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Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P_3 and polarity during neutrophil chemotaxis

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Neutrophils exposed to chemoattractants polarize and accumulate polymerized actin at the leading edge. In neutrophil-like HL-60 cells, this asymmetry depends on a positive feedback loop in which accumulation of a membrane lipid, phosphatidylinositol (PI) 3,4,5-trisphosphate (PI[3,4,5]P_3), leads to activation of Rac and/or Cdc42, and vice versa. We now report that Rac and Cdc42 play distinct roles in regulating this asymmetry. In the absence of chemoattractant, expression of constitutively active Rac stimulates accumulation at the plasma membrane of actin polymers and of GFP-tagged fluorescent probes for PI(3,4,5)P_3 (the PH domain of Akt) and activated Rac (the p21-binding domain of p21-activated kinase). Dominant negative Rac inhibits chemoattractant-stimulated accumulation of actin polymers and membrane translocation of both fluorescent probes and attainment of morphologic polarity. Expression of constitutively active Cdc42 or of two different protein inhibitors of Cdc42 fails to mimic effects of the Rac mutants on actin or PI(3,4,5)P_3. Instead, Cdc42 inhibitors prevent cells from maintaining a persistent leading edge and frequently induce formation of multiple, short lived leading edges containing actin polymers, PI(3,4,5)P_3, and activated Rac. We conclude that Rac plays a dominant role in the PI(3,4,5)P_3-dependent positive feedback loop required for forming a leading edge, whereas location and stability of the leading edge are regulated by Cdc42.

Introduction

Leukocytes interpret gradients of chemoattractant to crawl toward sites of tissue injury and inflammation. Differentiated HL-60 cells, which look and behave like neutrophilic leukocytes (neutrophils), orient their polarity in response to such a gradient and migrate in the right direction (Servant et al., 1999, 2000; Wang et al., 2002; Weiner et al., 2002). Like amoebae of *Dictyostelium discoideum* (Funamoto et al., 2002; Iijima and Devreotes, 2002), both differentiated HL-60 cells and normal blood neutrophils polymerize actin in lamellae at the leading edge, or pseudopod, in a process that depends upon activity of phosphatidylinositol (PI)* 3′-kinases (PI3Ks) and activation of Rho GTPases by PI 3,4,5-trisphosphate (PI[3,4,5]P_3) (Benard et al., 1999), a lipid product of PI3Ks. PI(3,4,5)P_3 accumulates preferentially in membranes at the leading edge (Weiner, 2002). PI3K activity is required for attractants to activate two GTPases, Rac and Cdc42, in neutrophils (Benard et al., 1999). Paradoxically, however, activities of Rho GTPases are reciprocally required to support stimulation of PI(3,4,5)P_3 accumulation (Servant et al., 2000; Wang et al., 2002). Indeed, asymmetric accumulation of PI(3,4,5)P_3 and actin at the leading edge depends upon a positive feedback loop in which accumulation of the lipid activates Rho GTPases, whereas activated Rho GTPases and (probably) polymerized actin in turn increase accumulation of the lipid (Wang et al., 2002; Weiner et al., 2002).

In fibroblasts, Rho GTPases coordinate many cellular responses, often by regulating formation of different actin assemblies. Rac triggers extension of lamellipodia containing arborized actin polymers, whereas Cdc42 induces extension of filopodia, finger-like protrusions formed by parallel arrays of actin filaments. Rho itself induces formation of stress fibers and stimulates contractility, which is mediated by activation

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*Abbreviations used in this paper: fMLP, formyl-methionine-leucine-phenylalanine; GEF, guanine nucleotide exchange factor; LT, lethal toxin; PAK, p21-activated kinase; PBD, p21-binding domain; PI, phosphatidylinositol; PI(3,4,5)P_3, PI 3,4,5-trisphosphate; PI3K, PI 3′-kinase; WASp, Wiskott-Aldrich Syndrome protein.

Key words: actin; PI 3-kinase; chemoattractant; pseudopod; positive feedback

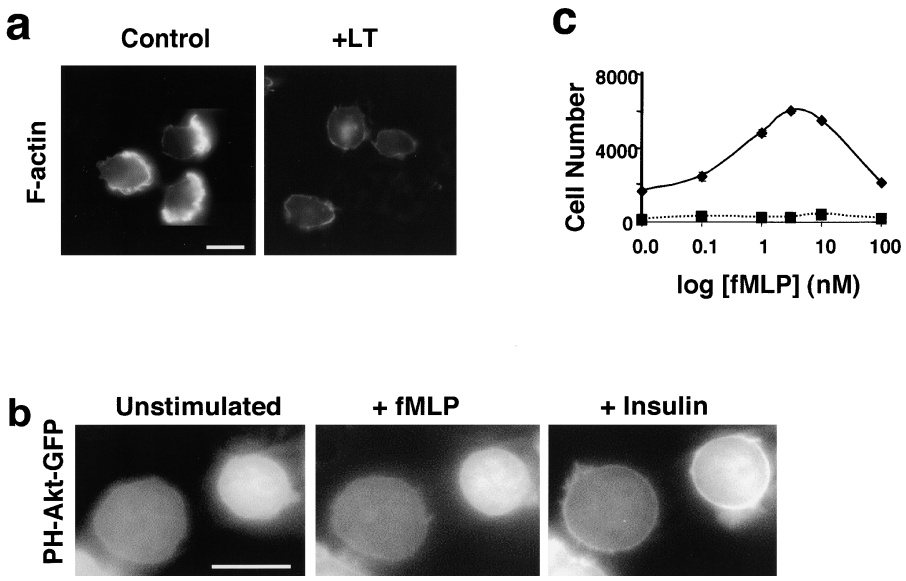

Figure 1. **LT inhibits polarization, actin polymerization, chemotaxis, and PI(3,4,5)P_3 production.** (a) F-actin localization in differentiated HL-60 cells stimulated by a uniform concentration of fMLP (100 nM) without pretreatment (HL-60, left) or after pretreatment with LT (200 ng/ml for 24 h; +LT, right). Bar, 10 μm. (b) Fluorescence microscopic images of differentiated HL-60 cells stably expressing PH-Akt-GFP. Cells pretreated with LT (unstimulated, left) were exposed to a uniform concentration (100 nM) of fMLP for 2 min (+fMLP, middle) followed by stimulation with insulin (100 μg/ml for 2 min; +insulin, right). Bar, 10 μm. Note that 31 of 40 cells (80%) showed PH-Akt-GFP translocation in response to insulin; none of these cells showed translocation in response to fMLP. (c) Migration in the transwell assay of differentiated HL-60 cells treated (dotted line) or not treated (solid line) with LT. Cell counts indicate cells that crossed the transwell membrane.

of complexes of actin and myosin (Tapon and Hall, 1997). Inhibition of Rac or Cdc42 has been shown to disrupt polarity or chemotaxis in polarized epithelial cells (Kroschewski et al., 1999), fibroblasts (Nobes and Hall, 1999), T cells (Haddad et al., 2001), and macrophages (Allen et al., 1998). Here we use bacterial toxins, dominant interfering point mutants, and fluorescent probes to explore the roles of Rac and Cdc42 in regulating PI(3,4,5)P_3 accumulation, polarity, and chemotaxis in differentiated HL-60 cells. Our experiments identify distinct roles for Rac and Cdc42 in creating and maintaining neutrophil polarity.

Results

Effects of lethal toxin

We reported previously (Servant et al., 2000) that *Clostridium difficile* toxin, which inhibits all known Rho GTPases (Sehr et al., 1998), blocks PI(3,4,5)P_3 accumulation at the plasma membrane of differentiated HL-60 cells. Fig. 1 shows that *Clostridium sordellii* lethal toxin (LT) similarly prevented a neutrophil chemoattractant, formyl-methionine-leucine-phenylalanine (fMLP), from stimulating actin polymerization and formation of pseudopods (Fig. 1 a), and membrane translocation of a fluorescent PI(3,4,5)P_3 probe (Fig. 1 b), the PH domain of Akt, tagged with GFP (PH-Akt-GFP). In addition, LT almost completely blocked fMLP-triggered migration across transwell filters (Fig. 1 c). LT ADP-ribosylates Rac and Cdc42 and inhibits their activities without affecting those of Rho (Just et al., 1996). In other experiments (unpublished data), a toxin that specifically inactivates Rho (C3 toxin) did not prevent PI(3,4,5)P_3 accumulation. These effects do not reflect damage to the cells' intrinsic ability to accumulate PI(3,4,5)P_3 as shown by the ability of the fMLP-unresponsive LT-treated cells to translocate PH-Akt-GFP to membranes in response to insulin (Fig. 1 b), a stimulus that activates PI(3,4,5)P_3 accumulation by mechanisms that depend on tyrosine phosphorylation rather than on activation of a G protein–coupled receptor. These results confirm our previous inference (Servant et al., 2000) that Rho GTPases are required for polarity and PI(3,4,5)P_3 accumulation and indicate that Rac or Cdc42, but not Rho, mediates both these responses to fMLP. We reported previously (Wang et al., 2002) that immunofluorescent staining with an mAb against PI(3,4,5)P_3 showed a distribution identical to that of PH-Akt-GFP; this antibody shows 30-fold selectivity for PI(3,4,5)P_3 versus PI(3,4)P_2 (Chen et al., 2002).

We developed a transient transfection protocol that allowed us to identify distinct roles of Rac and Cdc42 in regulating polarity and PI(3,4,5)P_3 accumulation, in determining subcellular distribution of activated Rac, and in directing cell migration (Figs. 2–6). Dominant interfering mutant proteins and fluorescent probes were transiently expressed in differentiated HL-60 cells, with transfection efficiencies that varied from 10 to 20%. In populations cotransfected with two constructs, >80% of cells expressing one also expressed the other (for details see Materials and methods). Although they migrate slightly less rapidly (see Materials and methods) than do cells stably expressing PH-Akt-GFP (Wang et al., 2002), cells transiently expressing the lipid probe showed unchanged responses to fMLP, including morphologic polarity, formation of actin polymers, directed motility, and asymmetric accumulation of the probe for activated Rac (Figs. 2, 3 a, 5 a, and 6 a).

Role of Rac as a mediator of PH-Akt-GFP translocation and polarity

We assessed effects of Rho GTPase mutants on morphology, actin accumulation, and PH-Akt-GFP translocation at 1 or 3 min after adding fMLP (Table I or Fig. 2, respectively). In 17 of 19 control cells (90%) at the earlier time point (Table I; photomicrographs in Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1), fMLP induced robust recruitment of PH-Akt-GFP to the plasma

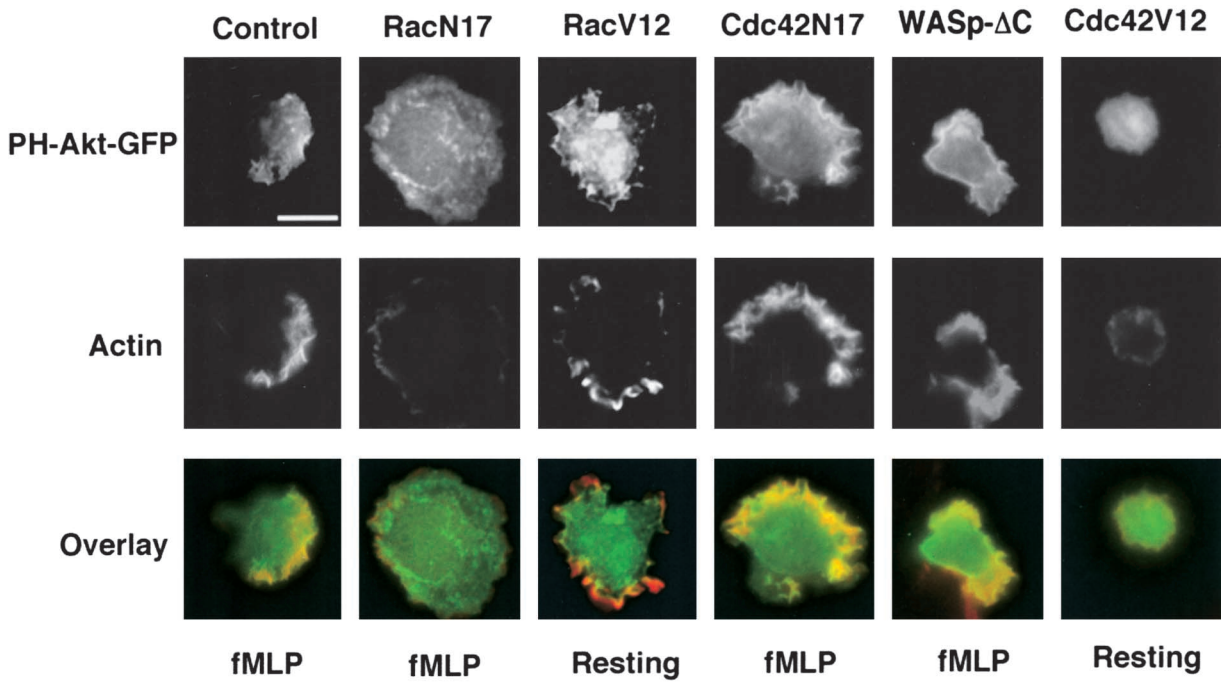

Figure 2. **Rac but not Cdc42 stimulates accumulation of PI(3,4,5)P_3 and actin polymers.** Spatial localization of PH-Akt-GFP (top images) or polymerized actin (middle images) in transfected differentiated HL-60 cells; bottom images show overlay of PH-Akt-GFP (green) and actin polymerization (red). Cells were transiently transfected to express either PH-Akt-GFP alone (control) or in combination with RacN17, RacV12, Cdc42N17, WASpΔC, or Cdc42V12 as indicated. Cells were unstimulated or stimulated for 3 min with a uniform concentration of fMLP (100 nM) and fixed. Bar, 10 μm. Note that the Cdc42V12 construct was normally functional despite its failure to induce actin assemblies in HL-60 cells. Indeed, transient expression of this Cdc42 mutant in COS-7 cells was associated with constitutive Cdc42 activity (result not shown) as assessed in a PAK-PBD pull-down assay (Benard et al., 1999).

membrane. By 3 min, control cells showed asymmetric recruitment of PH-Akt-GFP to the leading edge where it colocalized with polymerized actin (Fig. 2).

Similarly, fMLP caused localization to the pseudopod of a fluorescent probe that detects activated Rac (Fig. 3 a), PAK-PBD-YFP, the YFP-tagged p21-binding domain (PBD) of p21-activated kinase (PAK). Although in vitro this PBD binds the activated, GTP-bound forms of both Rac and Cdc42 (Manser et al., 1994), its YFP-tagged counterpart (PAK-PBD-YFP) predominantly detects Rac-GTP rather than Cdc42-GTP as shown by other data in Fig. 3. Overexpression of the constitutively active Rac mutant, RacV12, caused PAK-PBD-YFP to localize in lamellae surrounding the circumference of flat, spread-out cells (Fig. 3 d) in a distribution similar to that of actin and PH-Akt-GFP in RacV12-expressing cells (Fig. 2). Moreover, a myc epitope attached to RacV12 virtually superimposed on PAK-PBD-YFP fluorescence, suggesting that the fluorescent probe faithfully detects the GTP-bound form of Rac (Fig. 3, d–f). In contrast, the constitutively active Cdc42 mutant, CdcV12, caused no localization of PAK-PBD-YFP to the cell periphery (Fig. 3, g–h); instead, PAK-PBD-YFP appeared to localize with myc-tagged Cdc42V12 in a punctate cytoplasmic distribution. Although PAK-PBD-YFP might be expected to sequester GTP-bound forms of Rac, Cdc42, or both, its expression did not detectably alter neutrophil responses to fMLP. This probably reflects relatively higher concentrations of Rac-GTP versus PAK-PBD-YFP in activated HL-60 cells. Indeed, inhibition of ruffling in fibroblasts was seen only with high concentrations of PAK-PBD-YFP (Kraynov et al., 2000), making it possible to use lower concentrations in studying Rac-PAK-PBD-YFP with fluorescence resonance energy transfer.

Rac stimulates actin polymerization by several mechanisms in different cell types (Ridley, 2001). Chemoattrac-

Table I. **Rac but not Cdc42 participates in PI(3,4,5)P_3 accumulation**

	Stimulation	PH-Akt-GFP recruitment	
		Cell numbers	% of cells
Control	fMLP	17/19	90
RacN17	fMLP	16/48	33
Cdc42N17	fMLP	10/11	91
WASpΔC	fMLP	13/15	87
Control	Insulin	13/16	81
RacN17	Insulin	25/30	83
Control	None	0/20	0
RacV12	None	13/18	72
RacV12	Latrunculin B	3/19	16
Cdc42V12	None	0/12	0

Membrane recruitment of PH-Akt-GFP in transiently transfected differentiated HL-60 cells expressing either PH-Akt-GFP alone (controls) or in combination with the mutant construct indicated. Cells were fixed either before stimulation or after stimulation for 1 min with a uniform concentration of fMLP (100 nM) or insulin (100 μg/ml). Unstimulated or fMLP-stimulated cells were immunostained to identify cells expressing the mutant constructs. Immunopositive cells were scored for PH-Akt-GFP recruitment. For insulin stimulation, cells expressing RacN17 were identified based on their morphology (see Materials and methods). For each treatment, numbers and percentages summarize three separate sets of observations, each performed on cells transfected and studied on different days.

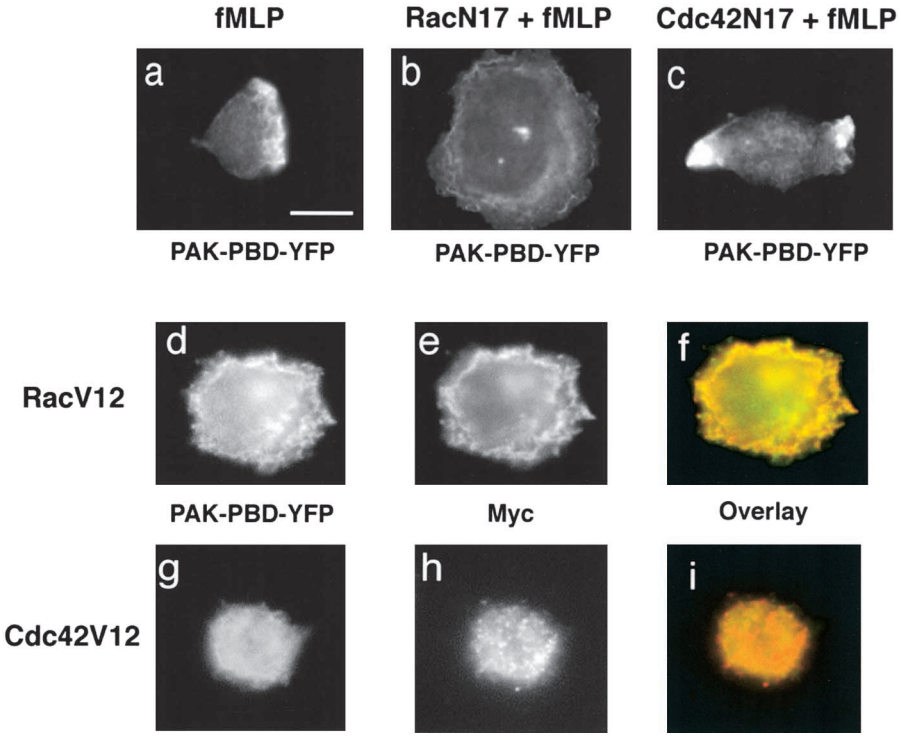

Figure 3. **PAK-PBD-YFP reflects localization of activated Rac at the plasma membrane but not that of activated Cdc42.** Localization of PAK-PBD-YFP in cells exposed for 3 min to 100 nM fMLP (a–c); in addition to PAK-PBD-YFP, the cell in b coexpressed RacN17 and the cell in c coexpressed Cdc42N17. Cells in d–f and g–i expressed constitutively active Rac or Cdc42, respectively; after 3 min exposure to fMLP, these cells were fixed and imaged for PAK-PBD-YFP fluorescence or immunofluorescence of the myc-tagged Rho GTPase as indicated. Bar, 10 μm.

tant-induced actin polymerization is impaired in neutrophils of mice lacking Rac2 (Roberts et al., 1999) or of a patient carrying a dominant inhibitory Rac2 mutation (Ambruso et al., 2000). In accord with these observations, the effects of overexpressed Rac mutants (Table I and Fig. 2) indicated that Rac plays an essential role in stimulating actin polymerization, pseudopod formation, motility, and PI(3,4,5)P_3 accumulation in differentiated HL-60 cells.

The inhibitory effect of RacN17 on activity of endogenous Rac is thought to result from sequestering the guanine nucleotide exchange factors (GEFs) that activate the endogenous Rho GTPase (Feig, 1999). Resting (untreated) RacN17-expressing cells were flat and spread-out (Figs. 2 and 3 b), unlike resting control cells. Further, RacN17 blocked PH-Akt-GFP translocation responses to fMLP and markedly reduced accumulation of polymerized actin (Fig. 2 and Table I; see also Fig. S1). After exposure to fMLP for 3 min, HL-60 cells failed to polarize or to accumulate PAK-PBD-YFP in pseudopods or even at the cell periphery (Fig. 3, b versus a). However, failure to translocate PH-Akt-GFP to membranes did not indicate inability to synthesize PI(3,4,5)P_3 as shown by the fact that insulin stimulated PH-

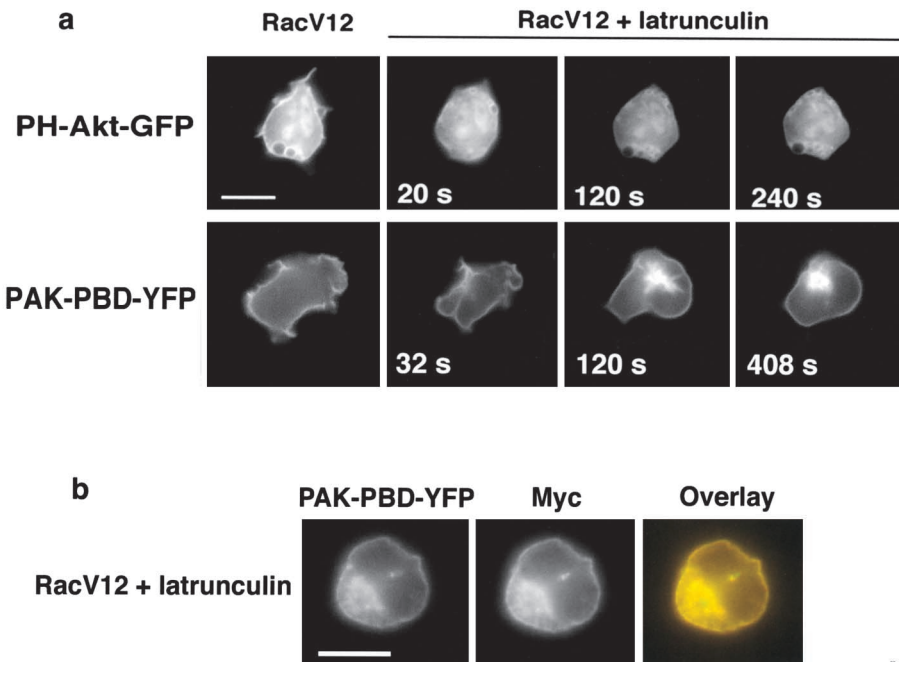

Figure 4. **Actin polymerization is necessary for Rac-dependent PI(3,4,5)P_3 accumulation.** (a) Spatial distribution of PH-Akt-GFP (top) or PAK-PBD-YFP (bottom) in cells expressing RacV12 before and after exposure to latrunculin B (20 μg/ml) for the indicated times. (b) PAK-PBD-YFP and myc-tagged RacV12 show persistent colocalization at the membrane even after treatment with latrunculin B for 10 min. Bars, 10 μm.

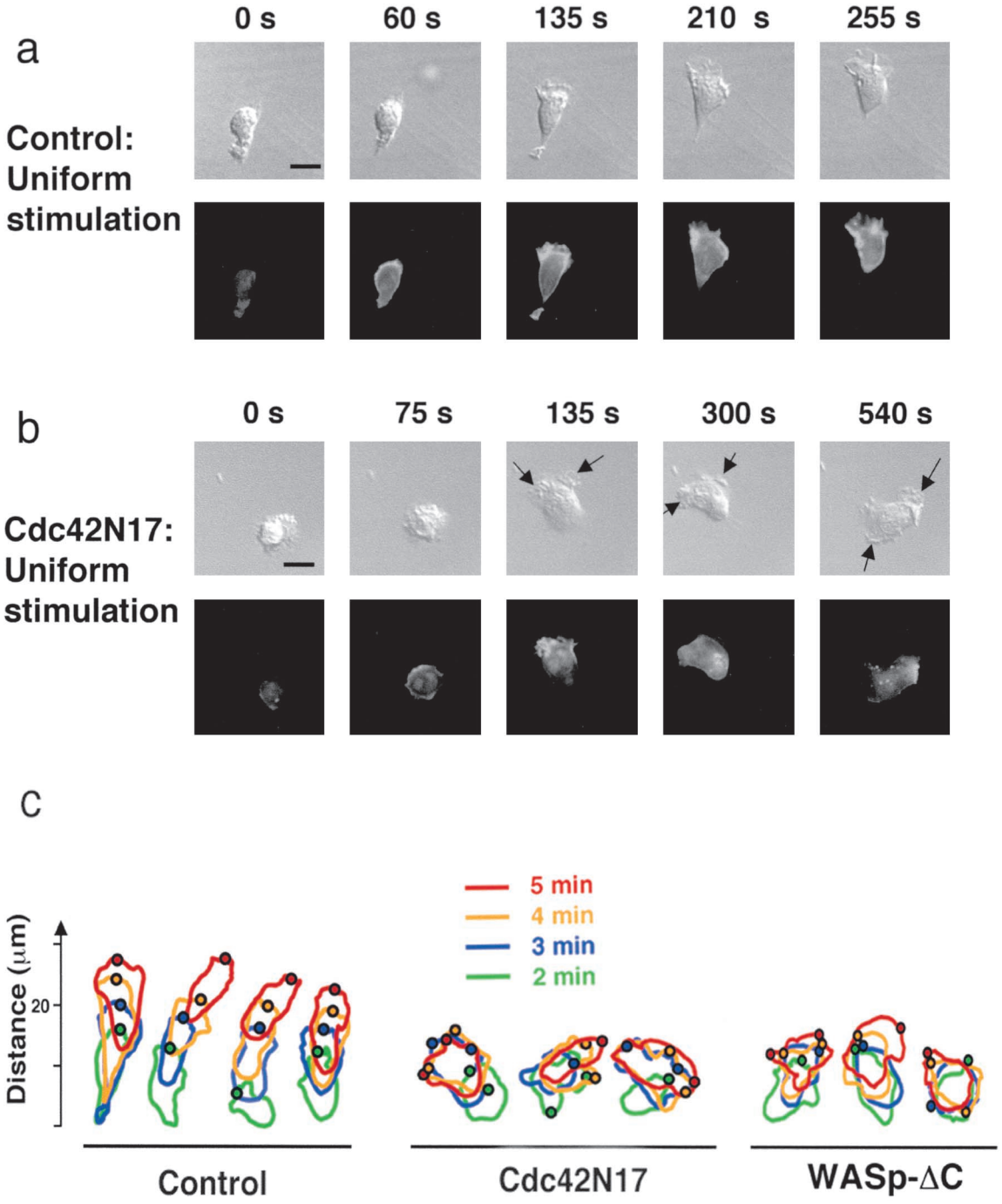

Figure 5. **Inhibition of Cdc42 activity prevents consolidation of a single leading edge upon stimulation by a uniform concentration of chemoattractant.** (a and b) Migration of differentiated HL-60 cells in a uniform concentration (100 nM) of fMLP. Cells expressed PH-Akt-GFP alone (a) or in combination with Cdc42N17 (b). (c) Outlines of cells migrating in a uniform concentration (100 nM) of fMLP. Each set of outlines represents a cell observed at 1-min intervals (denoted by colors as indicated), from 2 to 5 min after exposure to fMLP. Small circles in each outline represent the center of a PH-Akt-GFP–containing lamella at the cell periphery. The first control cell (left) and the first Cdc42N17-expressing cell (middle) are the same cells as those depicted in a and b, respectively. In a and b, the top images show the morphology of a single cell by Nomarski microscopy at the indicated times after chemoattractant stimulation; the bottom images show spatial localization of PH-Akt-GFP in the same cell at the same time points. Arrows identify leading edges. Bars, 10 μm. The cells in a and b with the outlines are shown in videos 1 and 2, respectively, available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1.

Akt-GFP accumulation equally well in control and RacN17-expressing cells (83 versus 81%; Table I and Fig. S1). A minority (33%) of RacN17-expressing cells did translocate PH-Akt-GFP in response to fMLP (Table I). We were unable to quantitate RacN17 expression in individual cells to test the possibility that this mutant was expressed less strongly in cells that showed PH-Akt-GFP responses to fMLP. Presence or absence of the actin response to fMLP did track reliably with presence or absence, respectively, of PH-Akt-GFP translocation.

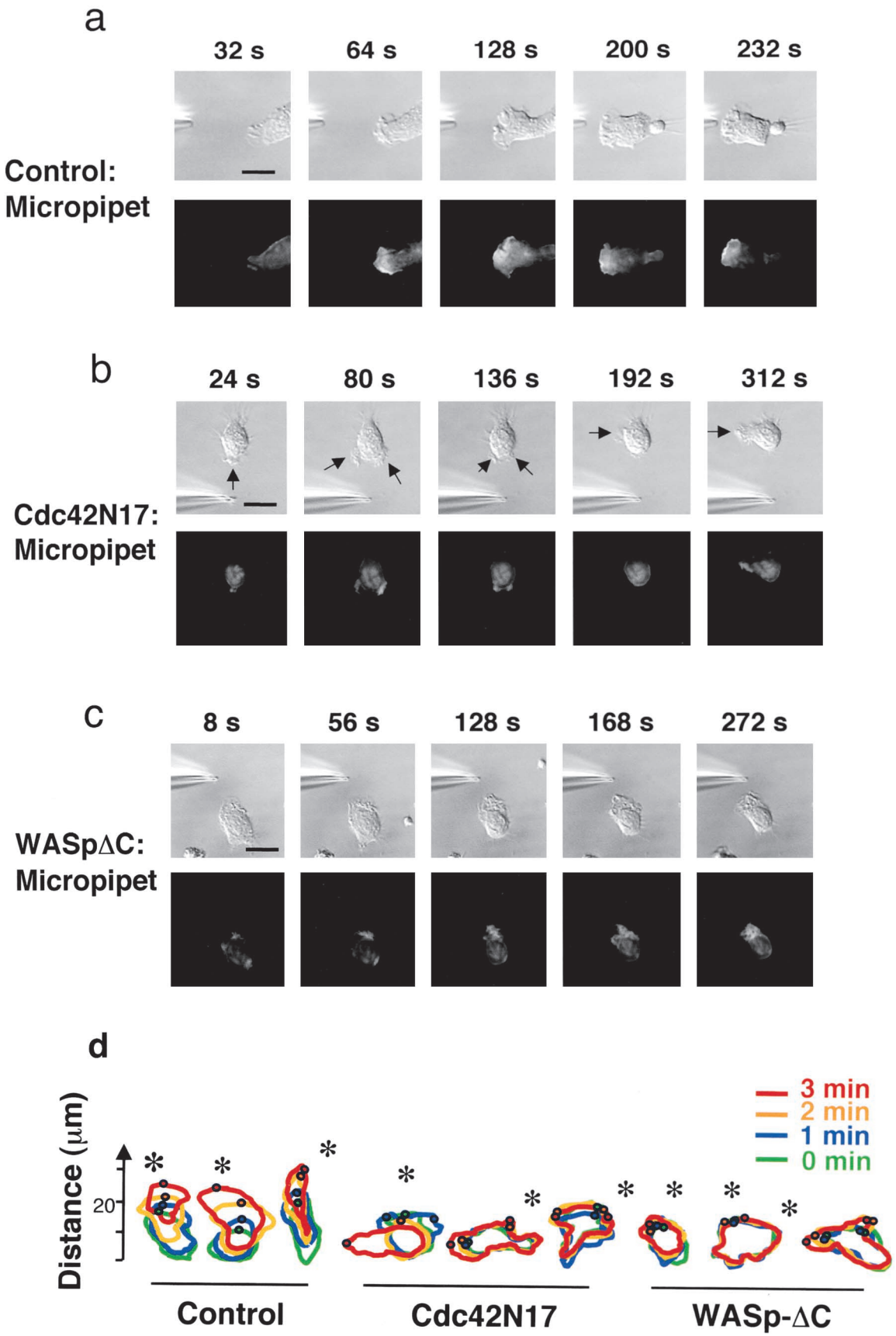

Figure 6. **Inhibition of Cdc42 activity blocks migration toward a point source of chemoattractant.** (a–c) Migration of cells toward a point source of fMLP (10 μM fMLP in a micropipette). Cells expressed PH-Akt-GFP alone (a) or in combination with Cdc42N17 (b) or WASpΔC (c). The top images show the morphology of a single cell by Nomarski microscopy at the indicated times after chemoattractant stimulation; the bottom images show spatial localization of PH-Akt-GFP in the same cell at the same time points. Arrows identify leading edges. Bars, 10 μm.

As expected, inhibition of polarity and actin polymerization by RacN17 was accompanied by severely impaired motility, both in a uniform concentration of chemoattractant and in response to a gradient of fMLP supplied by micropipette (Fig. S2, a and c, available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1). Again as expected, expression of constitutively active Rac (RacV12) caused formation of actin-containing ruffles and translocation of PH-Akt-GFP to the cell periphery in 72% of cells expressing the mutant (Table I and Fig. 2). Note that RacV12, unlike fMLP, did not induce cell polarity or migration; instead, actin and PI(3,4,5)P_3 appear to have been distributed more or less uniformly about the cell periphery.

When exposed to fMLP, many RacV12-expressing cells showed a paradoxical response: disappearance of ruffles and translocation of membrane PH-Akt-GFP back to the cytoplasm (unpublished data). Overexpression of activated Rac for 24 h appears not simply to have prevented fMLP from acting but instead has converted a positive response to a negative one. A satisfying explanation for this paradox awaits results of future experiments (see Discussion).

RacV12-induced PI(3,4,5)P_3 translocation requires actin polymers

Although PI(3,4,5)P_3 is required for fMLP-stimulated Rac activation in neutrophils (Benard et al., 1999), effects of the Rac mutants in HL-60 cells indicate that the converse is also true: active Rac is also both sufficient and necessary to stimulate accumulation of PI(3,4,5)P_3 (Fig. 2 and Table I). We have proposed (Wang et al., 2002; Weiner et al., 2002) that reciprocal activation of one another by Rho GTPases and PI(3,4,5)P_3 constitutes a positive feedback mechanism that amplifies polarity and formation of actin polymers in response to fMLP. Such a feedback loop should be susceptible to activation at multiple points within the loop as shown by the ability of exogenous PI(3,4,5)P_3 to stimulate PI3K- and Rho GTPase-dependent actin polymerization, polarity, and motility in HL-60 cells (Weiner et al., 2002). It is likely that RacV12 mimics one arc of this loop to stimulate PI(3,4,5)P_3 accumulation.

We suggested previously that actin plays an important role in Rac-stimulated PI(3,4,5)P_3 accumulation based on effects of latrunculin B, a protein that sequesters monomeric actin and stops de novo formation of actin polymers (Wang et al., 2002). Exposure of latrunculin-treated cells to a uniform concentration of fMLP did stimulate translocation of PH-Akt-GFP to the plasma membrane, but the translocation was short lived and did not go on to polarize in an asymmetric distribution, unlike that seen in cells capable of polymerizing actin. By inference, formation of actin polymers is necessary for sustained accumulation of PI(3,4,5)P_3 in response to fMLP.

To ask whether Rac-stimulated PI(3,4,5)P_3 accumulation does depend on actin polymerization, we tested the effect of exposing RacV12-expressing cells to latrunculin in the absence of fMLP stimulation. Latrunculin caused PH-Akt-GFP to translocate back from plasma membrane to cytosol (Fig. 4 a). This effect was reproducible: 72% of RacV12-expressing control cells examined showed PH-Akt-GFP located at the plasma membrane, whereas after treatment with latrunculin only 16% of RacV12-expressing cells did so (Table I). In contrast, latrunculin did not cause PAK-PBD-YFP, the probe for activated Rac, to return to the cytoplasm (Fig. 4 a). Instead, PAK-PBD-YFP remained at the membrane where it colocalized with myc-tagged RacV12 (Fig. 4 b). We infer that, at least in the absence of fMLP, stimulation of membrane PI(3,4,5)P_3 accumulation by Rac is mediated by (or depends on) actin polymerization.

Cdc42 controls the number and stability of pseudopods

Results in Figs. 2, 5, and 6 indicate that Cdc42 can control the stability, number, and directionality of pseudopods but in contrast to Rac is neither necessary nor sufficient for actin polymerization or PI(3,4,5)P_3 accumulation. Effects of inhibiting endogenous Cdc42 with the Cdc42N17 mutant, described below, were unexpected. For this reason, we tested a second Cdc42 inhibitor, WASpΔC, which is thought to act by a different mechanism, that is, by sequestering Cdc42-GTP rather than by titrating and inhibiting Cdc42 GEFs. WASpΔC, a fragment of Wiskott-Aldrich Syndrome protein (WASp), binds the GTP-bound form of Cdc42 but lacks COOH-terminal sequences necessary for interacting with downstream components responsible for Cdc42-dependent polymerization of actin (Symons et al., 1996). Effects of WASpΔC and Cdc42N17 proved indistinguishable, making it much more likely that the functional phenotypes were caused by reduced amount or activity of Cdc42-GTP. Unlike the effect of RacN17, inhibition of Cdc42 failed to block three characteristic responses to a uniform concentration of fMLP: membrane translocation of PH-Akt-GFP (Table I), accumulation of F-actin in lamellae (Fig. 2), and accumulation of PAK-PBD-YFP in lamellae (Fig. 3 c).

However, inhibiting Cdc42 did profoundly change the polarized morphology of cells observed 3 min after exposure to fMLP (Fig. 2). In contrast to the relatively stereotyped polar morphology of control cells treated with fMLP, cells expressing Cdc42N17 or WASpΔC often showed multiple lamellae containing F-actin and PH-Akt-GFP; some exhibited a single pseudopod, which was usually wider and less well defined that of control cells. (For photomicrographs of actin and PH-Akt-GFP distribution in six Cdc42N17 expressing cells, see Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1; Fig. S2 b shows a second WASpΔC-expressing cell.)

Videomicroscopy revealed that the diverse morphologies of Cdc42-inhibited cells treated with fMLP reflected marked instability of their pseudopods (Fig. 5; videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1). Fig. 5 a shows the response of a typical control cell to uniform fMLP: the cell polarized, formed

The experiments in a and b are shown in videos 3 and 4, respectively, available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1. (d) Outlines of migrating cells observed after exposure to an fMLP-containing micropipette (locations indicated by asterisks). As in Fig. 5 c, each set of outlines represents a cell observed at 1-min intervals (denoted by colors as indicated), from 0 to 3 min after exposure to the micropipette. Small circles in each outline represent the center of a PH-Akt-GFP–containing lamella at the cell periphery.

a single pseudopod, and crawled efficiently in one direction for several minutes. Fig. 5 b shows the quite different response of a Cdc42N17-expressing cell to uniform fMLP: this cell formed a somewhat broad pseudopod, which then split into two distinct lamellae, followed by formation of a lamella at its opposite edge. Consequently, the cell migrated only a short distance compared with the control. Fig. 5 c summarizes the migration and shape changes of randomly picked control and Cdc42-inhibited cells over a 3-min period (2–5 min after exposure to uniform fMLP). Unlike the four control cells, Cdc42-inhibited cells formed unstable and occasionally multiple pseudopods and migrated in an inefficient, vacillating manner (net movement of <2–3 μm over the 3-min period of observation).

It appeared possible that a gradient of the attractant would produce normal (or nearly normal) polarity and migration in a Cdc42-inhibited cell. As reported previously (Servant et al., 2000; Wang et al., 2002), a control cell formed a single well-defined pseudopod and crawled straight toward the fMLP-containing micropipette (Fig. 6 a; video 3, available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1). (The micropipette produces an fMLP gradient that decreases by ~15% over a distance of 10 μm [Servant et al., 2000; Wang et al., 2002].) In contrast, the Cdc42N17-expressing cell in Fig. 6 b first displayed two leading edges on the up-gradient side, then retracted both, and finally protruded a new leading edge in a direction away from the gradient (see also video 4, available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1). The WASpΔC-expressing cell shown in Fig. 6 c exhibited a milder chemotaxis defect: this cell translocated PH-Akt-GFP into a pseudopod that first extended toward the micropipette and then retracted; net migration over 5 min was ~5 μm (versus ~20–25 μm for controls). The results in Fig. 6, b and c, span the spectrum of severity of the Cdc42-inhibited phenotype in micropipette experiments but do not reflect differences between the two inhibitory constructs. Cell outlines over 3 min of exposure to micropipette gradients show direct pointing and migration of control cells, whereas Cdc42N17- and WASpΔC-expressing cells pointed in multiple directions and failed to migrate toward the source of attractant (Fig. 6 d). In every cell examined, inhibition of Cdc42 induced confused interpretation of the gradient, substantially reducing net movement toward the micropipette in comparison to control cells.

The constitutively active Cdc42 mutant (Cdc42V12), unlike the corresponding RacV12 mutant, failed completely to stimulate formation of actin polymers or translocation of PH-Akt-GFP (Fig. 2 and Table I) or to promote localization of the target probe, PAK-PBD-YFP, to the cell periphery (Fig. 3, d–f and g–i, for RacV12 and Cdc42V12, respectively). Moreover, RacV12 largely colocalized with PAK-PBD-YFP at the plasma membrane but Cdc42V12 did not (Fig. 3). In addition, the Cdc42V12 mutant did not trigger formation of actin-containing filopodia like those it induces in fibroblasts (Hall, 1998) and macrophages (Allen et al., 1998).

The reason for failure of this mutant to induce any responses in HL-60 cells is unknown, but the Cdc42V12 construct was functional (see the legend to Fig. 2), and it did exert an effect on the cells in which it was expressed: transfection with myc- or GFP-tagged Cdc42V12 produced robust immunofluorescent (Fig. 3 h) or GFP fluorescent signals (unpublished data), respectively, and Cdc42V12-expressing cells failed to show any morphologic response or PH-Akt-GFP translocation in response to fMLP (unpublished data). Like the puzzling effect of RacV12 on fMLP responses, this result does not suggest a straightforward explanation and merits further investigation.

Discussion

Role of Rac in the positive feedback loop at the leading edge

We have presented evidence (Table I and Fig. 2) that Rac activity, but not that of Cdc42, is necessary and sufficient for chemoattractant-stimulated accumulation of $PI(3,4,5)P_3$ and actin polymers in HL-60 cells. We can now assign to a single Rho GTPase, Rac, but not Rho or Cdc42, a dominant role in the positive feedback loop that amplifies polarized asymmetry of $PI(3,4,5)P_3$ and actin polymers at the leading edge. We inferred previously existence of this $PI(3,4,5)P_3$- and Rho GTPase-dependent positive feedback loop from several observations. First, exogenous $PI(3,4,5)P_3$ induced PI3K-dependent polarity and migration of human neutrophils (Niggli, 2000). In HL-60 cells, exogenous $PI(3,4,5)P_3$ induced, in addition to polarity and migration, accumulation of actin polymers and translocation of PH-Akt-GFP at the leading edge (Weiner et al., 2002). These effects were blocked not only by pharmacologic inhibition of PI3Ks but also by a toxin (*C. difficile* toxin B) that inhibits Rho GTPases.

More complete understanding of the detailed molecular mechanisms underlying the feedback loop awaits future experiments. One arc of the loop, in which $PI(3,4,5)P_3$ activates Rac, probably involves one or more $PI(3,4,5)P_3$-dependent GEFs found in leukocytes, such as pRex-1 (Welch et al., 2002), Vav (Han et al., 1998), or SWAP-70 (Shinohara et al., 2002). Molecular mechanisms mediating the reciprocal arc in which Rac-GTP promotes accumulation of $PI(3,4,5)P_3$ are even less well defined. The fact that latrunculin-promoted disappearance of F-actin in RacV12-expressing cells causes PH-Akt-GFP to translocate from membranes back into the cytoplasm (Fig. 4) strongly implicates actin polymers at the leading edge as direct or indirect mediators of this arc of the feedback loop. In this arc of the loop, F-actin can clearly act downstream of Rac-GTP and upstream of $PI(3,4,5)P_3$ accumulation, as shown by the ability of latrunculin to induce PH-Akt-GFP to translocate back into the cytoplasm of RacV12-expressing cells, whereas localization of RacV12 and the probe for activated Rac remain at the plasma membrane (Fig. 4). How polymerized actin promotes $PI(3,4,5)P_3$ accumulation, however, is not so clear: it may promote formation of scaffolds that increase $PI(3,4,5)P_3$ synthesis or decrease its degradation, for instance, by recruiting or facilitating activation of one or more PI3Ks (e.g., PI3Kγ by Gβγ [Stephens et al., 1997] or other PI3Ks via their p85 adaptor subunit [Chan et al., 2002]), by preventing access to $PI(3,4,5)P_3$ of an inactivating phosphatase, such as PTEN (Funamoto et al., 2002; Iijima and Devreotes, 2002), or (as in platelets [Tolias et al., 2000]) by promoting activation of PI-4P-5-kinase, thereby increasing

the concentration of the PI3K substrate, PI 4,5-bisphosphate. Although a role for actin in promoting $PI(3,4,5)P_3$ accumulation has not been established in *Dictyostelium*, membrane accumulation of $PI(3,4,5)P_3$ in this organism is regulated by changes in both synthesis and degradation of the phospholipid (Funamoto et al., 2002; Iijima and Devreotes, 2002). For this reason we suspect that both processes will be found to participate in the actin-to-$PI(3,4,5)P_3$ arm of the positive feedback loop in neutrophils.

Although Rac serves as the central Rho GTPase in the $PI(3,4,5)P_3$-dependent positive feedback loop that organizes the leading edge, this loop by itself cannot suffice to explain polarity or directional migration. Two sets of observations make this clear. First, expression of RacV12 does not trigger morphologic polarity but instead produces a cell surrounded by actin-containing ruffles and $PI(3,4,5)P_3$ (Fig. 2). Second, inhibiting Cdc42 profoundly destabilizes pseudopods and disrupts directional migration (Figs. 2, 5, and 6) but does not appear to interfere with the feedback loop because fMLP can still stimulate formation of discrete pseudopods containing $PI(3,4,5)P_3$ and Rac-GTP indicated, respectively, by localization of PH-Akt-GFP (Figs. 2 and 4) and PAK-PBD-YFP (Fig. 3).

RacV12 does not induce polarity

RacV12 disrupts polarity in macrophages (Allen et al., 1998) and in HL-60 cells fails to mimic the polarity- and motility-inducing effects of uniform extracellular concentrations of fMLP (Servant et al., 2000; Wang et al., 2002) or exogenous $PI(3,4,5)P_3$ (Weiner et al., 2002). The latter two stimuli can promote polarized asymmetry of newly formed actin polymers and $PI(3,4,5)P_3$ even when the stimuli themselves are presented to the cell symmetrically. In contrast, symmetrical distribution of activated Rac (that is, of RacV12) does not promote polarized asymmetric responses. From this discrepancy we may infer that both fMLP and exogenous $PI(3,4,5)P_3$ trigger the polarity-inducing mechanism by mechanisms upstream of or in addition to Rac activation. If so, localized Rac activity (as revealed by the distribution of PAK-PBD-YFP in Fig. 3 a) constitutes a "read-out" of the polarity mechanism, its location presumably controlled either by localized activation of appropriate Rac GEFs or by localized prevention of Rac inactivation.

In keeping with this idea, exposure of neutrophils to a uniform concentration of attractant first induces formation of ruffles around the entire cell periphery followed a minute or so later by development of clear morphologic polarity with ruffles confined to a single pseudopod (Zigmond et al., 1981). HL-60 cells respond similarly to either uniform fMLP (Wang et al., 2002) or to exogenous $PI(3,4,5)P_3$ (Weiner et al., 2002). In each case, ruffles and membrane $PI(3,4,5)P_3$ appear first in a symmetrical distribution and later concentrate at one side of the cell. Thus, it seems likely that fMLP or exogenous $PI(3,4,5)P_3$ do not simply induce Rac activation but instead also set in motion signals that limit spatial distribution of Rac activity. Indeed, neutrophil polarity may resemble the self-organizing polarity of the actin–myosin cytoskeleton observed in fish keratoplasts perturbed by a mechanical stimulus (Verkhovsky et al., 1999). Signals responsible for self-organizing polarity are not well defined but probably include contraction of actin–myosin complexes at the rear of keratoplasts (Verkhovsky et al., 1999), *Dictyostelium* amoebae (Chung and Firtel, 1999), and neutrophils (Cassimeris et al., 1990). In HL-60 cells, contraction of actin–myosin complexes could limit formation of arborized actin polymers, thereby restricting lamellae to one side of the cell. Such a process could amplify and consolidate $PI(3,4,5)P_3$ accumulation at the leading edge because de novo formation of actin polymers promotes and may even be necessary for $PI(3,4,5)P_3$ accumulation (Fig. 3).

We speculate that a similar inhibition of actin polymerization and $PI(3,4,5)P_3$ accumulation underlies the paradoxical ability of overexpressed RacV12 to block responses to fMLP (see Results). We propose the following speculative scenario: an activated Rac that does not depend on activation by GEFs subverts the normal Rac-dependent positive feedback mechanism, making it impossible (perhaps by soaking up limiting components) for the attractant to exert its normal effect on PH-Akt-GFP accumulation and actin polymerization; at the same time, fMLP exerts a direct negative effect on both actin polymerization and $PI(3,4,5)P_3$ accumulation by stimulating activity of a Rho GTPase (Rho rather than Cdc42 or Rac) that promotes formation of actin–myosin complexes, which in turn reduce $PI(3,4,5)P_3$ responsiveness to fMLP. In support of the latter part of this scenario, our unpublished experiments indicate that: (a) the pathway for fMLP-triggered Rho activation diverges quite early from fMLP-triggered Rac activation, actin polymerization, and $PI(3,4,5)P_3$ accumulation (pertussis toxin inhibits the latter pathway but not the former) and (b) Rho-dependent signals can effectively turn off fMLP-dependent $PI(3,4,5)P_3$ accumulation and polymerization of actin. Thus, it seems reasonable to propose that Rho activated by fMLP might strongly inhibit a Rac-dependent response that is intrinsically unable to amplify itself (that is, RacV12, as opposed to the Rac-GTP that accumulates in response to fMLP in control cells).

Cdc42 stabilizes polarity

Cdc42 does not participate directly in the $PI(3,4,5)P_3$ feedback loop as indicated by the failure of Cdc42N17 to inhibit accumulation of F-actin and $PI(3,4,5)P_3$ or localization of PAK-PBD-YFP (Figs. 2 and 4 c). However, Cdc42 does appear to constrain where $PI(3,4,5)P_3$, Rac-GTP, and F-actin accumulate and thereby consolidate formation of a single stable pseudopod. Inhibiting Cdc42 severely impairs stable polarity and motility: pseudopods appear, disappear, or move from one position on the cell surface to another within minutes, and cells migrate poorly or not at all (Figs. 5 and 6; see also videos 2 and 4).

Previous studies have shown that Cdc42 inhibitors impair polarity and/or chemotaxis in several cell types, including polarized epithelial cells (Kroschewski et al., 1999), T lymphocytes (Haddad et al., 2001), fibroblasts (Nobes and Hall, 1999), and macrophages (Allen et al., 1998). In these cells, the ability to polymerize actin was not affected, but localization of $PI(3,4,5)P_3$ and activated Rac were not examined. The time scales (hours) of all these experiments differed remarkably from the minute-to-minute behavior observed in HL-60 cells. In a wound-healing assay, Cdc42-inhibited fibroblasts showed loss of polarity and formed lamellae around

much of the cell circumference (Nobes and Hall, 1999), but the cells moved very slowly at a rate of ~1 cell diameter per h. In macrophages (Allen et al., 1998), expression of Cdc42N17 induced formation of broad leading lamellae and impaired interpretation of the chemoattractant gradient as in HL-60 cells. In contrast to HL-60 (Fig. 6), such macrophages migrated faster than control cells (Allen et al., 1998), but neither type of macrophage moved more than 1.5–2 cell diameters in 3 h. Thus, our experiments show for the first time that Cdc42 can control polarity and migration by regulating cellular events that take place in minutes. These results make it unlikely, for instance, that Cdc42 controls polarity primarily by events that require synthesis of new proteins (as is certainly the case in yeast cells [Nern and Arkowitz, 2000]).

We can only speculate about the mechanisms by which Cdc42 stabilizes the pseudopod in HL-60 cells and promotes oriented polarity in other eukaryotes. Cdc42 could limit actin polymerization and accumulation of $PI(3,4,5)P_3$ at the lateral edges of a pseudopod, perhaps by regulating formation of scaffolded complexes that inhibit Rac or promote activation of Rho at these locations. Alternatively, Cdc42 could regulate localization of pseudopods indirectly by controlling vesicle traffic and membrane localization of (for instance) enzymes that synthesize or degrade $PI(3,4,5)P_3$. In yeast, Cdc42 stabilizes the location of a bud site, and localized Cdc42 activity polarizes the actin cytoskeleton and secretion of newly synthesized proteins (Nern and Arkowitz, 2000).

Materials and methods

Materials

LT was prepared as described (Baldacini et al., 1990). Human fibronectin was from BD Biosciences. Latrunculin B was from Calbiochem. Human albumin (low endotoxin), DMSO, fMLP, and insulin were from Sigma-Aldrich. The mouse monoclonal anti-$PI(3,4,5)P_3$ antibody (Chen et al., 2002) was from Echelon. Mouse mAb against the 9E10 (Myc) epitope tag was purchased from Roche, Texas red–conjugated goat anti–mouse was from Jackson ImmunoResearch Laboratories, and rhodamine- and Alexa 647–conjugated phalloidin were from Molecular Probes. Transwell plates were from Corning. Femtotips were from Eppendorf. Electroporation cuvettes were obtained from Labrepco. The Electroporation chamber (model series 1600) was from GIBCO-BRL.

DNA constructs

Myc-tagged V12 and N17 mutants of Rac and Cdc42 were cloned in pTET7 downstream of a tetracycline repressor binding sequence as described (Kalman et al., 1999a). To activate expression of the mutant proteins, these constructs were cotransfected with a plasmid constitutively expressing a tetracycline repressor VP16 fusion protein. WASpΔC was as described (Kalman et al., 1999b). PAK-PBD-YFP (Kraynov et al., 2000) was a gift from Klaus Hahn (Scripps Institute of Immunology, La Jolla, CA); encoded in a pcDNA3 vector, this construct comprised residues 65–150 of human PAK1 with EYFP linked to its COOH terminus by a four-residue linker (Leu-Ser-Gly-Arg).

Cell culture, transfection, and toxins

Procedures for cultivation and differentiation of HL-60 and HL-60 cells stably expressing PH-Akt-GFP have been described (Servant et al., 2000; Wang et al., 2002). For transient transfections, differentiated HL-60 cells (on day 6 after addition of DMSO) were washed once in RPMI-HEPES and resuspended in the same medium to a final concentration of 10^8 ml^{-1}. DNA was then added to the cells (30 μg of PH-Akt-GFP or PAK-PBD-YFP DNA plus 50 μg of other constructs to be cotransfected), the cell DNA mixture was incubated for 10 min at RT, transferred to electroporation cuvettes, and subjected to an electroporation pulse on ice at 310 V, 1180 μF and low resistance. Transfected cells were allowed to recover for 10 min at RT and then transferred to 20 ml complete medium. Subsequent assays were performed 16–24 h after transfection. Although cells transiently transfected with GFP or PH-Akt-GFP migrated more slowly in response to fMLP than stably transfected cells (~5 versus ~7 μm per min; unpublished data), their abilities to polarize and move toward a micropipette source were unaffected.

Differentiated HL-60 cells cultured in differentiation medium were pretreated with LT (200 ng/ml) for 24 h at 37°C. For the latrunculin experiment, cells transiently expressing RacV12 and PH-Akt-GFP were allowed to adhere to fibronectin-coated coverslips and were then treated with 20 μg/ml latrunculin B as described (Wang et al., 2002).

Pull-down assay for activated Cdc42

COS-7 cells were transfected with Cdc42N17 or empty vector using the Lipofectamine-Plus. The pull-down assay for active Cdc42 was based upon a previously reported procedure (Benard et al., 1999). After 24 h incubation in DME-H21 with 0.2% FBS, cells were trypsinized and washed with serum-free medium and resuspended at 8×10^6 ml^{-1} in HBSS medium with FISH buffer containing protease inhibitors. Cells were lysed, and the lysate were incubated for 45 min with glutathione-sepharose beads linked to PAK-PBD-GST. Immunoblots of the bead fractions were blotted with anti-Cdc42 rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) and anti–rabbit IgG/HRP (Amersham Biosciences).

Assays and microscopic analysis

Transwell assays were performed as described (Laudanna et al., 1998). For spatial localization of fluorescent probes (PH-Akt-GFP or PAK-PBD-YFP) and actin in fixed cells, cells were subjected to one of the following stimulation conditions as described: no stimulation (basal), stimulation with fMLP (100 nM) for 1 or 3 min, or insulin (100 μg ml^{-1}) for 3 min. Cells were then fixed and immunostained for the myc epitope tag fused to the NH_2 terminus of mutant Rac and Cdc42. The myc antibody was used at a dilution of 1:1,000, and immunostaining was conducted as described previously (Servant et al., 1999). F-actin was assessed by incubating for 15 min with 10 U ml^{-1} rhodamine-phalloidin or Alexa 647–phalloidin as described (Wang et al., 2002). For all experiments in fixed cells, except those involving insulin stimulation, expression of mutant proteins was confirmed by immunostaining. In the case of insulin stimulation, even in control cells the weak PH-Akt-GFP recruitment was lost upon permeabilizing cells during the immunostaining protocol.

Live cells were imaged after stimulation either with a uniform concentration of chemoattractant (fMLP, 100 nM) or by a point source of chemoattractant from a micropipette containing 10 μM fMLP (generated by Femtotips) as described (Servant et al., 1999). Since >80% of the cells expressed both constructs, fluorescence associated with PH-Akt-GFP was used to infer coexpression of the mutant protein in living cells. Time-lapse videomicroscopy was performed as described (Servant et al., 1999).

Online supplemental material

The time-lapse experiments shown in Figs. 5 and 6 are also viewable as Quick-Time videos available at http://www.jcb.org/cgi/content/full/jcb.200208179/DC1. Videos 1 and 2 show the responses of transiently transfected differentiated HL60 cells expressing either PH-Akt-GFP alone or in combination with Cdc42N17 to a uniform concentration of chemoattractant. Videos 3 and 4 show cells expressing the same constructs responding to a point source of chemoattractant from a micropipette. Fig. S1 shows the early membrane recruitment of PH-Akt-GFP in transfected HL60 cells different mutant Rho GTPases upon stimulation by a uniform concentration of fMLP or insulin. Fig. S2 shows the migration of differentiated HL60 cells expressing RacN17 or WASpΔC in response to a uniform concentration of fMLP or to fMLP supplied by micropipette. Fig. S3 shows the range of phenotypes observed upon inhibition of Cdc42 activity.

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