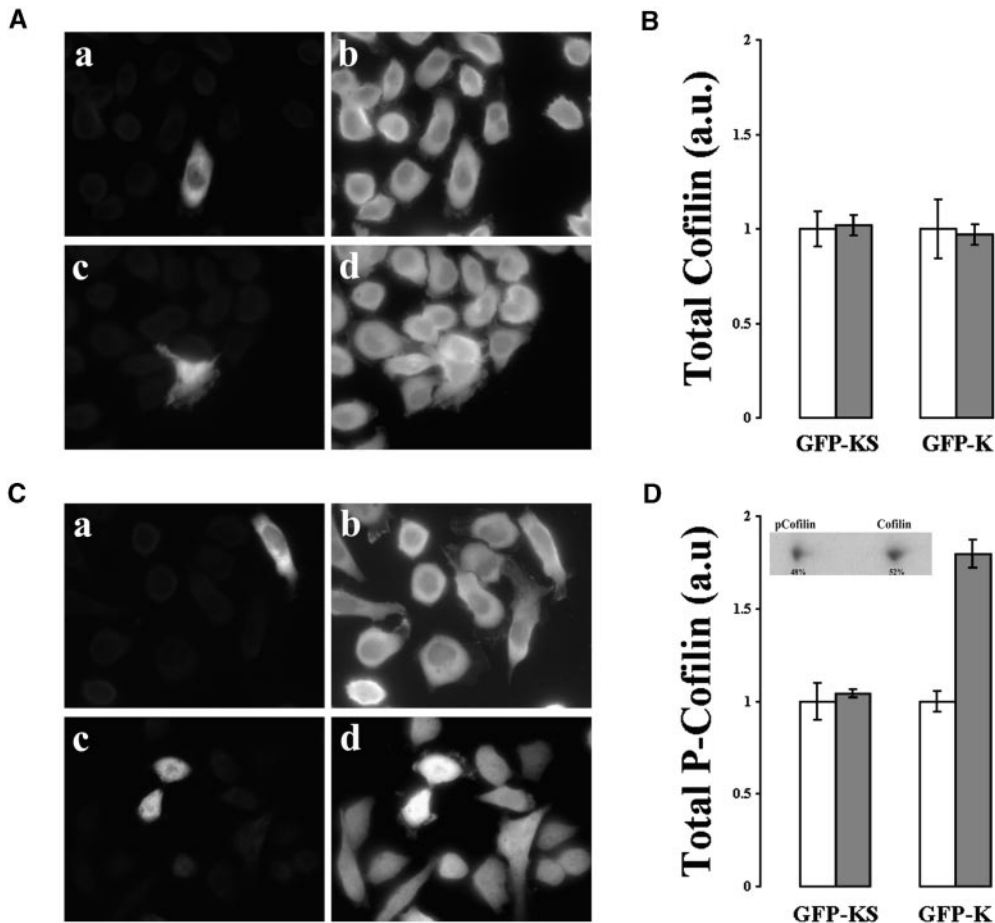
**Figure 1.** Expression of the GFP-K induces aggregation of F-actin and formation of stress fibers without significantly increasing the F-actin content. (A) A schematic diagram is shown of the GFP-tagged constructs used in this study. (B) Cells were subdivided into three categories and scored accordingly: cells exhibiting similar phalloidin staining compared with (a, normal F-actin) transgene-negative cells; (b, prominent F-actin) cells with thicker stress fibers; and (c and d, F-actin aggregates) cells containing F-actin aggregates. (C) Quantitative analysis of the three categories in cells expressing the kinase domain of LIM kinase 1 ( $n = 176$ ) or the corresponding inactive construct GFP-KS ( $n = 64$ ). (D) Total F-actin was measured in phalloidin-stained cells expressing GFP-K ( $n = 118$ ) and GFP-KS ( $n = 64$ ). Fluorescence intensities are plotted as mean  $\pm$  SEM here and in all figures. The data are representative for three experiments. The same results were obtained here and in all figures, regardless of the tag used (GFP or Myc), of the method used to deliver the DNA constructs (lipofection or microinjection), and whether the cDNA-microinjected cells were incubated in the absence of serum before fixation.

ganization of the actin cytoskeleton in MTLn3 cells (Fig. 1). GFP-K or, as a control, the deletion mutant of the kinase domain lacking kinase activity (GFP-KS) were expressed in MTLn3 cells before F-actin staining with bodipy-phalloidin. As shown in Fig. 1, expression of GFP-KS caused no significant change in the overall F-actin organization, compared with neighboring cells that do not express the construct. In contrast, expression of GFP-K induced aggregation of F-actin and/or the formation of more F-actin-containing stress fibers (Fig. 1 B). In cells expressing GFP-KS, 98.4% presented the normal F-actin distribution compared with neighboring cells (63 out of 64 cells analyzed). Out of 176 cells expressing GFP-K, only 0.6% presented the normal distribution of F-actin, whereas 32.4% showed more F-actin stress fibers and the majority showed aggregation of F-actin (67.0%). A more dramatic phenotype where cells showed only F-actin aggregates without any detectable stress fibers (Fig. 1 B, d) was observed in <1% of the total cell population expressing GFP-K.

To determine if the EGF receptor signaling pathway is still active after transfection with GFP-K, we analyzed the effect of EGF stimulation on the activation of the MAP kinase pathway, by following the appearance of phosphorylated forms of MAP kinase (Lange et al., 1998; McCawley et al., 1999). Using immunofluorescence, both transfected

and nontransfected cells, in the same field, exhibited a significant and equal increase in the level of phospho-MAP kinase, indicating that the EGF receptor is fully active and that EGF stimulation of the parallel signaling pathway is normal (data not shown). Thus, the expression of the LIM-kinase construct does not affect the EGF receptor activity.

To evaluate the effect of GFP-K expression on the amount of F-actin in the cell, the total F-actin content per cell before stimulation was measured as fluorescence intensity after staining with bodipy-phalloidin. The concentration of G-actin at the time when barbed ends first appear is relevant here. Barbed ends appear immediately after stimulation and peak by 50 s (Chan et al., 2000), whereas the stimulated increase in F-actin lags far behind, achieving less than a 20% change by 50 s (Chan et al., 1998). As shown in Fig. 1 D, expression of GFP-K resulted in a maximum increase of 10% in the total amount of F-actin ( $1.1 \pm 0.08$ ,  $n = 118$ ), compared with neighboring transgene-negative cells and cells expressing GFP-KS ( $n = 64$ ). Furthermore, immunofluorescence experiments using mAb C4 against actin showed that expression of the kinase domain of LIM-kinase 1 has no effect on the amount of total actin present compared with neighboring cells that do not express the construct (data not shown). Since the concentration of both F- and G-actin in resting MTLn3



**Figure 2.** Expression of GFP-K phosphorylates almost all cofilin in vivo without changing cofilin expression levels. MTLn3 cells microinjected with expression vectors for (a and b) GFP-KS or (c and d) GFP-K were immunostained with antibodies to (A) cofilin or to (C) phosphocofilin/ADF. Cofilin and phosphocofilin/ADF staining are shown in b and d, and transgene-positive cells are visualized in a and c. (B and D) Fluorescence intensities (mean  $\pm$  SEM) for GFP-positive cells (solid bars) were normalized against neighboring transgene-negative cells (open bars). The data presented are representative for at least three experiments. The inset in D is presented as a reference showing the relative amounts of cofilin (52%) versus phosphocofilin (48%) in MTLn3 cells.

cells is 75  $\mu$ M (Edmonds et al., 1996), these results indicate that the G-actin concentration remains almost constant and above 67  $\mu$ M upon expression of GFP-K.

### Expression of GFP-K Increases Cofilin Phosphorylation Levels

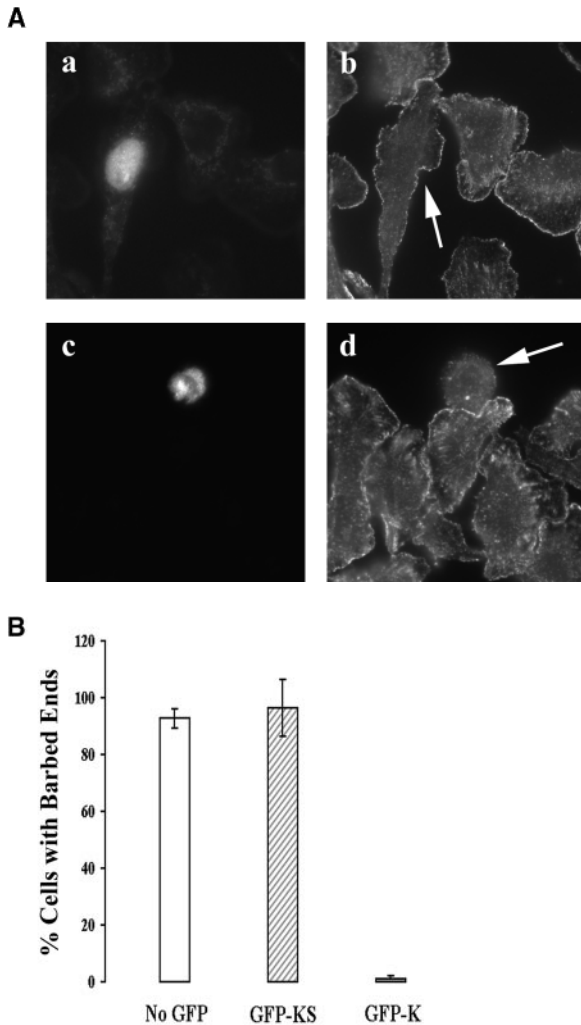
To assess the effect of GFP-K on the phosphorylation level of cofilin in vivo, we expressed GFP-K, or its inactive counterpart GFP-KS, in MTLn3 cells and monitored the level of phospho-cofilin by immunofluorescence using a phospho-epitope specific antibody. In parallel experiments, we analyzed the total amount of cofilin, using antibody Ab287. As shown in Fig. 2, expression of either GFP-KS or GFP-K does not affect the level of expression of cofilin (in both cases, the relative total pixel intensities were not significantly different from 1.0). In contrast, when the phospho-epitope antibody was used, only those cells that express GFP-K (Fig. 2 C) showed a dramatic increase in phosphorylation of cofilin. Further quantitative analysis of phospho-epitope staining for 33 GFP-KS cells and 104 GFP-K cells demonstrated that no significant change was observed in GFP-KS-expressing cells, whereas GFP-K expression induced a 1.8-fold increase in the level of phosphorylation of cofilin. Analysis of cell lysates from a monolayer of MTLn3 cells on two-dimensional gels, using nonequilibrium pH gradient electrophoresis (NEpHGE, pH 3–10) in the first dimension followed by Western blotting using Ab287, shows that the amount of phospho-cofilin in resting (untransfected) cells is 48% (Fig. 2 D, inset). Taken together, these results demonstrate that in MTLn3

cells expressing GFP-K, 86% of cofilin is phosphorylated, compared with 48% in transgene-negative cells.

### Phosphorylation of Cofilin Abolishes the Generation of Actin Barbed Ends in Response To EGF

To address the importance of cofilin activity in the generation of barbed ends quantitatively, we expressed GFP-K as a quantitative inhibitor of cofilin in vivo. MTLn3 cells were microinjected with either GFP-K or GFP-KS expression vectors and assayed for EGF-stimulated actin nucleation activity. Under the experimental conditions, only endogenous barbed ends incorporate labeled actin, which allows quantification of the number of barbed ends per cell (Bailly et al., 1999; Chan et al., 1998). As shown in Fig. 3 A, cells expressing the GFP-KS exhibited similar barbed end formation at the leading edge compared with neighboring transgene-negative cells. In contrast, cells expressing GFP-K failed to generate barbed ends under the same conditions, whereas neighboring cells that were not expressing the exogenous kinase generated barbed ends at the cell periphery.

Quantitative analysis of EGF-induced barbed end formation in 96 cells expressing GFP-K, 28 cells expressing GFP-KS, and transgene-negative cells from the same experiments demonstrate that the majority of transgene-negative cells ( $92.7 \pm 3.3\%$ ), as well as the majority of cells expressing GFP-KS ( $96.4 \pm 10\%$ ), incorporate exogenous actin at the leading edge upon EGF stimulation, indicating the normal number of EGF-induced barbed ends. In contrast, only  $1.1 \pm 1.2\%$  of cells expressing GFP-K show in-



**Figure 3.** Expression of the GFP-K abolishes EGF-induced actin barbed end generation at the leading edge. (A) MTLn3 cells were microinjected with either (a and b) GFP-KS or (c and d) GFP-K cDNAs and assayed for EGF-induced actin nucleation activity. Incorporation of exogenous-labeled actin in the cell cortex is seen as a thin rim of fluorescence at the cell periphery. (A, a and c, and arrows in A, b and d) Cells expressing exogenous kinase were detected by direct visualization of the GFP green fluorescence. Due to saponin-permeabilization before fixation, the cytoplasmic GFP-kinase is extracted, leaving behind GFP-kinase that is mostly nuclear and perinuclear in location. (B) The percent of cells showing incorporation of exogenous actin at the leading edge was determined for GFP-K cells ( $n = 96$ ), GFP-KS cells ( $n = 28$ ), and transgene-negative cells. The data presented is representative of at least three experiments.

corporation of exogenous actin at the leading edge, indicating nearly complete inhibition of barbed end formation (Fig. 3). That is, phosphorylation of 86% of the cofilin in the continued presence of abundant endogenous G-actin is sufficient to inhibit barbed end generation.

### Phosphorylation of Cofilin Abolishes EGF-induced Lamellipod Extension

Lamellipod extension is driven in part by the polymerization of actin (Condeelis, 1993). In rat adenocarcinoma cells, EGF stimulation mobilizes free barbed ends from which this polymerization occurs (Segall et al., 1996; Bailly et al.,

1998; Chan et al., 1998). As a consequence, inhibition of barbed end mobilization by phosphorylation of cofilin should inhibit lamellipod extension. To test this prediction, we compared the kinetics of lamellipod extension in cells expressing GFP-K or GFP-KS to that of neighboring transgene-negative cells (Fig. 4). EGF stimulation induced similar lamellipod extension in cells expressing the control inactive kinase (GFP-KS), compared with other cells in the same field that did not express the exogenous fusion protein. However, cells expressing GFP-K failed to extend lamellipods in response to EGF, whereas neighboring cells responded normally and showed the usual extension.

As described earlier by Segall et al. (1996), EGF treatment of MTLn3 cells results in a dose-dependent lamellipod extension peaking at 5 min, with an optimum EGF concentration of 5 nM. As shown in Fig. 4, all cells in GFP-KS transfection experiments, regardless of their expression status, as well as cells that did not express the active kinase in GFP-K transfection experiments, exhibited a normal EGF-induced lamellipod extension response. The maximum relative cell area in these cells ranged from  $1.62 \pm 0.11$  to  $1.77 \pm 0.14$ . However, GFP-K-expressing cells did not show any protrusive activity. The cell area remained unchanged during the time course of the experiment, with a maximum value of  $1.01 \pm 0.02$ . Taken together, the data presented in Figs. 2–4 show that inhibition of EGF-induced barbed ends at the leading edge, by an increase in cofilin phosphorylation level, prevents subsequent actin polymerization and lamellipod extension.

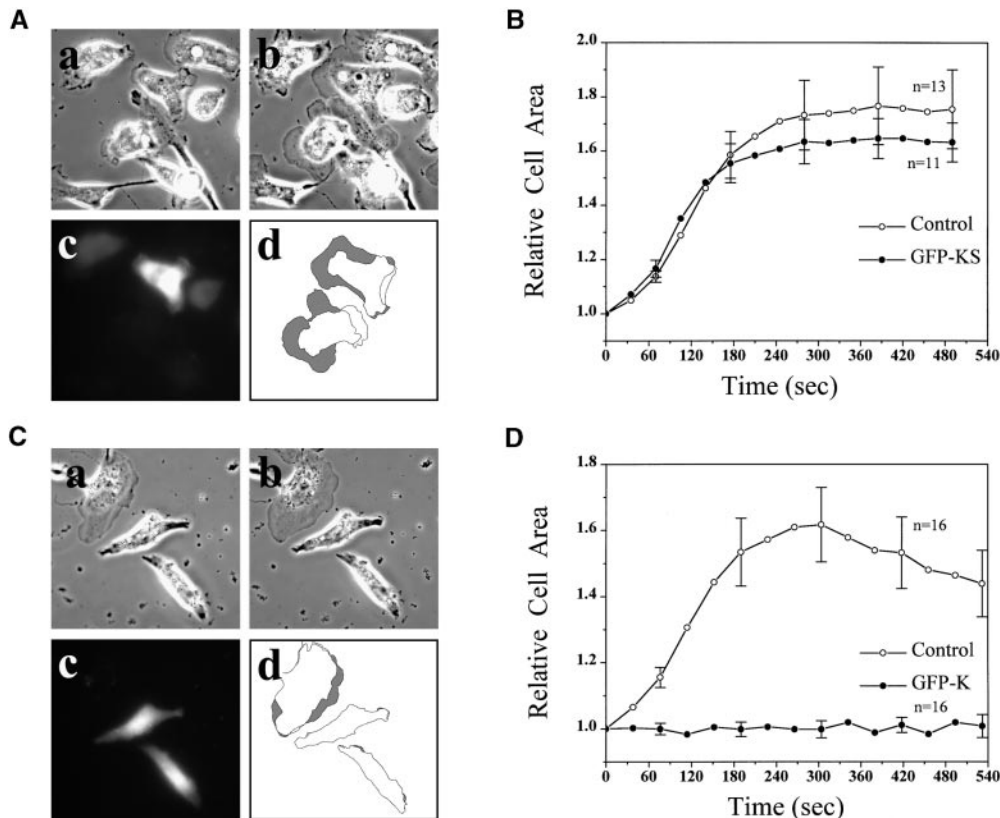
### Expression of the S3A Mutant of Cofilin Rescues the Inhibition of Barbed Ends and Lamellipod Extension by GFP-K

To investigate if the effects observed upon expression of GFP-K are specific to the phosphorylation of cofilin, we co-expressed the kinase domain together with S3A cofilin in MTLn3 cells. The S3A cofilin mutant is constitutively active and not able to be phosphorylated, since its unique phosphorylation site on serine 3 has been mutated to alanine (Moriyama et al., 1996; Nagaoka et al., 1996). When injected into living C2 myoblasts, S3A cofilin distribution overlaps endogenous cofilin (Moriyama et al., 1996), suggesting that the two proteins play similar roles in vivo. Furthermore, in yeast, it has been shown that disruption of the cofilin gene (COF1) is lethal and that S3A cofilin, but not S3D, is able to rescue cofilin null cells (Moriyama et al., 1996).

As shown in Fig. 5 A, cells expressing GFP-K alone have the same amount of cofilin as controls, whereas cells expressing both GFP-K and S3A (GFP-K/S3A) have more than twice ( $2.4 \pm 0.21$ ) the normal amount of cofilin. Since the wild-type cofilin does not increase in cells expressing GFP-K alone (Fig. 2), this indicates that the extra cofilin is the S3A form.

Comparison of these same populations of cells for cofilin phosphorylation level shows that cells expressing GFP-K alone or in combination with S3A cofilin have 1.8 times more phospho-cofilin compared with transgene-negative cells (Fig. 5 B), indicating that the same elevated level of phosphorylation of the wild-type cofilin exists in S3A-expressing cells as that observed in cells not expressing S3A (Fig. 2).

To determine the effects of S3A cofilin on actin dynamics in cells expressing GFP-K, the amount of F-actin, barbed ends, and lamellipod protrusion were measured in



**Figure 4.** Expression of the GFP-K abolishes EGF-induced lamellipod extension. MTLn3 cells were transiently transfected with either (A and B) GFP-KS or (C and D) GFP-K. 16–24 h after transfection, the cells were starved for 3 h and stimulated with 5 nM EGF. In A (GFP-KS) and C (GFP-K), live-phase contrast images were taken (a) before and (b) 4 min after EGF was added to the cells. Tracing of select cells from a and b were merged to show (d, grey area) the EGF-induced cell area increase, and (c) the GFP fluorescence is shown to indicate transgene-positive cells. In B (GFP-KS) and D (GFP-K), continuous time lapse digital images were analyzed using DIAS software. The cell area is a measure of lamellipod extension.

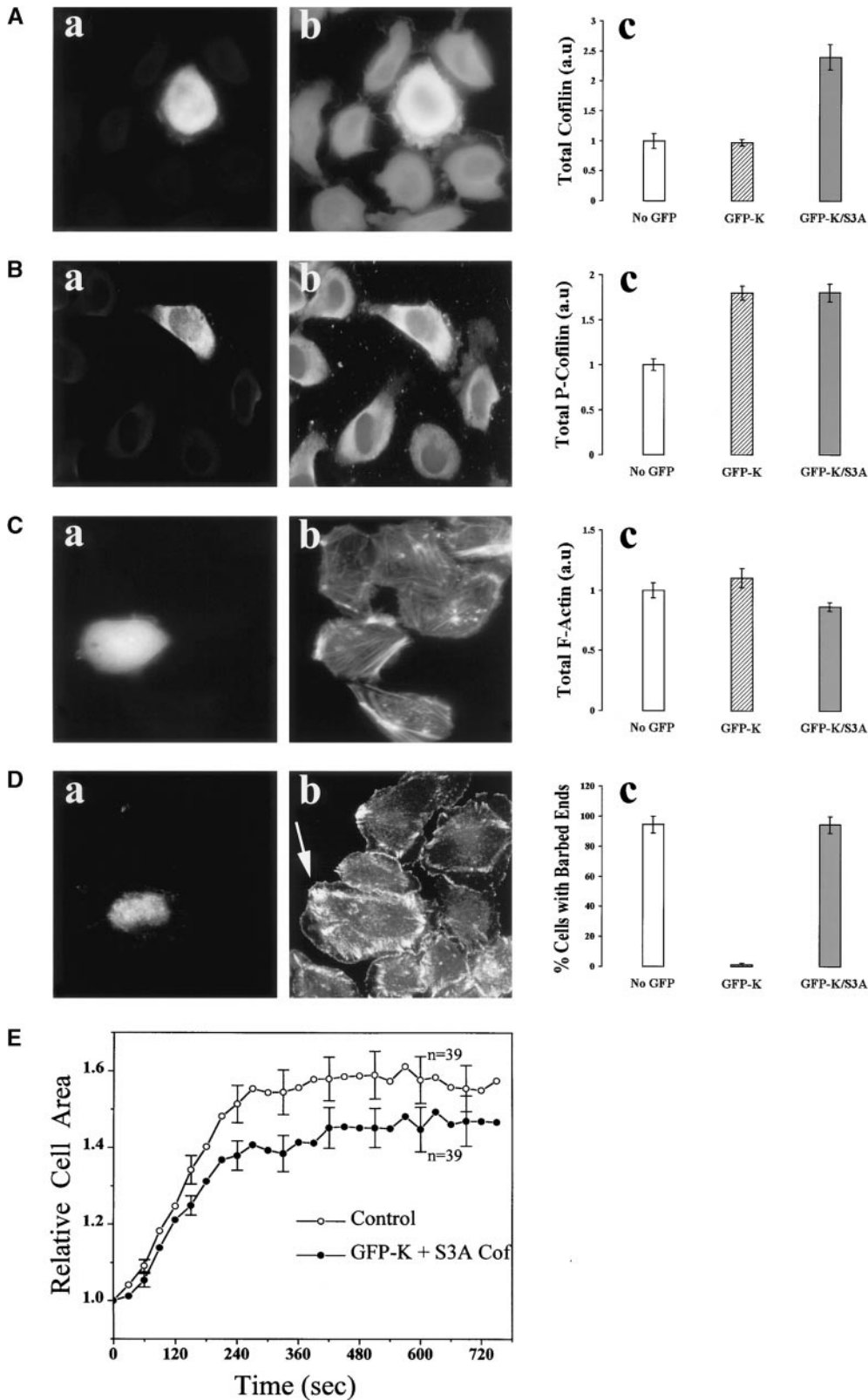
cells expressing either GFP-K alone or GFP-K plus S3A cofilin. As shown in Fig. 5 C, the amount of F-actin present in cells expressing GFP-K was approximately the same as control cells, whereas cells expressing both GFP-K and S3A had slightly less F-actin (normalized total pixel intensity =  $0.86 \pm 0.04$ ) (Fig. 5 C, c). In addition, expression of S3A in GFP-K positive cells prevents the appearance of more stress fibers and F-actin aggregation (Fig. 5 C, a and b) caused by the expression of GFP-K alone (Fig. 1).

The expression of S3A cofilin rescued the EGF-stimulated generation of actin barbed ends at the leading edge of GFP-K-positive cells, as shown in Fig. 5 D. Cells expressing both GFP-K and S3A had the same number of barbed ends as control cells, whereas cells expressing only GFP-K had none (Fig. 5 D, c).

The expression of S3A cofilin in MTLn3 cells also rescued the inhibition of EGF-induced lamellipod extension caused by expression of GFP-K (Fig. 5 E). GFP-K/S3A-positive cells (solid circles) extended lamellipods in response to EGF at the same rate as neighboring transgene-negative cells (Fig. 5 E, open circles), reaching a plateau 4–5 min after EGF was added. The amplitude of lamellipod extension was slightly lower in transgene-positive cells compared with nonmicroinjected cells. This difference in the amplitude of lamellipod extension was also observed in cells expressing inactive GFP-KS (Fig. 4 B), suggesting that it is probably due to overexpression of the exogenous recombinant protein independently from its kinase activity. The concomitant rescue of barbed end generation and lamellipod extension by S3A supports the proposed cause and effect relationship between actin polymerization and protrusion, discussed above.

## Discussion

Here, we illustrate the use of the kinase domain of LIM-kinase to probe the function of cofilin in vivo. We found several novel results. (a) Expression of the kinase domain of LIM-kinase 1 increases the levels of phospho-cofilin in vivo to the point where little active cofilin remains. This is the first time that the extent of phosphorylation of cofilin in vivo in response to ectopic expression of LIM-kinase has been measured. (b) Kinase expression does not alter the G/F-actin ratio significantly in vivo. This result is consistent with previous work in which the G/F-actin ratio was not altered by overexpression of ADF/cofilin in neurites (Meberg and Bamberg, 2000). Furthermore, since there is no significant change in G-actin levels in MTLn3 cells, the effects observed cannot be due to the activation of the serum-response factor pathway and resulting changes in transcription (Sotiropoulos et al., 1999). (c) Phosphorylation of the majority of the cell's cofilin is sufficient to inhibit the appearance of barbed ends, even in the continued presence of abundant G-actin. (d) Inhibition of cofilin activity is sufficient to inhibit lamellipod extension. This result is consistent with the increase in protrusive activity caused by ADF/cofilin overexpression in neurites (Meberg and Bamberg, 2000) and *Dictyostelium* (Aizawa et al., 1996). (e) Expression of S3A rescues all phenotypes resulting from expression of the kinase domain, indicating that the effects observed are mediated by the phosphorylation of cofilin. These results indicate a direct role for cofilin in the generation of the barbed ends that are required for lamellipod extension in EGF-stimulated cells.



**Figure 5.** Expression of the S3A mutant of cofilin rescues the inhibition of barbed ends and lamellipod extension caused by the kinase domain. MTLn3 cells were microinjected either with GFP-K or a 1:1 mixture of GFP-K and S3A cofilin cDNAs and analyzed 5–8 h later. (A–C) Cells were immediately fixed and processed for staining with (A, b) anti-cofilin antibody Ab287, (B, b) anti-phosphocofilin/ADF antibody, or (C, b) bodipy-phalloidin. (D and E) Cells were starved for 3 h before adding EGF. (D) Barbed end generation was measured as described in the legend to Fig. 3. (E) Lamellipod extension was scored as described in the legend to Fig. 4. (A–D, a, and D, b, arrow) Expressing cells are identified by direct visualization of the GFP fluorescence. Quantitative analyses are shown in A–D, c. “No-GFP” are control transgene-negative cells. The number of GFP-K/S3A cofilin-expressing cells (solid bars) analyzed in A–D (c) is 27, 63, 78, and 26, respectively. The data presented are representative of at least three experiments.

### Possible Mechanisms for Cofilin Function at the Leading Edge

Cofilin has been proposed to function at the leading edge as a depolymerization factor to increase the concentration of actin monomers to support the formation and polymer-

ization of new barbed ends (Carrier, 1998). This mechanism assumes that G-actin is limiting and that depolymerization is tightly coupled to polymerization. In a situation where actin monomers are abundant, this mechanism would not predict any change in either the number of

barbed ends or lamellipod extension upon inhibition of cofilin activity. The observation reported here, that barbed end appearance and lamellipod extension in response to stimulation with EGF are blocked by phosphorylation of cofilin, even in the continued presence of abundant G-actin, is inconsistent with this proposed mechanism for cofilin function. An alternative mechanism proposes that cofilin severs F-actin to create new barbed ends, which are used for nucleation of polymerization of the new filaments (Moon and Drubin, 1995; Chan et al., 2000). This mechanism predicts a direct dependence on the number of barbed ends on cofilin's severing activity, regardless of the concentration of G-actin present, and is consistent with the results of our study.

### *Is the Function of Cofilin the Same in Constitutive and Stimulated Cell Motility?*

Our results demonstrate that cofilin's severing activity plays a direct role in the generation of free actin barbed ends at the leading edge of cells stimulated with EGF. However, cofilin may have different functions, depending on cell type and the extent of motility exhibited in the absence of stimulation. For example, cells that exhibit constitutive cell motility, defined here as continuous locomotion in the absence of exogenous stimulation, are exhibiting an unregulated form of cell motility in which the depolymerization of actin may be tightly coupled to protrusion. This type of motility is exhibited by locomoting fibroblasts, keratocytes, and bacterial pathogens in the cytoplasm of host cells. Evidence for the tight coupling of depolymerization to protrusion comes from experiments with jasplakinolide in which inhibition of actin depolymerization immediately blocks protrusion and slows bacterial motility (Cramer, 1999). Additional evidence comes from oligonucleotide-mediated disruption of  $\beta$ -actin mRNA targeted to the leading edge of fibroblasts and loss of subsequent localized actin synthesis, which is correlated with decreases in the rate of locomotion (Kislauskis et al., 1997). On the other hand, chemotactic cells such as MTLn3 exhibit stimulated motility where the initiation and subsequent continuous movement require increasing exogenous stimulation. MTLn3 cells are intrinsically unpolarized and fail to show significant locomotion in the absence of stimulation with EGF (Shestakova et al., 1999). In these cells, the resting G-actin concentration is not limiting (Edmonds et al., 1996) and the depolymerization of actin does not appear to be coupled to protrusion (Segall et al., 1996; Chan et al., 1998). Nonlocomoting fibroblasts also exhibit protrusion that is uncoupled from actin depolymerization, as demonstrated by the greatly delayed inhibition of protrusion by jasplakinolide (Cramer, 1999).

In cells exhibiting continuous constitutive cell motility where G-actin is limiting, cofilin may function primarily as a depolymerization factor to increase the rate of filament turnover, thereby supplying G-actin for barbed end formation and polymerization (Carlier, 1998). The distribution of cofilin in such cells could be localized on any population of filaments adjacent to polymerizing filaments. This mechanism is consistent with the distribution of cofilin at the base of lamellipods in constitutively locomoting keratocytes directly opposite from the leading edge membrane (Svitkina and Borisy, 1999). On the other hand, in cells

that are at rest where G-actin is not limiting, cofilin may function as a generator of barbed ends by severing preexisting filaments at the plasma membrane. This mechanism is consistent with the distribution of cofilin found in MTLn3 cells where it is localized directly within the polymerizing filament population at the tip of the leading edge during stimulation (Chan et al., 2000).

The authors wish to thank J.R. Bamburg for providing the anti-phospho cofilin/ADF antibody, the Analytical Imaging Facility staff and J. Wyckoff for help with light microscopy and image analysis, and members of the Condeelis and Segall laboratories for their constructive discussions.

This work was supported by the Harrison Foundation and grants from the National Institutes of Health.

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