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Cofilin is a pH sensor for actin free barbed end formation: role of phosphoinositide binding

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Introduction

At the leading edge of motile cells, increased assembly of a branched actin filament network is a driving force for membrane protrusion (Pollard and Borisy, 2003). Key events acting synergistically to generate this actin network are filament severing to increase the abundance of actin free barbed ends (Carlsson, 2006), and filament nucleation and branching by the Arp2/3 complex (Pollard, 2007). Regulation of Arp2/3 complex nucleating activity by Rho family GTPases and nucleation-promoting factors of the Wiskott-Aldrich syndrome protein (WASP) family has been extensively studied (Goley and Welch, 2006; Stradal and Scita, 2006). Less is known about control of actin filament severing in motile cells. Although actin free barbed ends act as nuclei for filament assembly and can be generated by Arp2/3 complex nucleating activity and by uncapping barbed ends of preexisting filaments (Condeelis, 2001), filament severing by the actin-binding protein cofilin generates a rapid increase in free barbed ends in motile cells and is critical for membrane protrusion (Chan et al., 2000; Ghosh et al., 2004; Mouneimne et al., 2004).

Growth factors induce a rapid biphasic increase in actin free barbed ends, and we found both phases absent in fibroblasts lacking H+ efflux by the Na-H exchanger NHE1. The first phase is restored by expression of mutant cofilin-H133A but not unphosphorylated cofilin-S3A. Constant pH molecular dynamics simulations and nuclear magnetic resonance (NMR) reveal pH-sensitive structural changes in the cofilin C-terminal filamentous actin binding site dependent on His133. However, cofilin-H133A retains pH-sensitive changes in NMR spectra and severing activity in vitro, which suggests that it has a more complex behavior in cells. Cofilin activity is inhibited by phosphoinositide binding, and we found that phosphoinositide binding is pH-dependent for wild-type cofilin, with decreased binding at a higher pH. In contrast, phosphoinositide binding by cofilin-H133A is attenuated and pH insensitive. These data suggest a molecular mechanism whereby cofilin acts as a pH sensor to mediate a pH-dependent actin filament dynamics.

Abbreviations used in this paper: ADF, actin-depolymerizing factor; HSQC, heteronuclear single quantum coherence; MD, molecular dynamics; NMR, nuclear magnetic resonance; pHi, intracellular pH; P(1,4,5)P2, phosphatidylinositol-4,5-bisphosphate; PI(4,5)P2, phosphotidylinositol-4,5-bisphosphate; PI3-kinase, phosphoinositide 3-kinase; WT, wild type.

The online version of this paper contains supplemental material.
et al., 1998; Chen et al., 2004), and in wounded fibroblasts, increased pH is necessary for ADF- and coflin-regulated actin dynamics (Bernstein et al., 2000). H⁺ efflux mechanisms at the leading edge of motile cells have been speculated (Bailly and Jones, 2003; Bernstein and Bamburg, 2004) but have not been confirmed to spatially regulate coflin activity.

We find here that H⁺ efflux by the mammalian Na-H exchanger NHE1 promotes a coflin-dependent increase in actin free barbed ends in response to migratory cues. NHE1 catalyzes phosphotidylinositol-4,5-bisphosphate (PI(4,5)P₂) (Yonezawa et al., 1990; Ojala et al., 2001; Gorbatyuk et al., 2006; van Rheenen et al., 2007) or an increase in intracellular pH (pHᵢ), presumably by deprotonation of His133 in the F-site (Pope et al., 2004), may be necessary, which suggests that coflin acts as a coincidence detector with its activation, requiring several independent regulatory events. The activity of coflin in most species is recognized to be pH sensitive. Coflin activity in vitro increases at neutral and higher pH (Hawkins et al., 1993; Maciver et al., 1998; Chen et al., 2004), and in wounded fibroblasts, increased pHᵢ is necessary for ADF- and coflin-regulated actin dynamics (Bernstein et al., 2000). H⁺ efflux mechanisms at the leading edge of motile cells have been speculated (Bailly and Jones, 2003; Bernstein and Bamburg, 2004) but have not been confirmed to spatially regulate coflin activity.

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an electroneutral exchange of extracellular Na\(^+\) for intracellular H\(^+\), and its activity increases in response to migratory cues, including monolayer wounding (Franz et al., 2007), growth factors (Putney et al., 2002; Frantz et al., 2007), and integrin engagement (Schwartz et al., 1991; Tominaga and Barber, 1998). In motile fibroblasts (Denker and Barber, 2002) and Dictyostelium discoideum cells (Patel and Barber, 2005), NHE1 localizes at the distal margin of membrane protrusions, and its H\(^+\) efflux is necessary for directed migration of mammalian fibroblasts (Denker and Barber, 2002), leukocytes (Ritter et al., 1998), and epithelial (Klein et al., 2000; Reshkin et al., 2000) and melanoma cells (Stock et al., 2005), and for chemotaxis of Dictyostelium cells (Patel and Barber, 2005). In Dictyostelium cells with a targeted deletion of nhe1, actin filament assembly in response to a chemotaxant is attenuated (Patel and Barber, 2005).

Our current findings indicate that motile fibroblasts expressing a mutant NHE1 lacking H\(^+\) efflux have attenuated de novo actin filament assembly and no increase in actin free barbed ends compared with fibroblasts expressing wild-type (WT) NHE1. A rapid first phase of actin free barbed end formation was restored by expression of pH-insensitive Acanthamoeba castellanii actophorin or mutant cofilin-H133A but not by unphosphorylated cofilin-S3A. Computational modeling, nuclear magnetic resonance (NMR) spectroscopy, and functional studies revealed the significance of His133 in pH-sensitive cofilin activity and actin free barbed end formation. However, purified cofilin-H133A retained pH-sensitive conformational changes and severing activity. We found that Pl(4,5)P2 binding to WT cofilin is pH sensitive, with increased binding at pH 7.5, compared with pH 6.5, but Pl(4,5)P2 binding to cofilin-H133A is pH-insensitive, with binding similar to WT at pH 7.5. Although pH and Pl(4,5)P2 regulate cofilin activity, our data indicate that these two controls are related and suggest that pH-dependent Pl(4,5)P2 binding by cofilin regulates actin-severing activity. Additionally, our findings support an emerging role for pH-sensitive His switches in phosphoinositide binding.

Results

H\(^+\) efflux by NHE1 is necessary for increased actin filament assembly in response to migratory cues

In mammalian fibroblasts (Denker and Barber, 2002) and in Dictyostelium cells (Patel and Barber, 2005), H\(^+\) efflux by NHE1 is necessary for efficient directed migration. In chemotaxing Dictyostelium cells, NHE1 also is necessary to suppress lateral pseudopods and to promote de novo actin assembly at the cell front (Patel and Barber, 2005). We asked whether NHE1 regulates actin filament assembly in motile fibroblasts by using NHE1-deficient cells stably expressing WT NHE1 (WT cells) or a mutant NHE1 containing an isoleucine substitution for glutamine 266 that lacks H\(^+\) efflux (E266I cells; Denker et al., 2000). WT and E266I cells at the edge of a wounded monolayer had a time-dependent increase in cortical F-actin after wounding; however, at all time points, the abundance of F-actin was significantly lower in E266I cells compared with WT cells (Fig. 1, A and B).

Conversely, the abundance of cortical F-actin before wounding (not depicted) and immediately after wounding (Fig. 1 A) was similar in E266I and WT cells. De novo increases in F-actin in quiescent subconfluent cells treated with 50 ng/ml PDGF were also attenuated in E266I cells compared with WT cells (Fig. 1 C). With PDGF, pH\(_i\) increases in WT cells from 7.15 ± 0.03 at quiescence to 7.47 ± 0.05, but pH\(_i\) in E266I cells does not change and is 7.03 ± 0.05 in the absence and presence of PDGF (Franz et al., 2007). Like confluent cells, the abundance of F-actin in quiescent E266I cells was similar to WT cells. However, with 50 ng/ml PDGF, total F-actin increased significantly in WT cells at 1 min (P < 0.05), 3 min (P < 0.05), and 6 min (P < 0.01) but not in E266I cells (P > 0.1 at 1, 3, and 6 min; Fig. 1 C). These data suggest that H\(^+\) efflux by NHE1 is not necessary for steady-state F-actin abundance but is required for a rapid increase in actin filament assembly in response to migratory cues.

H\(^+\) efflux by NHE1 is necessary for biphasic actin free barbed end formation

The assembly of new actin filaments is enhanced by severing of existing filaments to increase the abundance of actin free barbed ends (Falet et al., 2002; Mouneimne et al., 2004; Carlsson, 2006). In motile epithelial cells (Mouneimne et al., 2004), macrophages (Cox et al., 1996), and Dictyostelium cells (Hall et al., 1989), the generation of new free barbed ends is biphasic. WT fibroblasts treated with PDGF also had a biphasic increase in the number of actin free barbed ends (Fig. 2, A and B). A rapid and transient first phase was maximal at 1 min, with a twofold increase, and returned to near control levels at 2 min. A second phase included a maximal increase at 3 min that was smaller than the first phase, and at 4 min, the number of actin free barbed ends was at control levels. The maximal increase in free barbed end formation was at ~0.5 µm of the submembranous region (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200804161/DC1). In quiescent cells, the number of actin free barbed ends was similar in WT and E266I cells. However, in E266I cells, there was no increase in free barbed ends with PDGF, and at 1 and 3 min, the abundance of free barbed ends was significantly less than in WT cells (P < 0.001 and P < 0.01, respectively; Fig. 2, A and B). Hence, H\(^+\) efflux is not necessary for the number of actin free barbed ends at steady state but is necessary for increased formation in response to PDGF.

We also found a pH-dependent regulation of actin free barbed ends in NHE1-deficient PS120 cells stably expressing the system N1 transporter (SN1 cells). SN1 is a plasma membrane amino acid transporter expressed in the central nervous system that couples uptake of extracellular glutamine with efflux of intracellular H\(^+\) efflux. When expressed in PS120 cells, SN1 is uniformly localized along the plasma membrane, and H\(^+\) efflux is dependent on the concentration of extracellular glutamine (Chaudhry et al., 1999). Although short-term (4 h) incubation with extracellular glutamine between 1 and 400 µM does not change the pH\(_i\) of WT cells (Srivastava et al., 2008), it significantly increased the pH\(_i\) of SN1 cells (Fig. 2 C). With 1 µM glutamine, pH\(_i\) was 7.06 ± 0.03 and similar to E266I cells; with 25 µM glutamine, pH\(_i\) was 7.18 ± 0.02 and similar to quiescent WT cells; and with 400 µM glutamine, pH\(_i\) was 7.38 ± 0.04 and similar to
but not sufficient in the absence of PDGF to increase actin free barbed end formation. Moreover, because SN1 is not clustered at lamellipodia like NHE1, these data suggest that the abundance of cortical free barbed ends can be regulated by global increases in pH$_i$ and that local changes may not be necessary.

The first but not second phase of increased free barbed ends in epithelial cells treated with growth factors is generated predominantly by severing activity of coflin (Mouneimne et al., 2004), which is recognized to be pH-dependent (Bamburg and Wiggan, 2002). Because the pH$_i$ of 7 in E266I cells treated with growth factors is significantly lower than the pH$_i$ of 7.5 in WT PDGF-stimulated WT cells. In three SN1 cell preparations, PDGF induced a biphasic increase in actin free barbed ends with 400 μM glutamine, with significant increases at 1 min (P > 0.001) and 3 min (P > 0.01); with 25 μM glutamine, there was a significant increase at 1 min (P > 0.05) but not at 3 min (Fig. 2 D). In two SN1 cell preparations incubated with 1 μM glutamine, there was no increase in free barbed ends with PDGF (Fig. 2 D). However, decreasing extracellular glutamine from 400 to 1 μM had no effect on the relative number of free barbed ends in the absence of PDGF. In addition to confirming pH dependence, data with SN1 cells indicate that increasing pH$_i$ from 7 to ~7.4 is necessary but not sufficient in the absence of PDGF to increase actin free barbed end formation. Moreover, because SN1 is not clustered at lamellipodia like NHE1, these data suggest that the abundance of cortical free barbed ends can be regulated by global increases in pH$_i$ and that local changes may not be necessary.

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cells (Yan et al., 2001; Frantz et al., 2007), we speculated that coflin-induced free barbed end formation might be inhibited at the lower pH of E266I cells. Although most species of coflin are activated by pH > 7, the activity of A. castellanii coflin actophorin is pH-insensitive (Maciver et al., 1998). The number of free barbed ends in WT fibroblasts expressing actophorin was not significantly different in the absence of PDGF (10.42 ± 2.13 arbitrary units) compared with untransfected (control) WT cells (9.56 ± 1.85; Fig. 3, A and B; P > 0.5; n = 3). With PDGF, there was a twofold and 1.5-fold increase in barbed ends at 1 and 3 min, respectively, that was similar to increases in cells without actophorin (Fig. 3, A and B). In E266I cells, actophorin had no effect on the relative number of free barbed ends in the absence of PDGF (9.97 ± 1.90 and 8.42 ± 1.06 with and without actophorin, respectively) and nearly restored the number of free barbed ends in the first phase to values in WT cells (P > 0.1; E266I at 1 min compared with WT at 1 min, n = 3), but had no effect on attenuated free barbed end formation in the second phase (Fig. 3, A and B).

Cofilin activity also increases with dephosphorylation of Ser3 (Bamburg and Wiggan, 2002). Transient expression of human coflin-S3A in WT cells had no effect on the number of free barbed ends in the absence of PDGF (11.15 ± 1.48 and 10.72 ± 0.89 with and without coflin-S3A, respectively;
E266I cells (Fig. S1, B and C). Hence, H+ efflux by NHE1 is not necessary for coflin expression or the regulated dephosphorylation of coflin. Although coflin-S3A has been found to be constitutively active in cells (Moriyama et al., 1996; Zebda et al., 2000; Ghosh et al., 2004), previous studies used cells with normal pH homeostasis. Our data suggest a pH-dependent activation of coflin for generating actin free barbed ends that is distinct from regulation by dephosphorylation of S3.

Structural models for regulation of coflin by phosphorylation and by pH
To understand how phosphorylation and pH independently regulate coflin-severing activity, we performed a series of molecular dynamics (MD) simulations. First, we performed explicit solvent MD on human coflin, starting from the NMR structure (Pope et al., 2004), with Ser3 either phosphorylated or unphosphorylated. In the simulation of phosphorylated Ser3, the N-terminal portion interacted with the longest helix in the protein (α4), which forms part of the G-site implicated in binding to both G-actin and F-actin (Fig. 4 A, left). In particular, pSer3 forms salt-bridging interactions with Lys126 and Lys127 (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200804161/DC1). Other than this newly identified interaction, the overall structure of coflin largely remains unperturbed. In contrast, the N terminus of unphosphorylated coflin remained unstructured (Fig. 4 A, right), which is in agreement with the experimental structure. These results appear to be broadly consistent with existing functional data showing that phosphorylation of Ser3 abrogates binding of G-actin and with structural data from Pope et al. (2004) that indicate significant chemical shift perturbations in coflin-S3D compared with WT coflin. Gorbatyuk et al. (2006) also described chemical shift perturbations in the N terminus as well as α4 upon phosphorylation of chick coflin by LIM kinase. These results are inconsistent, however, with data on actophorin (Blanchoin et al., 2000), where the phosphorylated residue is located at the immediate N terminus (Ser1). In that case, the protein structure was solved crystallographically in both the phosphorylated and unphosphorylated forms; pSer1 was not visualized in the electron density, and little conformational change was seen in the rest of the protein.

To understand how increased pH might modulate coflin activity independent of unphosphorylated Ser3, constant pH MD (CpHMD) simulations were performed on human coflin at pH values between 6 and 8. The solution structure of human coflin determined by NMR spectroscopy suggests that at pH 6, a solvent-exposed salt bridge forms between His133 and Asp98 (Pope et al., 2004). Our data corroborated this finding (Fig. 4 B, left) and also showed that at higher pH values, deprotonation of His133, the only histidine in human coflin, weakens the interaction with Asp98, which instead tends to interact with Lys96 (Fig. 4 B, right). In the absence of a structure for coflin bound to F-actin, the relationship of this pH-dependent conformational change to pH-dependent binding to F-actin is uncertain, but the His133 protonation state change and accompanying conformational changes could in principle modulate binding affinity at the F-site. Other conformational changes accompanying the change in pH are relatively minor, occurring primarily in the immediate vicinity of His133, as well as α1 and α4.

We also used NMR to monitor pH-dependent changes in coflin. Comparison of 2D 15N heteronuclear single quantum coherence (HSQC) spectra of recombinant human coflin at pH 6.5 and 7.5 showed chemical shift changes for residues in the vicinity of His133, which indicates changes in their chemical environments (Fig. S2 B). These findings are again consistent with chemical shift perturbations reported previously (Pope et al., 2004). Our data corroborated this finding (Fig. 4 B, left) and also showed that at higher pH values, deprotonation of His133, the only histidine in human coflin, weakens the interaction with Asp98, which instead tends to interact with Lys96 (Fig. 4 B, right). In the absence of a structure for coflin bound to F-actin, the relationship of this pH-dependent conformational change to pH-dependent binding to F-actin is uncertain, but the His133 protonation state change and accompanying conformational changes could in principle modulate binding affinity at the F-site. Other conformational changes accompanying the change in pH are relatively minor, occurring primarily in the immediate vicinity of His133, as well as α1 and α4.
E266I cells may be necessary for the first phase of free barbed end formation with PDGF. Additionally, Pope et al. (2004) predict that His133 has an upshifted \( pK_a \) of 7.4, although this could not be measured directly because of absence of signals from His133 in NMR spectra. We confirmed that expression of a mutant coflin-H133A in E266I cells restored the first but not second phase of free barbed end formation. Compared with vector controls, WT cells expressing WT coflin and coflin-H133A had similar biphasic increases in the number of free barbed ends with PDGF, although the relative increase with WT coflin was significantly greater than with coflin-H133A (Fig. 5, A and B; et al., 2004). Collectively, our simulation and NMR results, in combination with prior structural and biochemical data, support a model in which phosphorylation of Ser3 blocks binding to actin at the G-site, whereas protonation of His133 modulates binding at the F-site.

**Cofilin H133A restores the first phase of free barbed end formation in E266I cells but retains pH dependence**

On the basis of structural data, we speculated that deprotonation of His133 at the pH of 7.5 in WT cells but not at the pH of 7 in E266I cells may be necessary for the first phase of free barbed end formation with PDGF. Additionally, Pope et al. (2004) predict that His133 has an upshifted \( pK_a \) of 7.4, although this could not be measured directly because of absence of signals from His133 in NMR spectra. We confirmed that expression of a mutant coflin-H133A in E266I cells restored the first but not second phase of free barbed end formation. Compared with vector controls, WT cells expressing WT coflin and coflin-H133A had similar biphasic increases in the number of free barbed ends with PDGF, although the relative increase with WT coflin was significantly greater than with coflin-H133A (Fig. 5, A and B; et al., 2004). Collectively, our simulation and NMR results, in combination with prior structural and biochemical data, support a model in which phosphorylation of Ser3 blocks binding to actin at the G-site, whereas protonation of His133 modulates binding at the F-site.

**Cofilin H133A restores the first phase of free barbed end formation and de novo actin filament assembly in E266I cells.**

(A) Relative abundance of barbed ends in the absence and presence of PDGF for the indicated times in WT and E266I cells transiently expressing WT coflin or coflin-H133A. Data are expressed relative to the abundance of barbed ends in quiescent vector-transfected WT cells and represent means ± SEM of three independent transfections, with at least 15 cells scored at each time point for each cell preparation. (B) Representative images of WT and E266I expressing coflin-H133A used for measuring actin free barbed ends. Bar, 10 \( \mu \)m. (C) Cortical F-actin at the leading-edge of wounded WT and E266I cells transfected with vector, WT coflin, or coflin-H133A. Cells were fixed at the indicated times and labeled with rhodamine-phalloidin, and F-actin at the leading-edge was quantified using National Institutes of Health Image. Data are expressed relative to the abundance of F-actin in vector-transfected WT cells immediately after wounding (initial) and represent means ± SEM of 20 to 30 cells for from two representative cell preparations.
Figure 6. NMR spectra and severing activity for WT and H133A coflin. (A) Comparison of $^{15}$N-HSQC spectra for the H133A mutant at pH 6.5 (red) and 7.5 (blue). Selected residue assignments are shown. These spectra show that peaks corresponding to several residues show chemical shift changes on changing the pH, which suggests that one or more side chains titrating in a physiological range are still present in the mutant. (B) Comparison of $^{15}$N-HSQC spectra for WT and H133A at pH 6 (selected residue assignments are shown). The H133A mutation introduces some structural changes but the protein remains well folded. (C) Superposition of MD snapshots for WT (green) and H133A (pink) coflin at pH 6.5. (D, top) Quantification of F-actin severing
P < 0.05; n = 3). Expressing WT cofilin in E266I cells did not restore free barbed end formation in either first or second phases, and the number of free barbed ends in both phases was not significantly different compared with quiescent cells (P > 0.1; n = 3; Fig. 5 A). However, in E266I cells expressing cofilin-H133A, the number of free barbed ends in the first phase was restored to that of WT cells expressing WT cofilin or cofilin-H133A, but the number of free barbed ends at quiescence and in the second phase were unchanged compared with untransfected cells (P > 0.1; n = 3). Additionally, cofilin-H133A but not WT cofilin partially restored an increase in cortical F-actin in E266I cells at the edge of a wounded monolayer (Fig. 5 C).

NMR was used to follow pH-dependent changes in the chemical environments of residues in H133A cofilin. 2D 15N-HSQC spectra of H133A cofilin at pH 6.5 and 7.5 showed that the mutant adopts a fold very similar to WT (Fig. 6, A and B). MD simulations of cofilin-H133A are consistent with this observation, with major conformational differences largely confined to the flexible loops (Fig. 6 C). For H133A, similar pH-dependent chemical shift changes or broadening of NMR signals, as seen in the WT protein, were also observed for residues in the vicinity of Ala133 (His133). These changes indicate the presence of one or more residues, probably in addition to His133, that titrate in a physiological range.

The NMR results suggested that cofilin-H133A may have a similar pH-dependent activity toward F-actin compared with WT. To determine whether cofilin-H133A retains pH-sensitive F-actin severing activity similar to the WT protein, a light microscopy severing assay was used that allows direct observation of severing in vitro (Ichetovkin, et al., 2000, 2002). The results clearly indicate that cofilin-H133A exhibits pH-sensitive severing of F-actin that is indistinguishable from WT (Fig. 6 D). H133A had minimal severing activity at pH 6 but a 25–30-fold increased activity at pH 7.

Deprotonation of His133 attenuates PI(4,5)P2 binding

In vitro, PI(4,5)P2 and F-actin competitively bind to cofilin (Yonezawa, et al., 1990) and actophorin (Van Troys, et al., 2000). In epithelial cells, activation of phospholipase Cγ, which hydrolyzes PI(4,5)P2, is necessary for the cofilin-dependent first phase of actin free barbed end formation (Mouneimne, et al., 2004), and in fibroblasts, PDGF induces an increase in phospholipase Cγ activity (Margolis, et al., 1990) and hydrolysis of PI(4,5)P2 (McNamee, et al., 1993). In cells, Föster resonance energy transfer analysis of the interaction between cofilin and PI(4,5)P2 demonstrates that cofilin is bound to PI(4,5)P2 and is released and activated in response to EGF stimulation by PI(4,5)P2 hydrolysis (van Rheezen, et al., 2007). Although NMR experiments implicate His133 in binding the PI(4,5)P2 head group (Gorbatyuk, et al., 2006), whether the protonation state of His133 regulates PI(4,5)P2 binding has not been reported. We used computational docking experiments to suggest a plausible model of interaction between PI(4,5)P2 and cofilin. When the head group of PI(4,5)P2 is docked to this site with His133 doubly protonated, the terminal phosphates interact closely with the His133 side chain (Fig. 7 A). When His133 is neutral, the head group does not dock in this pose and instead interacts with Lys125 (Fig. S3 B). This residue is part of a small group of residues (L1e124-Lys125-Lys126-Lys127-Leu128-Thr129) that had observed NMR spectral perturbations when bound to PI(4,5)P2 for a K132A/H133A double mutant (Gorbatyuk, et al., 2006). The three cationic residues (Lys125, Lys126, and Lys127) may represent a secondary interaction site for the PI(4,5)P2 head group. Similarly, PI(4,5)P2 binds to yeast cofilin, where Lys132 and His133 are absent, but residues Arg109 and Arg110, which are equivalent to Lys125 and Lys126, are important for binding PI(4,5)P2 (Ojala, et al., 2001). These results, in combination with the previous NMR studies with short-chain PI(4,5)P2 constructs (Gorbatyuk, et al., 2006), suggest that deprotonation of His133 might decrease binding but not abolish it entirely.

Discussion

Increased pH is an evolutionarily conserved but poorly understood mechanism promoting cytoskeleton assemblies and cell movement. Earlier work on the fertilization of sea urchin eggs (Begg and Rehbn, 1979), the acrosomal reaction in echinoderm sperm (Tilney, et al., 1978), and the motility of nematode sperm cells (King, et al., 1994; Italiano, et al., 1999) suggests that transient increases in pH are necessary for de novo assembly of cytoskeletal filaments. A necessary role for increased pH in directed migration has been shown in Dictyostelium cells (Van Duijn and Inouye, 1991) and in different mammalian cell types (Ritter, et al., 1998; Klein, et al., 2000; Reshkin, et al., 2000; Denker and Barber, 2002; Stock, et al., 2005). In growing plant pollen tubes, increased pH, at the distal cortex also promotes F-actin assembly at the tube tip (Lovely-Wheeler, et al., 2006). Our understanding of how pH regulates cytoskeleton dynamics and cell movement, however, is limited.

by WT cofilin and cofilin H133A at indicated pH. Each bar represents the percent increase in the number of filaments 4 min after treatment with cofilin, expressed as means ± SEM. (bottom) Sample images of F-actin severing by WT cofilin and cofilin H133A at indicated pH. The filaments were observed before (as 0 min) and 4 min after incubation with cofilin. Bar, 2 μm.
not been experimentally confirmed in other cell types. In Dictyostelium cells, the first phase of F-actin assembly is attenuated but not eliminated by inhibition of Rac1B (Park et al., 2004), RasG (Sasaki et al., 2004), and DdNHE1 (Patel and Barber, 2005). The second phase is thought to drive membrane protrusion. In Dictyostelium cells, the second phase is also dependent on DdNHE1 and on increased abundance of PI(3,4,5)P3 at the cell front, which is regulated by phosphoinositide 3-kinases (PI3-kinases) and the PI3-phosphatase PTEN. Inhibiting PI3-kinases suppresses the second phase of actin polymerization, but the first phase is retained (Funamoto et al., 2001; Chen et al., 2003). However, several findings challenge the role of increased PI(3,4,5)P3 in regulating actin kinetics. First, a gene knockout strain in Dictyostelium lacking PTEN and all five type-I PI3-kinases...
retains actin assembly (Hoeller and Kay, 2007). Second, a redundant phospholipase A2 pathway regulates actin kinetics independent of PI3-kinase activity (Chen et al., 2007; van Haastert et al., 2007). Third, mutant cells that lack a second phase of actin kinetics have decreased (Denker and Barber, 2002) or increased (Patel and Barber, 2005) PI(3,4,5)P3.

The generation of new free barbed ends is necessary for increased actin filament assembly (Condeelis, 2001; Falet et al., 2002; Carlsson, 2006) and has biphasic kinetics in response to migratory cues (Mouneime et al., 2004). We used different fibroblast models expressing an inactive NHE1-E266I or the SN1 glutamine-H+ transporter to show that H+ efflux is necessary for both phases of free barbed end formation in fibroblasts, with the first but not the second phase being dependent on pH-sensitive coflin activity. In epithelial cells, coflin activity is also necessary for the first phase of free barbed end formation, but the second phase is dependent on PI3-kinase activity (Mouneime et al., 2004), like the second phase of actin assembly in amoeboid cells. Although migrating E266I cells have decreased and mislocalized PI(3,4,5)P3 (Denker and Barber, 2002), whether this contributes to the absence of the second phase of free barbed end formation is uncertain. In Ddhn1-null Dictyostelium cells, which lack a second phase of actin filament assembly, the abundance of PI(3,4,5)P3 is increased, which suggests that NHE1 regulation of the second phase may be mediated by mechanisms independent of PI(3,4,5)P3.

The ability of coflin-H133A but not coflin-S3A to restore the first phase of actin free barbed end formation in E266I cells indicates the importance of coincidence regulation for coflin activity that requires S3 dephosphorylation and increased pH. Coincidence regulation has also been suggested by previous work on the solution structure of coflin (Pope et al., 2004) and by our computational modeling and NMR data. These data indicate distinct mechanisms for modulating actin binding that are determined by Ser3 dephosphorylation in the N terminus and by pH sensing in the C terminus. Changes in pH have little effect on the overall structure and dynamics of coflin. Rather, lowered pH likely inhibits interactions with actin at the F-site by directly modulating the binding affinity at that site through the change in His133 charge, and through localized conformational changes. In contrast, we propose that phosphorylation of Ser3 inhibits binding to the G-site by steric occlusion, specifically by ionic interactions between pSer3 and Lys126, and Lys127. Little conformational change is predicted in the remainder of the structure, which is consistent with previous NMR work on human coflin-S3D (Pope et al., 2004) and chick coflin-pSer3 (Gorbayuk et al., 2006).

Coincidence regulation by protons suggests that global changes in pH may be sufficient for spatially restricted regulation, and that local pH changes may not be necessary. Our data with SN1 cells support this possibility because SN1 is not localized at membrane protrusions like NHE1. In activated fibroblasts (Dawe et al., 2003) and platelets (Falet et al., 2005), dephosphorylated coflin is restricted to the distal cortex, which would spatially limit a second activation step by increased pH. Additionally, because PI(4,5)P2 and F-actin competitively bind coflin (Van Troys et al., 2000; Yonezawa et al., 1990), dephosphorylated coflin at the plasma membrane would be inactive when bound to PI(4,5)P2. Moreover, coflin can only bind to tropomyosin-free actin filaments, which are only present at the plasma membrane interface with the cytoplasm, further restricting the location of F-actin severing by coflin (DesMarais et al., 2002).

Our data support a mechanism whereby pH-dependent coflin activity in cells is determined primarily by regulating coflin binding to PI(4,5)P2. We predict that, at the lower pH of resting cells, coflin activity is inhibited by greater maximum binding to PI(4,5)P2. Increased pH, with migratory cues lowers the abundance of PI(4,5)P2 bound to coflin, and, with dephosphorylation of S3, increases coflin activity. Our finding that protonation of coflin His133 increases PI(4,5)P2 binding is significant for two reasons. First, for activation of coflin, it suggests that increased pH and release of PI(4,5)P2 are biochemically linked. Although pH and PI(4,5)P2 are recognized regulators of coflin activity (Baillie and Jones, 2003), pH-dependent binding of PI(4,5)P2 to coflin has not been shown. However, we speculate that pH-dependent release of PI(4,5)P2 would not be sufficient as a coincidence activator of coflin without dephosphorylation of S3. In SN1 cells, increased actin free barbed ends required a higher pH and growth factor.

Second, of general significance is an emerging theme of pH-dependent phosphoinositide binding at sites containing histidine residues. Phosphoinositide binding to FYVE domains is pH-sensitive and regulated by a histidine switch (Lee et al., 2005). Additionally, we recently showed pH-sensitive PI(4,5)P2 binding to the guanine nucleotide exchange factor Dbs (Dbl’s big sister), which contains a His (H843) in the predicted phosphoinositide-binding site (Frantz et al., 2007). In contrast, PI(4,5)P2 binding to the guanine nucleotide exchange factor intersectin, which binds phosphoinositides at a charged Lys/Arg cluster, is pH-insensitive. The PI(4,5)P2 binding site in actrophorin lacks a His (Van Troys et al., 2000), which could contribute to the pH-insensitive activity of A. castellanii coflin. A review of phosphoinositide-binding domains in the PDB indicates that although binding sites containing charged Lys/Arg-rich clusters are the most prevalent, many of these domains contain a His within predicted phosphoinositide-binding sites. As recently suggested (Gorbayuk et al., 2006), phosphoinositide binding is a likely negative regulator of most proteins that cap or sever actin filaments. We predict that if phosphoinositides bind at His residues in these proteins, binding and protein function may be pH-sensitive. Additionally, the hydrolysis of PI(4,5)P2 by PLC, which is required for the full release and activation of coflin in vivo (van Rheenen et al., 2007), would be facilitated by the weakened binding of coflin to PI(4,5)P2 resulting from a local increase in pH in the presence of activated NHE1.

Materials and methods

Cell culture

NHE1-deficient PS120 fibroblasts stably expressing WT NHE1 (WT cells), or a mutant NHE1 lacking ion translocation (E266I cells; Denker et al., 2000), or stably expressing SN1 (Chaudhry et al., 1999) have been described previously. Cells were maintained in DMEM/H21 medium supplemented with 5% FBS and penicillin-streptomycin (growth medium) at 5% CO2. For experiments with wounded cells, fibroblasts plated on glass coverslips were grown to confluence, wounded with a P1000 pipette tip, and...
maintained at 5% CO₂ in growth medium for 15 min. For experiments with subconfluent cells, cells were plated on glass coverslips at ~75% confluence, maintained for 24 h in DME containing 0.2% FBS (quiescent cells), and treated for the indicated times with 50 ng/ml PDGF-BB (Roche). For expression of WT and mutant cofilin or actophorin, cells were cotransfected with cherry-red histone pAG285 by electroporation (Amazan Bio-systems Nucleofector kit; Lonza), plated on glass coverslips in growth medium for 24 h, then transferred to DME containing 0.2% FBS for 24 h before using. SN1 cells were maintained in glutamine-free DME in the absence of FBS and supplemented with the indicated concentrations of glutamine 4 h before use.

F-actin abundance
Wounded monolayers plated on glass coverslips were fixed in 3.7% formaldehyde for 20 min, permeabilized in 0.1% Triton X-100, incubated with rhodamine-phalloidin (1:500; Invitrogen) for 30 min, and processed for imaging with mounting medium containing 50% glycerol and N-propyl gal-late. Images were collected at room temperature using a microscope (Axio-lab; Carl Zeiss, Inc.) and a Plan-neofluar 100x objective with 1.3 numerical aperture (Carl Zeiss, Inc.) adapted with a computer-driven cooled charge-coupled device Spot camera (RT slider model 2.3.0) and Spot advanced 4.1.5 acquisition software (Diagnostic Instruments, Inc.). Images were acquired in the linear range of the detector’s response at a sensitivity such that none of the pixels in the image were saturated. Factin at the leading edge was quantified using National Institutes of Health (NIH) Image software. For each cell, a line perpendicular to the wound edge was drawn bisecting the nucleus and extending to the leading-edge membrane. Fluorescence intensity was determined in an area from the membrane to 5 μm within the cortex, and four pixels to the left and right of the perpendicular line. For total F-actin, untransfected subconfluent cells in the absence and presence of PDGF were fixed and labeled with rhodamine-phalloidin as described previously. After washing, the rhodamine dye was extracted in methanol at -20°C, and fluorescence intensity was measured at Ex 554 and Em 575 using a SpectraMax M5 (MDS Analytical Discovery). Cortical Factin in transfected cells was determined as described for wound-edge cells.

Actin free barbed ends assay
The number of actin free barbed ends was determined by a modification of previously described methods (Chan et al., 1998). Quiescent cells plated in MatTek issues culture dishes (MatTek Corporation) were treated with 50 ng/ml PDGF for the indicated times and permeabilized in buffer containing biotin–G-actin, 20 μg biotin–G-actin (Cytoskeleton, Inc.) in 100 μl of dilution buffer (1 mM Hepes, pH 7.5, 0.2 mM MgCl₂, and 0.2 mM ATP) was centrifuged at 100,000 g for 20 min to remove aggregates. The mixture was diluted to a final concentration of 0.02 g/liter in permeabilizing buffer (5 mM KCl, 137 mM NaCl, 4 mM NaHCO₃, 0.4 mM KH₂PO₄, 1.1 mM Na₂HPO₄, 2 mM MgCl₂, 5 mM Pipes, pH 7.2, 2 mM EGTA, and 5.5 mM glucose) containing 0.04 g/liter saponin and 1% BSA. After permeabilizing cells for 45 s at 37°C in the presence of biotin–G-actin, the reaction was stopped by adding permeabilizing buffer without saponin and BSA, and cells were fixed for 5 min with 3.7% formaldehyde in permeabilizing buffer. Cells were then incubated for 10 min with permeabilizing buffer containing 0.1 M glycine; blocked for 20 min in TBS buffer containing 1% BSA, 1% FBS, and 5 μg/ml biotin–G-actin; and incubated for 1 h with FITC- or rhodamine-conjugated antibiotin antibody (1:50; Jackson ImmunoResearch Laboratories) and washed with TBS buffer containing 1% BSA. Coverslips from MatTek dishes were mounted with 0.1 M N-propyl gallate in 50% glycerol in TBS, pH 7.5, for imaging. Images for all experiments were collected using identical settings within the linear range of the detector’s response and at a sensitivity such that none of the pixels in the image were saturated. Digital images were then linearly converted in NIH Image and analyzed using macro analysis as described previously (Mounieinte et al., 2004). In brief, the software averages the fluorescence intensity in 29 consecutive annuli of 0.22 μm, beginning from each cell, a line perpendicular to the wound edge was drawn bisecting the nucleus and extending to the leading-edge membrane. Fluorescence intensity was determined in an area from the membrane to 5 μm within the cortex, and four pixels to the left and right of the perpendicular line. For total F-actin, untransfected subconfluent cells in the absence and presence of PDGF were fixed and labeled with rhodamine-phalloidin as described previously. After washing, the rhodamine dye was extracted in methanol at -20°C, and fluorescence intensity was measured at Ex 554 and Em 575 using a SpectraMax M5 (MDS Analytical Discovery). Cortical Factin in transfected cells was determined as described for wound-edge cells.

DNA subcloned into pET11b (EMD) was used to transform Rosetta BL21 DE3 bacteria cells, which were grown in Luria broth (LB) media with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol at 30°C. For HT2-labeled protein, LB was replaced by minimal medium at pH 7.4 (1x M9 salts, 2 mM MgSO₄, 100 μM CaCl₂, 1x MEM vitamin mix [Invitrogen], 1x [Sigma–Aldrich], 100 μg/ml ampicillin, and 1 μg/ml tetracycline) and treated for 20 min to remove aggregates. The mixture was diluted to a final concentration of 0.02 g/liter in permeabilizing buffer (5 mM KCl, 137 mM NaCl, 4 mM NaHCO₃, 0.4 mM KH₂PO₄, 1.1 mM Na₂HPO₄, 2 mM MgCl₂, 5 mM Pipes, pH 7.2, 2 mM EGTA, and 5.5 mM glucose) containing 0.04 g/liter saponin and 1% BSA. After permeabilizing cells for 45 s at 37°C in the presence of biotin–G-actin, the reaction was stopped by adding permeabilizing buffer without saponin and BSA, and cells were fixed for 5 min with 3.7% formaldehyde in permeabilizing buffer. Cells were then incubated for 10 min with permeabilizing buffer containing 0.1 M glycine; blocked for 20 min in TBS buffer containing 1% BSA, 1% FBS, and 5 μg/ml biotin–G-actin; and incubated for 1 h with FITC- or rhodamine-conjugated antibiotin antibody (1:50; Jackson ImmunoResearch Laboratories) and washed with TBS buffer containing 1% BSA. Coverslips from MatTek dishes were mounted with 0.1 M N-propyl gallate in 50% glycerol in TBS, pH 7.5, for imaging. Images for all experiments were collected using identical settings within the linear range of the detector’s response and at a sensitivity such that none of the pixels in the image were saturated. Digital images were then linearly converted in NIH Image and analyzed using macro analysis as described previously (Mounieinte et al., 2004). In brief, the software averages the fluorescence intensity in 29 consecutive annuli of 0.22 μm, beginning from each cell, a line perpendicular to the wound edge was drawn bisecting the nucleus and extending to the leading-edge membrane. Fluorescence intensity was determined in an area from the membrane to 5 μm within the cortex, and four pixels to the left and right of the perpendicular line. For total F-actin, untransfected subconfluent cells in the absence and presence of PDGF were fixed and labeled with rhodamine-phalloidin as described previously. After washing, the rhodamine dye was extracted in methanol at -20°C, and fluorescence intensity was measured at Ex 554 and Em 575 using a SpectraMax M5 (MDS Analytical Discovery). Cortical Factin in transfected cells was determined as described for wound-edge cells.

MD simulations
To study the effects of phosphorylation, we used explicit solvent MD. The 20 initial structures were taken from the NMR structure 1Q8G from the Protein Data Bank. For each of these 20 structures of the cofilin, we performed two different simulations: with Ser3 unphosphorylated and with Ser3 phosphorylated. The calculations were done with GROMACS (version 3.2.1; GNU General Public License) using the GROMOS96 force field for pser. All systems were solvated with the SPC water model in a periodic cubic cell. The solvated systems were subject to 2,500 steps of steap descent minimization to prepare the system for the MD simulations. The equilibration of the systems was done in two parts: the first part consisted of 50 ps of dynamics in which the temperature was raised from 0 to 300 K. The second part of the equilibration consisted of 1 ns with an NPT ensemble. Once the systems were equilibrated, we calculated one additional nanosecond in the NPT ensemble at 300 K using a thermostat (Berendsen) and particle mesh Ewald (PME) for the Coulombic interactions. The results from the 20 simulations starting from different NMR models were very similar, and we show one representative result.

Constant pH MD simulations were performed using the Amber 8 suite of programs (Case et al., 2005). The Amber parm99 force field (Wang et al., 2000) and generalized Born solvation model (Onufriev et al., 2004) were used. First, the system was energy minimized using six consecutive rounds of 800 steps of the steepest descent algorithm followed by 1,200 steps of the conjugate gradient algorithm, giving a total of 130,000 steps. Harmonic restraints applied to the NMR structures were slowly relaxed from 25 to 1 kcal/mol/A² by the end of the energy minimization step. To save computational time, a 15-Å cutoff for nonbonded interactions was used. The equilibration period in the MD simulations consisted of three stages. In the first one, the system was gradually heated from 100 K to 300 K for 30 ps at 100 K intervals, followed by 70 ps at 300 K. The remaining restraints were gradually reduced to zero in this stage. The second stage consisted of 50 ps of unrestrained equilibration. Finally, the third stage,
the constant pH MD (CpHMD) approach was applied (Mongan et al., 2004). The method implemented in AMBER addresses the dynamic dependence of pK, through Monte Carlo sampling of the Boltzmann distribution of protonation states concurrent with the MD simulations. The nature of the distribution is affected by the solvent pH, which is set as an external parameter. In the final equilibration stage, the pH variable was defined and the system was submitted to an MD run of 20 ps. The salt concentration was set at 0.1 M. The MD run was then continued for 10 ns at conditions of low (6.5) and high pH (8). Solute temperature was weakly coupled to a Berendsen temperature bath at 300 K, with a time constant of 2 ps. Bond lengths including hydrogens were constrained using the SHAKE algorithm. The time step was 1 fs. The center-of-mass motion was removed at regular intervals of 1,000 fs. The trajectories were saved every 1 ps. In the MD simulations, van der Waals cutoffs for nonbonded interactions was 12 Å. Analyses were performed primarily with the ptraj program (AMBER; distributed by the University of California, San Francisco).

Docking

The NMR structures for WT coflin (protein database ID No. 1QBG, Model 1) and its H133A mutant were used in the docking studies of Pl[4,5]P2. To generate the structure for the mutant, His133 in the NMR Model 1 was substituted by an alanine residue using Maestro (Schrodinger, LLC). The docking calculations were performed using the Induced Fit protocol (Sherman et al., 2006), a combination of Glide (Friesner et al., 2004; Halgren et al., 2004) and Prime (Jacobson et al., 2004) that accounts for both lipid and receptor side chain flexibility. Before the docking calculations, both WT and mutant proteins were submitted to a series of restrained, partial minimizations using the OPLS 2005 force field (Jorgensen et al., 1996; Kaninski et al., 2001). Pl[4,5]P2 was submitted to a preminimization with the MMFF94 force field using a “4Å” distance-dependent dielectric constant.

NMR

Experiments were performed on Avance DRX 500 and Avance 800 MHz spectrometers (Bruker) at 300 K. Data were processed using TopSpin 2.0 software (Bruker). Spectra were analyzed using ANSIG (Kraulis et al., 1994). Resonance assignments were transferred to spectra using values deposited in the Biological Magnetic Resonance Data Bank (http://www.bmbrc.wisc.edu/) under accession code BMRB-6004 (Zierler-Gould et al., 2004).

F-actin severing assay

The ability of coflin to sever F-actin was directly observed using an in vitro severing assay described previously (Ietchotkin et al., 2000; Ietchotkin et al., 2002). In brief, 2 μM actin containing 20% rhodamine-labeled and 10% biotin-labeled actin were polymerized at room temperature for 2 h in ISAP buffer [20 mM Pipes, pH 7, 1 mM ATP, 1 mM MgDT, 50 mM KCl, 5 mM EGTA, and 2 mM MgCl2] containing 0.2 μM phallolidin. Coverslips (50, 3, and 24 mm, no. 1.5) were coated with 15 ml of 0.1% nitrocellulose in 0.2 mg/ml glucose oxidase, 6 mg/ml glucose, and 100 mM DTT; Kron and Bernstein, 1986; Kron et al., 1991) and perfused into the chamber. After a 5-min incubation, free actin was washed off with two 30-μl rinses of antibleaching buffer A and then with two 30-μl rinses of antibleaching buffer B to a final concentration of 5 or 16 μM was perfused into the chamber. The resulting chamber holds a volume of around 30 μl. Antibiotin antibody was diluted to 30 mg/ml with wash buffer (ISAP buffer containing 0.5 mg/ml BSA) perfused into the chamber, and incubated for 5 min before washing twice with wash buffer. Rhodamine/biotin-labeled F-actin was diluted 30-fold to 0.067 μM with anticleaving buffer A (ISAP containing 5 mg/ml BSA and anticleaving components: 0.036 mg/ml catalase, 0.2 mg/ml glucose oxidase, 6 mg/ml glucose, and 100 mM MgDT; Kron and Spudich, 1986; Kron et al., 1991) and perfused into the chamber. After a 5-min incubation, free actin was washed off with two 30-μl rinses of anticleaving buffer A and then with two 30-μl rinses of anticleaving buffer B (coflin storage buffer containing 0.036 mg/ml catalase, 0.2 mg/ml glucose oxidase, 6 mg/ml glucose, and 100 mM MgDT). Cofilin diluted in anticleaving buffer B to a final concentration of 5 or 16 μM was perfused into the chamber, and images of bound filaments were taken with a microscope (Olympus) equipped with a charge-coupled device camera and scored for severing as described previously (Ietchotkin et al., 2000).

Phospholipid binding

Lipid micelles were prepared as described previously (Lebensohn et al., 2006) by using a mini-extractor (Avanti Polar Lipids, Inc.), and they contained phosphatidyl ethanolamine/phosphatidyl choline/Pl[4,5]P2 (71:25:4 molar ratio; Avanti Polar Lipids, Inc.). Vesicle suspensions were adjusted to the indicated pH with KOH or HCl. Quantitative binding and Ks were obtained by incubating 20 μM Pl[4,5]P2 with the indicated concentrations of coflin for 5 min at room temperature and then col-

lected by centrifugation at 100,000 g for 60 min. Supernatants and pellets were analyzed by SDS-PAGE and Coomassie staining. The amount of protein on the gel was determined by densitometry analysis using NIH Image. Specific binding was calculated as the abundance of protein bound to vesicles containing Pl[4,5]P2 minus binding to vesicles in the absence of Pl[4,5]P2. The abundance of protein bound to vesicles in the absence of Pl[4,5]P2 was minimal and pH-independent. pH-dependent dissociation constants were calculated from transformations of binding curves using GraphPad Prism 5 software (GraphPad Prism). Binding at pH 6.5 and 7.5 was also determined by incubating 20 μM Pl[4,5]P2 with 3 μM of recombinant WT coflin or coflin-H133A. To correct for variations in lipid vesicle preparations, data were expressed relative to binding at pH 6.5 for each determination.

Statistical analysis

Data were analyzed with GraphPad Prism software using an unpaired t test with 95% confidence intervals. The sample size represented the number of separate cell preparations, except for analysis of F-actin after monolayer wounding, which included data obtained from 40–60 cells in two separate cell preparations.

Online supplemental material

Fig. S1 shows spatially localized cortical actin free barbed and formation and the time-dependent phosphorylation of coflin in cells treated with PDGF. Fig. S2 shows predicted and measured structural changes in coflin with increasing pH within the physiological range. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200804161/DCC1.

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