Cofilin promotes stimulus-induced lamellipodium formation by generating an abundant supply of actin monomers

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Cofilin stimulates actin filament disassembly and accelerates actin filament turnover. Cofilin is also involved in stimulus-induced actin filament assembly during lamellipodium formation. However, it is not clear whether this occurs by replenishing the actin monomer pool, through filament disassembly, or by creating free barbed ends, through its severing activity. Using photolabile Dronpa-actin, we show that cofilin is involved in producing more than half of all cytoplasmic actin monomers and that the rate of actin monomer incorporation into the tip of the lamellipodium is dependent on the size of this actin monomer pool. Finally, in cofilin-depleted cells, stimulus-induced actin monomer incorporation at the cell periphery is attenuated, but the incorporation of microinjected actin monomers is not. We propose that cofilin contributes to stimulus-induced actin filament assembly and lamellipodium extension by supplying an abundant pool of cytoplasmic actin monomers.

Introduction

Actin filament dynamics are essential for various cell activities, including cell migration, morphological change, and polarity formation. These events are regulated by a variety of actin-binding proteins, which cooperatively act in the assembly/disassembly and reorganization of actin filaments in cells (Pollard and Borisy, 2003; Revenu et al., 2004). Cofilin/actin-depolymerizing factor (ADF) family proteins, ubiquitously expressed in eukaryotes, are key regulators of actin filament dynamics (Moon and Drubin, 1995; Welch et al., 1997; Bamberg et al., 1999; Pantaloni et al., 2001). In vitro studies have demonstrated that cofilin stimulates actin filament disassembly by accelerating the off rate of actin monomers from the pointed ends of actin filaments (depolymerization) and by severing actin filaments (Carlier et al., 1997; Rosenblatt et al., 1997; Lappalainen and Drubin, 1997; Maciver, 1998). Depletion or inactivation of cofilin in Drosophila melanogaster or mammalian cells results in aberrant F-actin accumulation, implicating cofilin in actin filament disassembly in the cell (Gunsalus et al., 1995; Arber et al., 1998; Yang et al., 1998; Chen et al., 2001; Hotulainen et al., 2005; Nishita et al., 2005).

Conversely, cofilin is required for actin filament assembly in the cell, as seen in the case of stimulus-induced lamellipodium formation (Chan et al., 2000; Zebda et al., 2000; Ghosh et al., 2004). The observations that cofilin preferentially binds to the ADP-bound actin in filaments and enhances actin filament disassembly from the pointed ends in the rear of the lamellipodium have led to the treadmilling model, where cofilin contributes to actin filament assembly by replenishing actin monomers for polymerization (Bamburg et al., 1999; Pantaloni et al., 2001; Pollard and Borisy, 2003). An alternative model has recently proposed that cofilin is involved in stimulus-induced actin filament assembly by severing actin filaments to create free barbed ends that are used as nucleation sites for actin polymerization (Condeelis, 2001; Ghosh et al., 2004; DesMarais et al., 2005). This model is based on the observation that in MTLn3 mammary adenocarcinoma cells cofilin inactivation inhibited EGF-induced barbed end formation and lamellipodium extension in the cell periphery, without changing the G/F-actin ratio in the cell (Chan et al., 2000; Zebda et al., 2000). However, in other types of cells, conflicting results have suggested that the G/F-actin ratio decreases after cofilin inactivation (Chen et al., 2001; Hotulainen et al., 2005). Thus, it remains unclear whether cofilin contributes to stimulus-induced actin filament assembly in the cells by supplying actin monomers through its depolymerizing/severing activity, creating free barbed ends through its severing activity,
or both of these two processes. To define the extent to which cofilin plays a role in these two possible processes in stimulus-induced actin filament assembly, it is essential to determine the G/F-actin ratio quantitatively in both cofilin-active and -inactive cells.

In this study, we have assessed the actin monomer pool in the cytoplasm of living cells by measuring the fluorescence decay of Dronpa (Dp)-labeled actin photoactivated in a small region of the cytoplasm. Dp is a GFP-like protein whose fluorescence can be reversibly switched off and on by photobleaching and photoactivation, respectively (Ando et al., 2004). Together with F-actin sedimentation assays, we provide evidence that cofilin is involved in the generation of more than half of the actin monomers in the cytoplasm. Using cofilin mutants, we also show that the severing activity, rather than the depolymerization activity, of cofilin is predominantly involved in maintaining the actin monomer pool in the cell. We also demonstrate that actin monomers in the cytoplasm are incorporated into the tip of the lamellipodium at the rate dependent on the actin monomer pool size in the cytoplasm. Furthermore, in cofilin-inactivated or -depleted cells, in which >80% of total cofilin is converted to the inactive phosphocofilin by LIM-kinase overexpression or total cofilin expression is decreased ~80% by RNA interference, stimulus-induced actin monomer incorporation at the cell periphery is attenuated, but the incorporation of microinjected actin monomers is not. Our results suggest that cofilin contributes to stimulus-induced actin filament assembly in the cell periphery by supplying an abundant pool of actin monomers to the cytoplasm.

### Results

#### Assessment of actin monomer population in the cytoplasm of living cells by measuring fluorescence decay of Dp-actin

To assess actin monomer pool size in the cytoplasm of living cells, we expressed Dp-labeled actin in COS7 cells and measured the fluorescence decay of Dp-actin in the cytoplasm. The level of Dp-actin expressed in COS7 cells was <1%, compared with endogenous actin, and the expression had no apparent effect on F-actin assembly (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). After the fluorescence of the whole cell was photobleached to the background level, Dp or Dp-actin was locally photoactivated in a small square region of the cytoplasm, and fluorescence images were acquired every 0.4 s. Both Dp (Fig. 1 A and Video 1) and Dp-actin (Fig. 1 B and Video 2 A) rapidly diffused from the photoactivated region throughout the cytoplasm. Quantitative analyses of the time-dependent changes in fluorescence intensity in the photoactivated region (Fig. 1 D) and the fluorescence decay at 0.8 s after photoactivation (Fig. 1 E) indicate that the rate of fluorescence decay of Dp-actin is slightly slower than that of Dp.

To determine whether the rate of fluorescence decay of Dp-actin is related to the actin monomer pool in the cytoplasm, we examined the effects of actin-modulating drugs on the diffusion of Dp-actin. When COS7 cells were pretreated with jasplakinolide (Jasp), a drug that induces actin polymerization, Dp-actin in the photoactivated region was almost immobile.

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**Figure 1.** The rate of fluorescence decay of photoactivated Dp-actin reflects the actin monomer population in the cytoplasm. (A–C) Fluorescence decay of Dp or Dp-actin. COS7 cells were transfected with Dp (A) or Dp-actin (B and C). Whole cells were photobleached, and a 5.8-μm² region (white box) was photoactivated. Fluorescence images were acquired every 0.4 s for 7.2 s at 37°C using a laser-scanning confocal imaging system (Videos 1 and 2 A, available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). (C) COS7 cells expressing Dp-actin were pretreated with 1 μM Jasp for 20 min before photoactivation (Video 2 B). Bar, 20 μm. (D) Time course of the fluorescence decay of Dp and Dp-actin in the photoactivated region with correction for photobleaching. The mean fluorescence intensity immediately after photoactivation (t = 0 s) was set to 100%. Data are means ± SEM of 24 (Dp), 68 (Dp-actin), 23 (Dp-actin; Jasp), and 22 cells (Dp-actin; LatA) from at least three independent experiments. (E) Fluorescence decay at 0.8 s after photoactivation from data in D. * P < 0.05, compared with the decay of Dp-actin in untreated cells.
(Fig. 1 C and Video 2 B), and the rate of fluorescence decay was correspondingly reduced (Fig. 1, D and E). In contrast, treatment of the cell with latrunculin A (LatA), a drug that induces actin filament disassembly, slightly accelerated the fluorescence decay rate of Dp-actin, compared with untreated cells (Fig. 1, D and E). These results suggest that the rate of Dp-actin fluorescence decay reflects the relative levels of G/F-actin in the cytoplasm, with fast decay rates corresponding to a high G-actin population. Although the rate of Dp-actin fluorescence decay theoretically also depends on the turnover rate of actin filaments, the half-life of actin filaments in the cell ranges from tens of seconds (in the leading edge of migrating keratocytes) to several minutes (in stress fibers; Amato and Taylor, 1986; Theriot and Mitchison, 1991, 1992; McGrath et al., 1998). Therefore, the rates of fluorescence decay of Dp-actin measured in this study, at least in the initial phase after photoactivation (less than a few seconds), appear to reflect primarily the G/F-actin ratio in the cytoplasm. Thus, the level of G-actin relative to total actin in the cytoplasm of living cells can be estimated by measuring the rate of fluorescence decay of Dp-actin immediately after photoactivation.

**Cofilin inactivation or knockdown markedly decreases the cytoplasmic actin monomer pool**

To examine the role of cofilin in regulating the actin monomer pool in the cytoplasm, we analyzed the effect of overexpression of LIM-kinase 1 (LIMK1), which inactivates cofilin by phosphorylation of Ser-3 (Yang et al., 1998), on the fluorescence decay of Dp-actin in COS7 cells. By expression of wild-type (WT) LIMK1, >80% of total cofilin was converted to phosphocofilin in COS7 cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). Expression of LIMK1 (WT) clearly reduced the mobility of photoactivated Dp-actin in the cytoplasm and the rate of fluorescence decay in the photoactivated region (Fig. 2, A and F; and Video 2 C). In contrast, expression of kinase-dead LIMK1(D460A) had no apparent effect on fluorescence decay (Fig. 2, B and F; and Video 2 D).

Figure 2. Effects of expression of LIMK1, cofilin, or their mutants on the fluorescence decay of Dp-actin. COS7 cells were cotransfected with Dp-actin and LIMK1 [A], LIMK1(D460A) [B], LIMK1 + cofilin[S3A] [C], LIMK1 + cofilin[WT] [D], or LIMK1 + Slingshot-1 [E]. Photobleaching, photoactivation, and fluorescence microscopy were conducted as in Fig. 1. White boxes indicate the photoactivated regions. See Videos 2 and 3 (available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). Bar, 20 μm. (F) Time course of the fluorescence decay of Dp-actin in the photoactivated region, measured as in Fig. 1 D. Data are means ± SEM of 44 (LIMK1), 33 (LIMK1[D460A]), 55 (LIMK1 + cofilin[WT]), 35 (LIMK1 + cofilin[S3E]), 47 (LIMK1 + cofilin[S3A,Y82F]), 57 (LIMK1 + cofilin[S3A,S94D]), and 30 cells (LIMK1 + Slingshot-1) from at least three independent experiments. (G) Fluorescence decay at 0.8 s after photoactivation from data in F. * P < 0.05, compared with cells expressing LIMK1[WT].
Figure 3. Cofi lin/ADF double knockdown decreases the rate of fluorescence decay of Dp-actin. [A] Suppression of endogenous cofi lin and ADF expression by siRNA. MCF-7 cells were cotransfected with cofi lin and ADF siRNAs plasmids or transfected with control siRNA plasmid. After 4 d of culture, cell lysates were analyzed by immunoblotting with antibodies specific for cofi lin, ADF, and actin. (B) Effects of cofi lin/ADF double knockdown on the fluorescence decay of Dp-actin. MCF-7 cells were cotransfected with Dp-actin and cofi lin/ADF siRNAs (top) or control siRNA (bottom). Photo-bleaching, photoactivation, and fluorescence microscopy were conducted as in Fig. 1. White boxes indicate the photoactivated regions. See also Video 4 (available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). Bar, 20 μm. [C] Time course of the fluorescence decay of Dp-actin in the photoactivated region, measured as in Fig. 1 D. Data are means ± SEM of 24 cells (cofi lin/ADF siRNA) and 25 (control siRNA) from three independent experiments. [D] Fluorescence decay at 0.8 s after photoactivation from data in C. *, P < 0.05, compared with control cells.

These results suggest that LIMK1-mediated cofi lin inactivation markedly reduced the G-actin pool in the cytoplasm. The fluorescence decay of Dp-actin in LIMK1-expressing cells was reduced by 59%, compared with control cells expressing Dp-actin alone (Fig. 2 G), which indicates that cofi lin contributes to the production of more than half of the G-actin pool in control cells. When a nonphosphorylatable, constitutively active cofi lin(S3A) mutant was coexpressed with LIMK1, it partially rescued the mobility of Dp-actin and increased the fluorescence decay rate, compared with cells expressing LIMK1 alone (Fig. 2, C, F, and G; and Video 3 A). The partial rescue is probably due to the low actin-disassembling activity of cofi lin(S3A) mutant (Fig. S3 B). In contrast, coexpression of cofi lin(WT) had no apparent effect (Fig. 2, D, F, and G; and Video 3 B), most likely because of its phosphorylation and inactivation by LIMK1. Similarly, the phosphorylation-mimic cofi lin(S3E) mutant had no effect (Fig. 2, F and G). Furthermore, coexpression of the cofi lin phosphatase Slingshot-1, which neutralizes LIMK1 activity (Niwa et al., 2002), almost completely blocked the inhibitory effect of LIMK1 on fluorescence decay (Fig. 2, E, F, and G; and Video 3 C). These observations suggest that the LIMK1-induced decrease in Dp-actin fluorescence decay is primarily caused by cofi lin phosphorylation and inactivation and that the cytoplasmic G-actin pool depends largely on cofi lin activity.

We also examined the effects of knockdown of cofi lin/ADF expression using siRNA in MCF-7 human breast carcinoma cells. Similar to LIMK1 overexpression in COS7 cells, double knockdown of cofi lin and ADF remarkably reduced the mobility of Dp-actin and the rate of fluorescence decay of Dp-actin in the cytoplasm of MCF-7 cells (Fig. 3 and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1), which further supports the theory that cofi lin plays a critical role in maintaining the G-actin pool in the cytoplasm.

The actin filament-severing activity of cofi lin plays a dominant role in increasing the actin monomer pool in the cytoplasm

Cofi lin stimulates depolymerization (dissociation of actin monomers from the pointed end) and severing of actin filaments. To elucidate which of these two activities plays a dominant role in increasing the G-actin pool in the cytoplasm, we expressed LIMK1 with (S3A,Y82F)- or (S3A,S94D)-cofi lin mutants in COS7 cells and examined the effects of these mutants on the LIMK1-mediated inhibition of Dp-actin diffusion. Similar to the reported characteristics of Y82F- and S94D-cofi lin mutants (Moriyama and Yahara, 1999, 2002), the in vitro analyses showed that cofi lin(S3A,Y82F), but not cofi lin(S3A,S94D), has severing activity (Fig. S3, C and D), whereas both mutants retain the ability to promote dilution-induced F-actin disassembly (which reflects the combined depolymerization and severing activities; Fig. S3 B). Cofi lin(S3A,Y82F), which retains the severing activity, significantly recovered the LIMK1-mediated inhibition of the fluorescence decay of Dp-actin, to the extent similar to cofi lin(S3A) (Fig. 2, F and G; and Video 3 D). In contrast, cofi lin(S3A,S94D), which exhibits no severing activity, did not block LIMK1 inhibition of fluorescence decay (Fig. 2, F and G; and Video 3 E). These results suggest that the severing activity of cofi lin plays a dominant role in increasing the actin monomer pool in the cytoplasm.

Quantification of the G/F-actin ratio by F-actin sedimentation assays

We also analyzed the effects of LIMK1(WT or D460A) expression on the G/F-actin ratio in cells using F-actin sedimentation assays. COS7 cells were cotransfected with plasmids for Myc-actin and LIMK1 at a molar ratio of 1:5, to ensure the coexpression of LIMK1 in almost all Myc-actin–expressing cells. Cell lysates
were centrifuged to separate G- and F-actin and analyzed by anti-Myc immunoblotting. Expression of LIMK1(WT), but not LIMK1(D460A), markedly reduced the ratio of G- to F-actin, compared with mock-transfected cells (Fig. 4 A). G-actin accounted for 41 and 18% of total actin (G- plus F-actin) in mock-transfected and LIMK1-expressing cells, respectively, thus indicating that 56% of the G-actin in control cells was shifted to F-actin by LIMK1 expression (Fig. 4 A). These data are consistent with results from the fluorescence decay of Dp-actin (Fig. 2 G). The LIMK1-induced reduction in actin monomer content was substantially blocked by coexpression of coflin(S3A) or coflin(S3A,Y82F), but not by coflin(WT), coflin(S3E), or coflin(S3A,S94D) (Fig. 4 B). Together with data from the Dp-actin fluorescence decay assays, these results strongly suggest that coflin contributes to the production of more than half of the G-actin in the cell and that the severing activity of coflin plays a dominant role in this process.

The rate of actin monomer incorporation into the tip of the lamellipodium is correlated to the actin monomer pool in the cytoplasm

We next examined whether the G-actin population in the cytoplasm is correlated to actin filament assembly in the lamellipodium. We analyzed the fluorescence decay of Dp-actin in the cytoplasm and its incorporation into the lamellipodium in the same cell. We used N1E-115 cells because they stably produced lamellipodia by expression of active Rac(V12). After cotransfection of the cells with Dp-actin and Rac(V12) and cell-wide photobleaching, Dp-actin was photoactivated in a rectangular region in the cytoplasm (Fig. 5 A). Dp-actin photoactivated in the cytoplasm was continuously incorporated into the tip of the lamellipodium and then flowed retrogradely toward the cell body (Fig. 5 A and Video 5 A, available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). Expression of LIMK1 slowed the fluorescence decay of the photoactivated Dp-actin in the cytoplasm and repressed its incorporation into the lamellipodium (Fig. 5 B and Video 5 B). Fig. 5 D shows the time courses of Dp-actin fluorescence decay in the photoactivated region. Fig. 5 E shows the rate of Dp-actin incorporation into the lamellipodium, measured as the length of Dp-actin fluorescence from the tip of the lamellipodium to the cell center per unit time after photoactivation. These analyses revealed that LIMK1 expression suppresses both the fluorescence decay of Dp-actin in the cytoplasm and its incorporation into the lamellipodium. The inhibitory effects of LIMK1 were substantially blocked by coexpression of coflin(S3A) (Fig. 5 C and Video 5 C), but not by coflin(WT) or coflin(S3E) (Fig. 5, D and E). The rate of Dp-actin incorporation into the lamellipodium is linearly correlated with the fluorescence decay in the cytoplasm in each cell (Fig. 5 F), which indicates that actin monomer assembly at the tip of the lamellipodium is highly dependent on the cytoplasmic actin monomer pool size. Because coflin elevates the cytoplasmic G-actin levels, coflin likely enhances actin filament assembly at the tip of the lamellipodium by increasing the G-actin level in the cytoplasm.

When Dp-actin in the front region of the lamellipodium was photoactivated, it flowed retrogradely and then rapidly diffused throughout the cytoplasm (Fig. 6 A and Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). The fluorescence intensity in the front region declined continuously until it reached a plateau at 15%, whereas the intensity in the rear of the lamellipodium initially increased, reached a maximum level at 20 s after photoactivation, and declined to a level similar to that in the front region (Fig. 6 B). These results suggest that most of the actin monomers that disassembled from the rear of the lamellipodium diffused into the cytoplasm.

Cofilin is required for EGF-induced actin filament assembly in the cell periphery

Previous studies have shown that EGF induced actin filament assembly in the periphery of MTLn3 carcinoma cells (Chan et al., 1998, 2000). As this EGF-induced assembly was suppressed by coflin inactivation without changing the G/F-actin ratio in the cell, it was suggested that coflin promotes actin filament assembly by creating new barbed ends through its severing activity (Chan et al., 2000; Zebda et al., 2000). We have shown here, however, that LIMK1-mediated inactivation or...
knock down of coflin/ADF led to a large decrease in the actin monomer population in the cytoplasm. We also showed that the rate of actin monomer incorporation into the cell periphery depends on coflin activity, as well as the cytoplasmic actin monomer population. We therefore hypothesized that coflin is involved in stimulus-induced actin filament assembly in the cell periphery by supplying actin monomers in the cytoplasm and that coflin inactivation inhibits actin filament assembly by decreasing the cytoplasmic actin monomer concentration to levels at which actin assembly is not feasible. To test this hypothesis, we analyzed the effects of LIMK1 overexpression on EGF-induced actin filament assembly by measuring the incorporation of Alexa Fluor 546–labeled actin (Alexa546-actin) into actin filaments in the cell periphery.

Figure 5. Dp-actin photoactivated in the cytoplasm is efficiently incorporated into the lamellipodium, and the rate of incorporation is dependent on the G-actin pool in the cytoplasm. (A–C) Dp-actin photoactivated in the cytoplasm is incorporated into the lamellipodium. NIE-115 cells were cotransfected with Dp-actin and RacV12 (A); Dp-actin, RacV12, and LIMK1 (B); or Dp-actin, RacV12, LIMK1, and coflin(S3A) (C). After cell-wide photobleaching, a 14.25- × 2.85-μm rectangular region (white box) was photoactivated, and fluorescence images were acquired every 2 s for 20 s at 37°C using a laser-scanning confocal imaging system to measure the fluorescence decay of Dp-actin (shown in D). The same cells were photobleached, and the same rectangular region was again photoactivated. Fluorescence images were acquired every 5 s for 40 s to measure the incorporation of Dp-actin into the tip of the lamellipodium (A–C; see Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). Bar, 20 μm. (D) Time course of the fluorescence decay of Dp-actin in the photoactivated region. Plasmids transfected into the cells are indicated on the right. (E) The rate of Dp-actin incorporation into the lamellipodium, measured as the distance of Dp-actin fluorescence advanced from the tip of the lamellipodium toward the cytoplasm for 20 or 40 s after photoactivation. Data in D and E are means ± SEM of 36 (Dp-actin), 20 (Dp-actin; LIMK1[WT]), 33 (Dp-actin; LIMK1[WT]; coflin[S3A]), 26 (Dp-actin; LIMK1[WT]; coflin[WT]), and 23 cells (Dp-actin; LIMK1[WT]; coflin[S3E]). *, P < 0.05, compared with cells expressing LIMK1[WT] alone. (F) The correlation between the fluorescence decay of Dp-actin at 2 s after photoactivation and the rate of Dp-actin incorporation into the lamellipodium. Each point represents an individual cell transfected with the plasmids indicated in D.
Microinjected actin monomers are effectively incorporated into the cell periphery after EGF stimulation in both coflin-active and -inactive cells

Previous studies have suggested that cell stimulation activates the Arp2/3 complex, which enhances de novo nucleation and arborization of actin filaments to generate dendritic actin filament structures (Takenawa and Miki, 2001; Pantaloni et al., 2001; Pollard and Borisy, 2003). Arp2/3 complex–mediated dendritic actin structure formation exponentially increases the number of barbed ends, and this process requires actin monomers for polymerization. To examine whether coflin contributes to EGF-induced barbed end formation directly, by severing actin filaments, or indirectly, by supplying actin monomers, we analyzed the effect of microinjection of Alexa546-actin monomers into cells expressing LIMK1(WT) or LIMK1(D460A). If coflin primarily contributes to EGF-induced barbed end formation by severing actin filaments, injected Alexa546-actin will be incorporated into the periphery of control cells, but not into the periphery of cells expressing LIMK1(WT), after stimulation with EGF. Alternatively, if coflin contributes to barbed end formation indirectly by supplying actin monomers for Arp2/3-mediated dendritic actin structure formation, we analyzed the effect of microinjection of Alexa546-actin monomers into cells expressing LIMK1(WT) or LIMK1(D460A). If coflin primarily contributes to EGF-induced barbed end formation by severing actin filaments, injected Alexa546-actin will be incorporated into the periphery of control cells, but not into the periphery of cells expressing LIMK1(WT), after stimulation with EGF. Alternatively, if coflin contributes to barbed end formation indirectly by supplying actin monomers, microinjected Alexa546-actin monomers will be incorporated into the periphery of both control and LIMK1(WT)-expressing cells in response to EGF stimulation. As shown in Fig. 8 A (top) and in Video 7 A (available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1), Alexa546-actin monomers injected into cells expressing LIMK1(WT) were initially diffuse in the cytoplasm and then incorporated into the distal cell margin 1–3 min after EGF stimulation. The YFP-actin distribution remained largely unchanged after EGF stimulation, probably because most YFP-actin was already assembled into F-actin as a result of coflin inactivation (Fig. 8 A, middle; and Video 7 A). Alexa546-actin injected into control cells expressing LIMK1(D460A) was similarly incorporated into the cell periphery after EGF stimulation (Fig. 8 B, top; and Video 8 A). In this experiment, YFP-actin
was also incorporated into the cell periphery after EGF stimulation, because YFP-actin monomers were abundant in the cell (Fig. 8B, middle). Quantitative analysis of Alexa546-actin fluorescence intensity in the cell margin revealed that Alexa546-actin incorporation into the cell margin significantly increased with time after EGF stimulation in both LIMK1(WT)- and LIMK1(D460A)-expressing cells (Fig. 8A and B, right). The time course of Alexa546-actin incorporation in LIMK1(WT)-expressing cells was comparable to that of LIMK1(D460A)-expressing cells.

To further examine the role of coflin in stimulus-induced actin assembly, we performed Alexa546-actin microinjection studies on neuregulin (NRG)-stimulated MCF-7 cells, in which coflin/ADF were knocked down by siRNA. Alexa546-actin monomers injected were efficiently incorporated into the cell periphery of both coflin/ADF and control siRNA MCF-7 cells.
in response to NRG stimulation (Fig. 8, C and D; and Videos 9 A and 10 A, available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1). Similar to the case of LIMK1 overexpression, YFP-actin was incorporated into the cell periphery in control siRNA cells, but it was almost unchanged in cofilin/ADF siRNA cells. In both LIMK1(WT)-expressing COS7 cells and cofilin/ADF siRNA MCF-7 cells, injected Alexa546-actin monomers were also incorporated into the cell periphery even in the absence of cell stimulation, although the level of incorporation was substantially lower than in stimulated cells (Fig. 8, A and C, graphs; Fig. S4, A and C; and Videos 7 and 9). This behavior probably reflects the fact that actin monomers are depleted in cofilin-inactive cells, and therefore, the ratio of the injected Alexa546-actin monomers is relatively high in the actin monomer pool available for polymerization. Thus, we conclude that actin monomers injected into the cytoplasm were effectively incorporated into actin filaments in the cell periphery in response to cell stimulation in both cofilin-active and -inactive cells. These results suggest that coflin is required for stimulus-induced barbed end formation and actin filament assembly in the cell periphery primarily by supplying actin monomers.

**Discussion**

Cofilin is known to stimulate actin filament disassembly by depolymerizing and severing actin filaments both in vitro and in vivo (Bamburg et al., 1999; Pantaloni et al. 2001). In contrast, it has also been reported that cofilin is involved in actin filament polymerization within the cell, as in the case of stimulus-induced lamellipodium formation (Chan et al., 2000; Ghosh et al., 2004). Because the rate of actin filament assembly at the leading edge depends on the concentrations of actin monomers and free barbed ends, and cofilin can both increase actin monomers by disassembling actin filaments and create free barbed ends by severing the filaments, the precise role of cofilin in actin filament assembly and disassembly in the cell is unclear. This study was conducted to understand comprehensively the cellular role of cofilin in actin filament assembly and disassembly, especially in stimulus-induced lamellipodium formation. Our results demonstrate that (1) inactivation or knock down of cofilin remarkably decreased the actin monomer pool in the cytoplasm, indicating that cofilin is responsible for the generation of the bulk of the actin monomer pool in the cytoplasm; (2) actin monomers in the cytoplasm were efficiently incorporated into the tip of the lamellipodium, and incorporation was dependent on both cofilin activity and the cytoplasmic actin monomer population; and (3) cofilin inactivation suppressed stimulus-induced actin monomer incorporation at the cell periphery, but microinjection of actin monomers into cofilin-inactivated cells rescued actin incorporation into the cell periphery, which indicates that defective actin monomer incorporation in cofilin-inactivated cells is due to the lack of actin monomers, not to the lack of free barbed ends. Together, these findings suggest that cofilin contributes to stimulus-induced actin filament assembly in the cell periphery by supplying a large amount of actin monomers to the cytoplasm.

**Role of cofilin in controlling the G-actin population in the cytoplasm**

In unstimulated cells, actin filaments are continuously assembled and disassembled at rates considerably faster than those of actin filament treadmilling in vitro (Pollard, 1986; Theriot and Mitchison, 1991). Cofilin is probably involved in this rapid turnover of actin filaments by stimulating actin filament disassembly. To understand the role of cofilin in actin filament dynamics within the cell, it is essential to quantify the G/F-actin ratios in cofilin-active and -inactive cells, but there have been only a few controversial reports measuring these ratios. Previous studies have reported that no significant change in F-actin content was observed after cofilin inactivation by LIMK1 expression or anti-cofilin antibody injection, by measuring the fluorescence intensity of MTLn3 cells stained by fluorescently labeled phalloidin, although they observed thick stress fibers and F-actin aggregates in cofilin-inactivated cells (Chan et al., 2000; Zebda et al., 2000). Based on these observations that cofilin inactivation did not change the G/F-actin ratio in the cell, these researchers proposed the barbed end creation model. In contrast, Hotulainen et al. (2005) analyzed the relative ratio of G/F-actin in NIH3T3 and B16F1 cells by estimating the content of G-actin, F-actin, and total actin using the fluorescence intensities of DNase I, phalloidin, and anti–β-actin antibody staining, respectively, and showed that the G-actin content decreased substantially in cofilin knockdown cells. However, phalloidin staining of F-actin is affected by association with actin-binding proteins, and the anti–β-actin antibody used to measure total actin does not stain stress fibers (Mies et al., 1998). Thus, more precise and quantitative analyses are needed to determine the G/F-actin ratio in cells. In this study, we assessed the actin monomer pool size in living cells by measuring the fluorescence decay of Dp-actin photoactivated in the cytoplasm. Using this method and F-actin sedimentation assay, we showed that cofilin inactivation or knock down markedly reduced the G-actin content in the cell. Thus, we conclude that cofilin critically contributes to the production of actin monomers in cells; more than half of the G-actin pool is generated by the action of cofilin. Considering the very low content of actin monomers in cofilin-inactivated cells, cofilin inactivation probably inhibits stimulus-induced actin filament assembly and lamellipodium formation by the lack of actin monomers required for polymerization.

Although the dendritic nucleation/array treadmill model proposes that actin monomers disassembled from the pointed ends of actin filaments by the help of cofilin activity are recycled for polymerization at the tip of the lamellipodium (Cramer, 1999; Pollard and Borisy, 2003), it has not been clarified whether actin monomers incorporated into the tip of the lamellipodium are self-sufficiently supplied from the pointed ends of actin filaments within the lamellipodium or indirectly via the cytoplasmic actin monomer pool. Here, we showed that actin monomers in the cytoplasm are efficiently incorporated into the tip of the lamellipodium at the rate correlated with the cytoplasmic actin monomer pool size. We also observed that Dp-actin photoactivated in the lamellipodium flowed retrogradely and rapidly diffused into the cytoplasm. Thus, most of the actin monomers
Figure 9. A model for the role of cofilin in stimulus-induced lamellipodium formation. (A) In control cofilin-active cells, cofilin contributes to production of the bulk of actin monomers in the cytoplasm and enhances actin filament turnover by stimulating actin filament disassembly. Stimulation of the cell with factors (such as EGF) activates the Arp2/3 complex and thereby stimulates the formation of dendritic actin filament structures, resulting in an increase in the number of barbed ends. Actin assembly onto the barbed ends near the cell edge pushes the membrane forward, leading to lamellipodial extension. Actin monomers required for dendritic actin filament assembly are supplied from the cytoplasmic actin monomer pool, which is maintained by cofilin activity. (B) In cofilin-inactivated or -depleted cells, actin monomers in the cytoplasm are decreased. Stimulation of the cell activates the Arp2/3 complex, but dendritic actin filament assembly is suppressed because sufficient actin monomers are not available. When actin monomers are exogenously injected into the cytoplasm, they are efficiently incorporated into the cell periphery, and actin filament assembly occurs even under conditions of cofilin depletion. P-cofilin, Ser-3-phosphorylated cofilin.

Role of cofilin in stimulus-induced actin filament assembly

During cell migration, actin filaments are assembled to form the lamellipodial protrusion at the leading edge of the cell. Addition of actin monomers to the barbed ends of actin filaments at the tip of the lamellipodium gives the force to propel the membrane forward. A previous study showed that EGF induces a large generation of actin barbed ends at the leading edge and cofilin inactivation inhibited EGF-induced barbed end formation, lamellipodium extension, and cell migration (Chan et al., 2000). By measuring the incorporation of extracellularly added Alexa546-actin into the periphery of permeabilized COS7 cells, we also observed that cofilin inactivation suppressed EGF-induced barbed end formation. Thus, cofilin plays a critical role in stimulus-induced barbed end formation at the cell periphery. In general, free barbed ends can be generated by severing or capping of preexisting actin filaments or de novo synthesis (and branching) of actin filaments. It is theoretically possible that cofilin is involved in barbed end formation either directly, by severing actin filaments, or indirectly, by supporting formation of branched actin filaments by increasing the actin monomer pool in the cell. Studies on EGF-stimulated MTLn3 carcinoma cells proposed the former model where cofilin creates new barbed ends by directly severing actin filaments (Condeelis, 2001; Ghosh et al., 2004; DesMarais et al., 2005). If cofilin dominantly acts by directly creating new barbed ends, microinjected actin monomers would not be effectively incorporated into the cell periphery of cofilin-inactivated cells after EGF stimulation, because the barbed end creation is blocked by cofilin inactivation. If cofilin dominantly acts by supplying actin monomers, injection of actin monomers would recover the inhibitory effect of cofilin inactivation on stimulus-induced actin filament assembly at the cell periphery. We demonstrated that Alexa546-actin monomers injected were effectively incorporated into the cell periphery after cell stimulation, in both control and cofilin-depleted cells, which suggests that cofilin contributes to stimulus-induced actin filament assembly by supplying actin monomers.

Based on these observations, we propose a model for the role of cofilin in stimulus-induced lamellipodium formation (Fig. 9). In resting cells, cofilin plays a critical role in maintaining the actin monomer pool in the cytoplasm by stimulating actin filament disassembly, thereby creating an abundant supply of actin monomers. Stimulation of the cell induces activation of the Arp2/3 complex, via Rac and WAVE activation (Takenawa and Miki, 2001), which stimulates de novo synthesis and arborization...
of actin filaments to form the dendritic actin structures. Cofilin contributes to this process by supplying actin monomers for polymerization. Arp2/3-assisted dendritic growth of actin filaments will exponentially increase the number of free barbed ends. In contrast, in cofilin-depleted cells, the actin monomer pool is substantially lower in resting cells; therefore, stimulus-induced dendritic actin polymerization and barbed end formation in the cell periphery are inhibited even when the Arp2/3 complex is activated. In conclusion, we have provided evidence that cofilin contributes to stimulus-induced actin filament assembly by supplying an abundant pool of actin monomers to the cytoplasm and that its severing activity is predominantly involved in this process.

Materials and methods

Materials

Jasp, LaiA, and Alexa Fluor 546 were purchased from Invitrogen. EGF and NRG were purchased from Sigma-Aldrich and R&D Systems, respectively. Actin was purified from rabbit skeletal muscle. G-actin was labeled with Alexa Fluor 546 C3 maleimide, according to the manufacturer's protocols (Invitrogen).

Plasmid construction

Plasmids coding for Dp and SECFP were provided by A. Miyawaki (Riken, Wako, Japan); Expression plasmids for GFP (pEGFP-C1) and DsRed-monomer (mDsRed-C1) were purchased from CLONTECH Laboratories, Inc. Plasmids for YFP-actin and CFP-SSH-1L were constructed as described previously (Endo et al., 2003; Kaji et al., 2003). Expression plasmids for Dp-actin, LIMK1-SECFP, LIMK1-mDsRed, and cofilin-mDsRed were constructed by subcloning PCR-amplified Dp, SECFP, mDsRed, β-actin, LIMK1, or cofilin cDNA into the pEGFP-C1 vector. The plasmid for Myc-actin was constructed by inserting β-actin cDNA containing a Myc epitope tag into the pEGFP-C1 vector. Human cofilin siRNA plasmid (target sequence GGAG-GATCCTGTTGTATAC, human ADF siRNA plasmid (target sequence GCAATGGGACAGAATG), and control siRNA plasmid (target sequence TCTCCCGCGAAGAAGATA, corresponding to the mutated human SSH-1L oligo) were constructed as described previously (Nishita et al., 2005).

Cell culture and transfection

Cells were cultured in DME supplemented with 10% (COS7 and MCF-7 cells) or 15% fetal calf serum (N1E-115 cells). Cells were transfected with expression plasmids using Lipofectamine 2000 (Invitrogen). Cells were used for various assays after being cultured for 18–24 h (COS7 and N1E-115 cells) or 4 d (MCF-7 cells) after transfection.

Photactivation and time-lapse fluorescence microscopy

Photactivation of Dp or Dp-actin and fluorescence imaging were performed using a laser-scanning confocal imaging system (LSM 510; Carl Zeiss Microimaging, Inc.) equipped with a PL APO 63 × oil-immersion objective lens (NA 1.3; Leica) and a cooled charge-coupled device camera (CoolSNAP HQ; Roper Scientific) and a fl uorescence microscope (DMIRBE; Leica) equipped with a PL APO 63 × oil-immersion objective lens (NA 1.3; Leica) and a cooled charge-coupled device camera (CoolSNAP HQ; Roper Scientific). Incorporation of Alexa546-actin into the cell periphery was quantified by measuring the mean fluorescence intensity in a region 2 μm from the cell edge, using a customized macro in ImageJ (Fig. 7 C).

Visualization of free barbed ends using a cell-permeabilization assay

Visualization of free barbed ends using a cell-permeabilization assay was performed using a fluorophore-tagged actin binding protein. Alexa546-actin monomers were injected into cells using a microinjection system (without phenol red) with 10% fetal bovine serum for 3 h before microinjection. The images were obtained using a fluorescence microscope (DMIRBE; Leica) equipped with a PL APO 63 × oil-immersion objective lens (NA 1.3; Leica) and a cooled charge-coupled device camera (CoolSNAP HQ; Roper Scientific) driven by Q550FW Imaging Software (Leica). Incorporation of Alexa546-actin into the cell periphery was quantified by measuring the mean fluorescence intensity in a region 2 μm from the cell edge using ImageJ (Fig. 3 C).

Online supplemental material

Fig. S1 shows the expression level and localization of Dp in COS7 cells. Fig. S2 shows the level of Pcofilin in LIMK1-expressing COS7 cells. Fig. S3 shows the biochemical characterization of cofilin mutants. Fig. S4 shows the fluorescence images of Alexa546-actin microinjected into COS7 cells expressing LIMK1 (WT or D460A) or MCF-7 cells transfected with cofilin/ADF siRNA or control siRNA, without cell stimulation. Videos 1 and 2 show the time-lapse fluorescence of Dp or Dp-actin in COS7 cells. Video 3 shows the time-lapse fluorescence of Dp-actin in COS7 cells coexpressing LIMK1 (WT) with cofilin mutants or SSH-1. Video 4 shows the time-lapse fluorescence of Dp-actin or Alexa546-actin in the MCF-7 cells transfected with cofilin/ADF or control siRNA. Videos 5 and 6 show the time-lapse fluorescence of Dp-actin transfected in the cytoplasm or in the lamellipodium in RacV12-expressing N1E-115 cells. Videos 7 and 8 show the time-lapse fluorescence of Alexa546-actin and YFP-actin in COS7 cells expressing LIMK1 (WT) or LIMK1(D460A) (Video 8) with or without EGF stimulation. Videos 9 and 10 show the time-lapse fluorescence of Alexa546-actin and YFP-actin in MCF-7 cells transfected
with cofilin/ADF siRNA (Video 9) or control siRNA (Video 10) with or without NRG stimulation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200610005/DC1.

We thank Dr. Atsushi Miyawaki for plasmids for Dp and SECFP and Sachiko Fujimura for technical assistance. This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18013007 and 17049002).

Submitted: 2 October 2006
Accepted: 5 April 2007

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