Regulation of chemotaxis by the orchestrated activation of Ras, PI3K, and TOR

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Abstract

Directed cell migration and cell polarity are crucial in many facets of biological processes. Cellular motility requires a complex array of signaling pathways, in which orchestrated cross-talk, a feedback loop, and multi-component signaling recur. Almost every signaling molecule requires several regulatory processes to be functionally activated, and a lack of a signaling molecule often leads to chemotaxis defects, suggesting an integral role for each component in the pathway. We outline our current understanding of the signaling event that regulates chemotaxis with an emphasis on recent findings associated with the Ras, PI3K, and target of rapamycin (TOR) pathways and the interplay of these pathways. Ras, PI3K, and TOR are known as key regulators of cellular growth. Deregulation of those pathways is associated with many human diseases, such as cancer, developmental disorders, and immunological deficiency. Recent studies in yeast, mammalian cells, and Dictyostelium discoideum reveal another critical role of Ras, PI3K, and TOR in regulating the actin cytoskeleton, cell polarity, and cellular movement. These findings shed light on the mechanism by which eukaryotic cells maintain cell polarity and directed cell movement, and also demonstrate that multiple steps in the signal transduction pathway coordinately regulate cell motility.

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Introduction

Chemotaxis, or directed cell movement up a chemical concentration gradient, is a fundamental cellular process and plays essential roles in development, tissue homeostasis, wound healing, innate immunity, and metastasis of tumor cells in higher organisms as well as finding food, repellent action, and forming a multicellular body in protozoa. The basic migratory systems are conserved from protozoa to vertebrates and can be dissected into two types of processes: (1) an amoeboid type crawling system, driven by filamentous-actin (F-actin) assembly induced force; (2) an adhesion receptor-mediated cell movement, driven by remodeling of the extracellular matrix, such as the adhesion receptor and integrin-mediated attachment. An adhesion receptor-mediated migration, which is slower than amoeboid migration, has been found in fibroblasts, myoblasts, and neural crest cells. The system depends on adhesion receptor and/or integrin-dependent cell–substrate interaction, and cells create substantial remodeling of the extracellular matrix, resulting in cellular migration towards...
newly occurring adhesion sites (Lauffenburger and Horwitz, 1996). On the other hand, the ameboid system depends on cytoskeleton-mediated cell movement, in which the assembly of a branched network of actin filaments provides the mechanical propulsion. This review focuses on an ameboid crawling system, which emerged about a billion years ago (Baldauf et al., 2000), and has been conserved throughout evolution between Dictostelium and leukocytes (Friedl, 2004; Parent, 2004; Pollard and Borisy, 2003). We will begin with a basic introduction to directional cellular movement and the cellular components that regulate the actin cytoskeleton, followed by recent findings on Ras/PI3K/TOR activation, regulation, and roles in chemotaxis.

Directional cellular movement

To respond to and migrate up a chemoattractant gradient, cells have to organize a series of complex processes: (1) receptors for a chemoattractant on the plasma membrane bind the chemoattractant; (2) receptors convert the extracellular cue into intracellular signaling; (3) signaling molecules undergo dynamic changes of their location and activation state, which cause asymmetric localization of cellular components; (4) polarized signaling molecules induce coordinated remodeling of the actin cytoskeleton and cell adhesion to the substratum, which produce the formation of a new pseudopod in the direction of the chemoattractant source, resulting in cellular movement. Numerous external stimuli, activating through various cell surface receptors and signal transduction pathways, can promote migration through this process. Cells are able to respond to chemoattractant gradients as shallow as a 2–5% difference between the anterior and the posterior of the cell. This is a surprising ability if one considers that some cells have a diameter of <10 μm. How can a cell sense the small differences in a shallow chemoattractant gradient? Cells must acquire an intracellular system that can convert and amplify this shallow gradient into a steep intracellular gradient of signaling and actin cytoskeletal components (Chung et al., 2001a; Merlot and Firtel, 2003; Parent and DeVreotes, 1999). Before discussing what is known about the cellular compass that senses and sets the direction of the cells, we briefly outline the multiple steps involved in the formation of the leading edge.

Asymmetry of the actin cytoskeleton

F-actin localizes to the leading edge, where it assembles to induce protrusion of pseudopodia, and to a lesser extent to the posterior (Figs. 1 and 2; Ridley et al., 2003; Weiner et al., 2002; Pollard and Borisy, 2003). Leading edges are enriched in actin-modifying enzymes, such as the Arp2/3 complex, WAVE/Scar, WASP, and ADF/cofilin (Mullins et al., 1997; Myers et al., 2005; Nozumi et al., 2003; Sukumvanich et al., 2004; Svitkina and Borisy, 1999; Welch et al., 1997). Myosin II is assembled at the cell’s posterior and lateral sides where it provides rigidity to the polarized cell through cortical tension. Contraction of myosin II in the uropod enables the uropod to lift off of the substratum and retract toward the forward-moving pseudopod. Cells lacking myosin II or the components that regulate its contraction cannot properly retract the uropod, resulting in chemotaxis defects (Chung et al., 2001a; DeVreotes and Janetopoulos, 2003; Worthylake and Burridge, 2003; Xu et al., 2003). In addition, microtubules play an important role in maintaining polarity in a number of cell types through directing vesicular traffic and cytoskeletal regulators (reviewed in Watanabe et al., 2005; Xu et al., 2005). The remodeling of the actin cytoskeleton is essential for cell motility. Treatment with an F-actin polymerization inhibitor, such as latrunculin A (LatA), induces a rounded shape and complete abrogation of cell motility (immobilization). Fig. 1 shows a cartoon of some of the molecules and the signaling networks that control leading edge function.

Remodeling of the cytoskeleton by Rho GTPases

The Rho family of small GTPases are key regulators of the actin/myosin cytoskeleton during chemotaxis (reviewed in Fukata et al., 2003; Millan and Ridley, 2005; Raftopoulos and Hall, 2004). The most well-known members of this family are Rho, Rac, and Cdc42 proteins, which are present in all mammalian cells. These proteins control three stages of cell migration: progression of the frontal edge, adhesion that stabilizes the frontal area, and de-adhesion and shifting of the uropod. Rac and Cdc42 are the best-characterized molecules that play pivotal roles in controlling cell polarization, lamellipodium formation and expansion, and organization of focal complexes. Rho regulates the contractile activity of the actin–myosin cytoskeleton outside of the frontal area, and thus contraction and de-adhesion of the uropod. Similar to other small GTPases, Rho GTPases act as molecular switches and cycle between active (GTP-bound) and inactive (GDP-bound) states. This activity is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (reviewed in Moon and Zheng, 2003; Olofsson, 1999; Peck et al., 2002; Ridley, 2001; Schmidt and Hall, 2002). Activated Rac and Cdc42 induce morphologically
distinct actin protrusions at the plasma membrane, lamellipodia, and filopodia, respectively (Nobes and Hall, 1995; Ridley and Hall, 1992). Mammalian cells have three Rac isoforms, Rac1, 2, and 3. Rac1 is expressed ubiquitously, and Rac2 is strictly expressed in hematopoietic cells. Rac3 is predominantly expressed in the developing nervous system and adult brain. Leukocytes (including neutrophils, B cells, and macrophages) from Rac2 knockout mice display markedly reduced F-actin assembly and chemotaxis. Although leukocytes lacking Rac2 still have Rac1 expression, Rac1 does not compensate for the deficiency (Gu et al., 2003; Li et al., 2002). However, both Rac1 and Rac2 are crucial for the regulation of actin-mediated function, because rac1 null neutrophils that have normal Rac2 expression display profound defects in directional movement (Glogauer et al., 2003; Sun et al., 2004), and Rac1 and Rac2 double null hematopoietic cells and neutrophils completely lose chemotactic response as well as stimuli-induced F-actin polymerization (Gu et al., 2003). Their distinct yet synergistic function could be partly explained by their subcellular localization. In Madin–Darby canine kidney (MDCK) cells, overexpressed GFP-tagged Rac1 predominantly localizes at the plasma membrane, whereas GFP-Rac2 mainly localizes in the cytosol (Michaelson et al., 2001). The importance of Rac in the human immune system was demonstrated by the discovery of a patient with a naturally occurring Rac2 mutation that functioned as a dominant negative Rac2. The patient suffered from severe bacterial infections and reduced wound healing due to the neutrophil dysfunction of chemotaxis, cell polarization, ingestion, degranulation, and superoxide anion production (Ambruso et al., 2000; Gu et al., 2001).

A chemotactic analysis of the Rac signal was conducted in a rac null Dictyostelium strain. The genome sequence revealed that Dictyostelium contains 15 Rho family member GTPases, of which Rac1a/b/c, RacF1/F2, and RacA, RacB, and RacC are categorized into the Rac subfamily (Eichinger et al., 2005; Rivero...
and Somesh, 2002). Although neither Rho subfamily members nor a true Cdc42 have been identified, locomoting Dictyostelium cells produce filopodial-like actin projections, suggesting a functional counterpart of Cdc42 must exist. A null mutation of RacB, one of probably several distinct Rac GTPases that participate in controlling the actin cytoskeleton, causes a significant reduction in chemotactant-induced F-actin polymerization and cell polarity without loss of directional motility (Park et al., 2004). As racB null cells still exhibit F-actin polymerization, we assume that other members of the Rac family play similar roles in controlling leading edge formation and cell polarity. It is unclear if the Racs are redundant or if they control different aspects of the F-actin response, either at different sites in the cells or through the control of different effectors. RacB activation occurs in response to a chemotactant with kinetics similar to those of actin polymerization. This RacB activation is partially under the control of PI3K signaling and it is also affected by F-actin polymerization, as RacB activation is severely reduced when F-actin polymerization is blocked by LatA. Intriguingly, Dictyostelium RacGEF1, which is a cognate GEF for RacB, localizes to the sites of F-actin polymerization in chemotaxing cells. Treatment of cells with LatA results in a cytoplasmic localization of RacGEF1, which reduces RacB activation. This observation suggests there is a feedback loop between RacB and RacGEF1 through F-actin polymerization (Park et al., 2004).

**Cdc42**

Mammalian cells have one Cdc42, and genetic analysis using Cdc42-lacking leukocytes remains to be done, although fibroblastoid cells lacking Cdc42 by conditional gene inactivation have been produced. On the basis of experiments using a dominant negative Cdc42 mutant, inhibition of Cdc42 does not block cell movement and pseudopodia formation, although it

**Fig. 2.** Cellular distribution and activation profile of signaling components. Upper panels depict fluorescent images of GFP-RBD (imaging Ras activation) and RFP-PHtau (imaging sites of PI(3,4,5)P3 accumulation) in a chemotaxing wild-type cell. An asterisk indicates the direction of the chemotactant source. Ras activation and PI(3,4,5)P3 accumulation occur exclusively at the leading edge. The diagram shows the localization of signaling molecules in polarized wild-type cells. The activation profile of each signaling molecule is shown at the bottom.
suppresses persistent leading edge formation and induces the non-directional F-actin projection that causes defective directional movement (Allen et al., 1998; Srivivasan et al., 2003). Cdc42 can induce Rac activation by recruiting and activating a complex of p21-activated kinase (PAK) and PAK-interacting exchange protein/cloned-out of library (PIXs/COOLs) (Cau and Hall, 2005; Manser et al., 1998; Obermeier et al., 1998). PIX was identified as a PAK-associated protein. There are two homologs, PIXz/Cool2 and PIXβ/Cool1 both of which have GEF activity toward Rac and Cdc42. PIXs can exist as monomers or dimers through the leucine zipper. Dimerized forms specifically bind to GDP-Rac and activate it; the βi subunit of heterotrimeric G-proteins induces the monomer form and facilitates GTP exchange of Cdc42 (Feng et al., 2004). A feedback activation of Cdc42 occurs through Gβγ-mediated PAK/PIX activation. This feedback activation seems essential for directional migration since neutrophils isolated from PIXz knockout mice display severely reduced PAK1 and Cdc42 activation and result in chemotaxis defects (Li et al., 2003). The role of Cdc42 is complex and is integrated with the function of other Rac GTPases. Fibroblastoid cells, a non-amoeboid cell type, lacking Cdc42 exhibit an only partially reduced directionality (Czuuchra et al., 2005). Expression of dominant negative Cdc42 in these cells leads to more severe defects, suggesting that other Racs under the regulation of RhoGEFs that are sensitive to the dominant negative Cdc42 may coordinate with Cdc42 in controlling directionality and lamellipod formation during normal chemotaxis and/or other Racs can partially compensate for Cdc42 in its absence.

**WAVE/Scar and WASP family proteins regulate the Arp2/3 complex**

Rac and Cdc42 induce morphologically distinct F-actin protrusion at the leading edge through Arp2/3 complex-mediated de novo actin nucleation. Arp2/3 is a heptameric complex and an activated Arp2/3 complex generates the branched actin filament by creating a nucleation core for actin polymerization. The Arp2/3-mediated branched actin filaments grow rapidly and provide the mechanical force to push the membrane forward (Pollard and Borisy, 2003). The Arp2/3 complex alone does not induce actin polymerization. Under the control of Rac and Cdc42, respectively, WAVE/Scar and Wiskott–Aldrich syndrome protein (WASP) family proteins activate the Arp2/3 complex to produce robust F-actin polymerization (reviewed in Bomard and Caron, 2004; Stradal et al., 2004; Takenawa and Miki, 2001). Thus, localized Rac and Cdc42 signaling drives cellular motility.

**WASP**

WASP is the first member of the WASP/WAVE/Scar family and it was identified as the causative gene of Wiskott–Aldrich syndrome (WAS) (Derry et al., 1994). Patients with WAS suffer from immune system disorders, including eczema, bleeding, and recurrent infections caused by a deficiency of the actin cytoskeleton of leukocytes and platelet cells (Thrasher, 2002). Leukocytes derived from WAS patients and mice lacking WASP reveal severely impaired cell migration up the chemoattractant gradient and a highly reduced cell polarization in response to chemoattractant stimulation (Badolato et al., 1998; Snapper et al., 2005; Zicha et al., 1998). Mammals have two WASPs, a hematopoietic specifically expressed WASP and a ubiquitously expressed N-WASP. N-WASP is an essential gene and a knockout results in an embryonic-lethal phenotype. Interestingly, N-WASP-deficient fibroblasts still form filopodia, whereas certain intracellular pathogens lose their motility in infected N-WASP-deficient cells (Lommel et al., 2001; Snapper et al., 2001).

Dictyostelium contains two WASP genes, WASP1 and WASP2. Mutants having WASP1 disruption and knockdown expression of WASP2 result in cell polarization defects and severely reduced chemoattractant-induced F-actin polymerization and chemotaxis. Interestingly, Dictyostelium WASP binds to both PI(4,5)P2 and PI(3,4,5)P3 via its basic domain and preferentially localizes at the leading edge in chemotaxing cells (Myers et al., 2005). WASPs contain a VCA region (verprolin homology domain, coflin homology domain, and acidic region) at the C-terminus. The VCA region binds monomeric actin (G-actin) and the Arp2/3 complex and stimulates a conformational change in Arp2/3 to nucleate and polymerize actin (Panchal et al., 2003). The VCA domain is essential and sufficient to induce Arp2/3-mediated actin polymerization. The activity of the VCA domain is masked and inhibited through intermolecular interaction with its N-terminus under resting conditions. The N-terminus of WASPs contains an Ena/Vasp homology1/WASP homology1 (EVH1/WH1) domain and a basic region, a Cdc42 and Rac interactive binding domain/GTPase-binding domain (CRIB/GBD domain), and a proline-rich region. The autoinhibition is relieved when GTP-Cdc42, PI(4,5)P2, or an SH3-containing molecule (such as NCK and Grb2) binds to CRIB/GBD, the basic domain, or the proline-rich domain, respectively. WASP activity is also regulated through phosphorylation. Phosphorylation of the VCA region increases the affinity towards the Arp2/3 complex (Cory et al., 2002; Cory and Ridley, 2002). Upon Cdc42 binding to CRIB/GBD, WASP is more accessible to be phosphorylated, and tyrosine phosphorylation in the CRIB/GBD occurs in response to various stimuli, which recruits an SH2- and an SH3-containing protein.
Recruitment of a member of the Src family of tyrosine kinases (e.g. Src, Lck) has been reported. The binding of the SH2-containing protein stabilizes the open conformation of WASP, and can mediate WASP activation without the binding of Cdc42-GTP to the WASP CRIB domain (Torres and Rosen, 2006). Until recently, the autoinhibition model was accepted widely. However, biochemical studies reveal that most WASPs bind, through the EVH1/WH1 domain, to members of the WIP (WASP-interacting protein) family proteins, WIP, CR16, and WICH/WIRE (WIP- and CR16-homologous protein/WIP-related) (Ho et al., 2001; Kato et al., 2002; Ramesh et al., 1997). WAS mutations are predominantly found in the EHV1/WH1 domain and disrupt the binding between WIP and WASPs (Volkman et al., 2002). WIP contains multiple domains involved in actin cytoskeleton regulation (Aspenstrom, 2005). The phenotype of WIP knockout cells is similar but not identical to that of WASP knockout cells (Anton et al., 2002). Furthermore, biochemical purification identified trans-inducer of Cdc42-dependent actin assembly (Toca-1), a Pombe Cdc15 homolog (PCH) family protein, as a binding partner and a crucial regulator of the N-WASP/WIP complex. Toca-1 specifically binds to GTP-loaded active Cdc42 but not to GDP-Cdc42 or active GTP-loaded Rac1 (Ho et al., 2004). An in vitro reconstitution assay determined that Toca-1 is essential for Cdc42/N-WASP-mediated F-actin polymerization. Activated Cdc42 needs to bind not only WASPs but also Toca-1 to relieve the inhibition of the WASP complex to activate the Arp2/3 complex. Although physiologic roles and the mode of action of Toca-1 remain to be studied, the identification of the Toca-1/WASp/WIP complex facilitates our understanding of the integrated signaling pathways that control WASP activity during chemotaxis.

WAVE

WAVE/Scar was first identified in a second site suppressor screen of a cAMP receptor null mutation in Dictyostelium. Cells lacking WAVE/Scar overcome the morphological defect caused by the lack of one of the chemoattractant receptors, cAR2 (Bear et al., 1998). Dictyostelium has a single gene encoding WAVE/Scar. The WAVE/Scar mutant cells are much smaller than wild-type cells and display decreased chemotactic ability. Drosophila also contains a single gene for WAVE/Scar. The dsRNA-mediated RNA interference analysis indicates that WAVE/Scar knockdown cells have significantly reduced lamellipodial formation and cell movement. Interestingly, the WAVE knockdown cells also display deficient filopodial formation, whereas knockdowns of WASP do not have significant defects (Biyasheva et al., 2004). This observation suggests that WAVE/Scar plays a dominant role in F-actin projection in Drosophila culture cell lines; however, the possibility remains that the knockdown of WASP was incomplete and that WASP still plays a role in this system.

Mammalian cells have three WAVE/Scar genes, WAVE1, WAVE2, and WAVE3. The expression of WAVE1 and WAVE3 is mainly restricted to the brain (Dahl et al., 2003; Suetsugu et al., 1999). WAVE2 is expressed ubiquitously with a high expression in peripheral blood leukocytes (Suetsugu et al., 1999). WAVE2/Scar2 knockout mice are embryonic-lethal, and have brain malformation and hemorrhaging (Yamazaki et al., 2003; Yan et al., 2003). WAVE2-deficient mouse embryonic fibroblasts (MEFs) show severely impaired lamellipodial formation in response to growth factors. Macrophages exclusively express the WAVE2 protein. The retrovirus-mediated infection of WAVE2-specific shRNA leads to a 50–70% reduction of WAVE2 protein. The knockdown cells have an ∼50% reduction of both chemoattractant-induced F-actin protrusion and cell migration to a chemoattractant in a transmigration chamber (Kheir et al., 2005). Interestingly, a different isoform-specific function has been observed in WAVE1- and WAVE2-deficient MEFs. WAVE1-deficient MEF can induce peripheral lamellipodia, as generally observed at the leading edge. However, the MEFs have decreased dorsal ruffles, which is defined as F-actin protrusions upward from the cellular surface. Conversely, WAVE2-deficient MEFs have normal dorsal F-actin protrusions, although the cells have severely impaired peripheral lamellipodial formation (Suetsugu et al., 2003).

Similar to WASPs, WAVE/Scar proteins contain a basic region, a proline-rich region, and a VCA domain that activates the Arp2/3 complex to mediate Rac-induced F-actin polymerization. However, unlike WASPs, WAVE/Scar contains neither an EVH1/WH1 domain nor a GTPase-binding domain. Purification of WAVE1 from bovine brain reveals WAVE1 is in a pentameric protein complex with p53-inducible messenger RNA/specifically Rac1-associated protein (PIR121/Sra1), Nap1/Hem2 (Nck-associated protein), Ab1 binding partner (Ab1), and an actin-stimulating peptide, heat shock protein C300 (HSRPC300) (Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004; Steffen et al., 2004). This complex is conserved and is found in plants as well as animals and Dictyostelium (Stovold et al., 2005; reviewed in Szymanski, 2005). Biochemical studies show that Rac binds to PIR121/Sra1, which causes WAVE-mediated Arp2/3 activation. Nap and Ab1 serve as a central core that brings PIR121/Sra, WAVE/Scar, and HSPC300 together (Gautreau et al., 2004). The complex is crucial for leukocyte motility and Dictyostelium chemotaxis (Blagg et al., 2003; Kheir et al., 2005; reviewed in Ibarra et al., 2005).
Knockdown of Abi1, PIR121, and Nap in fibroblast cells leads to a defect in lamellipodial formation reminiscent of that in WAVE knockdown cells (Innocenti et al., 2004; Steffen et al., 2004). Each component may integrate a differential signal into the WAVE function. For examples, WAVE cannot translocate in Abi-deficient fibroblast cells, in T cells in response to T cell receptor (TCR) stimulation, and in Drosophila S2 cells (Kunda et al., 2003; Leng et al., 2005; Zipfel et al., 2006). WAVE2 undergoes tyrosine phosphorylation in response to PDGF stimulation, and this phosphorylation is mediated through Abi1 (Leng et al., 2005). PDGF-induced tyrosine phosphorylation of WAVE2 does not occur in MEFs lacking Abi and Abi-related kinase, whereas expression of Abi1 in the mutant MEFs restores tyrosine phosphorylation. A major site of phosphorylation by Abi kinase has been mapped to tyrosine residue 150. Wild-type WAVE2 significantly increases F-actin polymerization in vitro after incubating Abi recombinant kinase, while Y150F-mutant WAVE2 does not show any enhancement by Abi kinase. In addition, WAVE is hyper-phosphorylated via an ERK-dependent process, which blocks interaction with Grb2 (Miki et al., 1999). Although Grb2 positively regulates WAVE, the physiological role of the Ser/Thr phosphorylation, identification of the responsible kinase, and the phosphorylation sites remain unclear. There are also conflicting views about the role of each component in the stabilization and activation of WAVE/Scar. Future analyses should clarify the role of the complex, how the different phosphorylations regulate WAVE activity, and the integral roles of individual components of the WAVE complex.

**Localized regulation of RhoGEFs, Cdc42/WASP, and Rac/WAVE**

It is clear that Cdc42/WASP and Rac/WAVE are pivotal and vital to reorganizing the actin cytoskeleton. The activation of Cdc42 and Rac must be coordinate regulated spatiotemporally. Fluorescence resonance energy transfer (FRET) analyses as well as GFP reporter studies of activated Cdc42 and Rac have revealed that Cdc42 and Rac are exclusively activated at the leading edge (Itoh et al., 2002; Kraynov et al., 2000; Srinivasan et al., 2003). This localized Rac activation is achieved by chemoattractant-mediated localized activation of RhoGEFs as well as an autonomous feedback circuit that we will discuss later. Classic RhoGEFs contain a pleckstrin homology (PH) domain adjacent to the Dbh homology (DH) domain, which is responsible for GEF activity. PH domains bind to phosphatidylinositol or proteins and regulate the subcellular localization or GEF activity. In addition to the PH and DH domains, RhoGEFs often contain other modular domains, including the SH2 domain, the SH3 domain, the GTPase-binding domain, GEFs for other GTPases, the CH domain, and/or the PDZ domains. These modular domains integrate upstream signaling into RhoGEF localization or activation (reviewed in Schmidt and Hall, 2002). The human genome contains >60 RhoGEFs and the *Dictyostelium* genome has >45 RhoGEFs (Eichinger et al., 2005; Schmidt and Hall, 2002). Several GEFs, such as P-REX 1, TIAM 1, VAV, Dbl, and SOS1 are activated upon the production of PI(3,4,5)P3 through their PH domain; therefore, PI3K activation is important for their biological activity (Das et al., 2000; Fleming et al., 2000; Hill et al., 2005; Welch et al., 2002). VAV has GEF activity toward Rac and Cdc42, and this GEF activity is controlled by PH-domain binding to PI(3,4,5)P3 and tyrosine phosphorylation (Han et al., 1998). A dominant negative mutant of VAV blocks macrophage chemotaxis toward M-CSF (Vedham et al., 2005). VAV1 expression is restricted to hematopoietic cells (Katzav et al., 1989). Primitive hematopoietic cells derived from VAV1 null mice show severely reduced chemotaxis speed toward SDF-1 and lysophospholipids (Whetten et al., 2003). A cell type-specific requirement of PI3K for the activation of VAV has been observed: tyrosine phosphorylation of VAV by integrin-induced Src or Syk activation. Hence, VAV can couple integrin signaling to T cell adhesion (Gakidis et al., 2004; Irie et al., 2005). Neutrophils derived from P-REX knockout mice show selective reduction of Rac2 but not Rac1 in response to chemotactic stimulation. P-REX1-deficient neutrophils can migrate up the chemoattractant gradient, although with severely reduced speed. The mutant neutrophils fail to accumulate at an inflammation site (Dong et al., 2005; Welch et al., 2005). Neutrophils derived from PI3Kγ (a major PI3K in neutrophils) knockout mice have defects similar to those of Rac2 knockout neutrophils. Whereas the phenotype of P-REX1 null neutrophils is far less severe than that of Rac2-deficient cells, it is likely there is another Rac activator that is regulated by PI3K signaling.

**The CD M and Zizimin families**

Rac and Cdc42 are also activated by the CDM (Ced-5, Dock180, Myoblast city) protein family and the Zizimin protein family, respectively (Meller et al., 2002, 2005; Reif and Cyster, 2002). They lack a DH domain, and harbor GEF activity through a module named Docker, CZH2, or the DHR2 domain. The CDM and Zizimin families are well conserved in yeast, plant, *Caenorhabditis elegans*, *Drosophila*, *Dictyostelium*, and mammals (reviewed in Meller et al., 2005). Zizimin has Cdc42-specific GEF activity, although the relationship between phosphoinositides and Zizimin regulation is not yet clear.
Animals carrying a mutation of Ced-5, a *C. elegans* orthologue of DOCK180, have defects in cell migration and phagocytosis (Wu and Horvitz, 1998). A *Drosophila* orthologue, Myoblast city, regulates myoblast fusion and dorsal closure that is due to a defect in border cell movement (Duchek et al., 2001; Erickson et al., 1997). DOCK2 is specifically expressed in hematopoietic cells (Fukui et al., 2001; Nishihara et al., 1999). T cells and B cells from DOCK2 knockout mice have a severe phenotype: strong reduction of chemoattractant-mediated Rac activation; and defective cell polarization, adhesion, and F-actin polymerization, which leads to the abrogation of chemotactic movement (Fukui et al., 2001; Sanui et al., 2003). DOCKs form a complex with ELMO and Crk, by which DOCKs can couple Rac activation to an upstream signal such as integrin activation, and to PI3K activation. PI3K-dependent and -independent functions of DOCK2 have been observed (Nombela-Arrieta et al., 2004). The direct molecular linkage between PI3K and DOCK protein was uncovered with the finding that DOCK180, and possibly all DOCKs, can specifically bind to PI(3,4,5)P3 through its DHR-1 domain. DOCK180 translocates to the cortex in response to chemoattractant stimulation and colocalizes with PI(3,4,5)P3 at the leading edge. This translocation is abolished by treatment with a PI3K inhibitor (Cote et al., 2005; Kobayashi et al., 2001). The DOCK180 mutant that lacks DHR1 fails to promote directed cell migration. Replacement of the PH domain from BMX/Etk tyrosine kinase that specifically recognizes and binds to PI(3,4,5)P3 acts like wild-type DOCK180. ELMO contains a PH domain that has been suggested to bind PI(3,4,5)P3 (Zhou et al., 2001). Overexpression of an ELMO N-terminal deletion mutant, which does not contain a PH domain, sequesters DOCK180 in the cytoplasm, implicating a possible involvement of membrane targeting of the DOCK/ELMO complex through the ELMO N-terminus (Grimsl et al., 2004). These observations open up the intriguing possibility that the DOCK/ELMO complex requires two PI(3,4,5)P3s to translocate the complex to the membrane, which may serve as a threshold to initiate PI(3,4,5)P3-mediated Rac activation. It is also possible that PI3K and other signaling cascades are required for DOCK translocation and activation in vivo.

**Recruitment of signaling molecules through PI3K signaling**

Cells evolutionally developed systems by which they can amplify a shallow extracellular gradient of chemoattractant into a steep intracellular gradient of PI(3,4,5)P3 at the leading edge. PI(3,4)P2 and PI(3,4,5)P3 are generated by PI3Ks at the inner phase of the plasma membrane and are degraded by the 3-phosphoinositide phosphatase PTEN. PI(3,4)P2 and PI(3,4,5)P3 can locally accumulate at high local concentrations, as their diffusion coefficients are low, and they diffuse 100 times slower than other second messengers, such as aqueous soluble small signaling molecules including cAMP, cGMP, and IP3 (Postma and Van Haastert, 2001). (Fig. 2 shows the spatial regulation of key signaling responses in chemotaxing cells.) Local sequestration models, such as phosphoinositide clustering via headgroup interaction, that explain this diffusion have been suggested (Janmey and Lindberg, 2004). The site of PI(3,4,5)P3 accumulation at the leading edge allows the cell to localize a number of integrated signaling pathways that control forward movement of chemotaxing cells, including RacGEFs and Rac/Cdc42 effectors like WAVE and WASP. The basic domain of WAVE2, and probably all WAVEs based on sequence similarity, is a module that recognizes PI(3,4,5)P3 with higher affinity than PI(4,5)P2; full-length WAVE2 binds to PI(3,4,5)P3 with a $K_d$ of 185 nM, while the $K_d$ for binding to PI(4,5)P2 is 1.2 μM. A constitutively active PI3K mutant recruits WAVE2 to the plasma membrane in the presence of dominant negative Rac (Oikawa et al., 2004). Intriguingly, the basic domain of N-WASP, found at the N-terminus of the protein, is able to bind PI(3,4,5)P3 in addition to PI(4,5)P2 (Oikawa et al., 2004), which is consistent with the observations on *Dictyostelium* WASP (Myers et al., 2005).

**PI3K**

The PI3K family proteins are defined as lipid kinases that phosphorylate the D-3 position of phosphatidylinositol or phosphatidylinositol phosphate. PI3Ks are classified into Class I, II, and III, based on their structures. Class I PI3K is best characterized by its function and regulation, and is primarily responsible for the production of PI(3,4,5)P3 in response to extracellular stimulation. Class II PI3K poorly phosphorylates PI(4,5)P2, and its biological function is not well understood, although researchers are beginning to clarify its function (Harada et al., 2005; Katso et al., 2001). Class III PI3K, Vps34, exists as a heterodimer with a Ser/Thr kinase, Vps15/p150, and regulates intracellular vesicle trafficking (reviewed in Odorizzi et al., 2000) and autophagy (Klionsky, 2005; Wurmser and Emr, 2002). As mammalian Class III PI3K is inhibited by wortmannin, an inhibitor widely used to study the role of Class I PI3Ks, at low nanomolar concentrations, some of the suggested functions attributed to Class I PI3Ks using wortmannin may overlap with those of Class III PI3Ks (Volinia et al., 1995). Recently, mammalian Vps34 was
shown to regulate mTOR activation in response to amino acids (Byfield et al., 2005; Nobukuni et al., 2005).

Mammalian Class I PI3K is composed of two subfamilies, Class IA and Class IB, based on the structural and functional differences (Fruman and Cantley, 2002; Katso et al., 2001), although Class IA and IB share some features of domain structure such as a Ras binding domain (RBD). Class IA PI3K is a heterodimer consisting of the catalytic subunit p110 and the regulatory subunit p85. All p85s, including the shorter isoforms, contain the SH2 domain by which Class IA PI3K is recruited and activated upon a growth factor- or cytokine-induced tyrosine phosphorylation event. The function and biological roles of Class IA PI3Ks are well reviewed (Fruman and Cantley, 2002; Katso et al., 2001; Vanhaesebroeck et al., 2005).

Class IB PI3K (PI3Kγ) is a heterodimer containing the p110γ catalytic subunit and a p101 or p84 regulatory subunit (Stephens et al., 1994, 1997; Stoyanov et al., 1995; Sui et al., 2005). PI3Kγ is the PI3K primarily responsible for producing PI(3,4,5)P3 in response to G-protein-coupled receptor (GPCR) activation in leukocytes. In mammalian (HEK) cells, overexpression of Gβγ subunits leads to PI3Kγ activation by interacting with the p110γ catalytic subunit and p101 regulatory subunits (Brock et al., 2003; Hirsch et al., 2000; Li et al., 2000). PI3Kγ is also under the control of Ras; GTP-Ras directly binds and activates PI3Kγ in vivo and in vitro (Pacold et al., 2000). Neutrophils and monocytes derived from mice lacking PI3Kγ do not have detectable PI(3,4,5)P3 production in response to a chemoattractant, FMLP, or C5a (Hannigan et al., 2002; Sasaki et al., 2000). The mutant cells display severe defects in cell polarity and reduced efficiency of chemotaxis and directionality. Pharmacological inhibition of PI3K in a variety of mammalian cell types blocks chemotaxis and tumor cell malignancy. An inhibitor specific to PI3Kγ has been developed and is the focus of current attention (Ward and Finan, 2003). Treatment with PI3Kγ inhibitors selectively blocks the chemotaxis of neutrophils and monocytes in vitro and in vivo, a phenotype similar to that of the pι3kγ null cells (Camps et al., 2005).

Dictyostelium has six homologs of PI3Kγ, PI3K 1–6. The knockouts of PI3K1 and PI3K2 result in defective cell polarization and directionality, and chemotactic speed is decreased (Funamoto et al., 2001, 2002). Cells lacking PI3K1, 2, and 3, which show a 95% reduction in Akt activation, exhibit more severe defects in polarity and chemotaxis speed (K. Takeda and R.A. Firtel, in preparation). Defects in directionality are exhibited at lower chemoattractant concentrations, suggesting that at higher concentrations, the remaining PI3K may be sufficient to mediate directional movement and/or additional pathways may play key roles in mediating directional sensing. A detailed study, which focused on the more physiologically relevant role of PI3K, indicates PI3K acts as amplifier of chemoattractant input. Cells lacking PI3K1/2 or LY294002-treated cells fail to chemotax properly to linear chemoattractant gradients that might be more physiological than exponential gradients produced by a point source (K. Takeda and R.A. Firtel, in preparation). As chemotactic behavior in vivo can be affected by the concentration of the chemoattractant gradient (Heit et al., 2002; Postma et al., 2003), we should consider whether the chemoattractant concentration we use is at an appropriate physiological level.

Akt and other downstream effectors

PI3K evokes signaling through downstream effector molecules, among which Akt is well known and one of the central players regulating cellular growth and survival (reviewed in Cantley, 2002; Hay, 2005; Luo et al., 2003). Growing evidence suggests that Akt regulates cell migration of various cell types as a part of a PI3K effector. In neutrophils, Akt co-localizes with F-actin and is under the control of PI3Kγ (Hannigan et al., 2002). The chemotactic ability is correlated with the Akt activation state (Heit et al., 2002; Rane et al., 2005). Overexpression of constitutively active Akt enhances cell migration and the invasive ability of the fibroblast cell line HT1080, whereas expression of kinase-dead Akt in cells has the opposite effect. Similarly, tumor cells with increased Akt expression have a more invasive and metastatic state (Scheid et al., 2002; Vivanco and Sawyers, 2002). Disruption of Akt/ PKB in Dictyostelium results in strong defects in cell polarity and chemotaxis (Meili et al., 1999, 2000). Akt/PKB regulates cell polarity at least partly through PAKa activation that plays a role in myosin II assembly (Chung and Firtel, 1999; Chung et al., 2001b). Recently, two independent groups identified a coiled-coil protein as a binding partner of Akt and named it Girdin/APE (girders of actin filaments/Akt phosphorylation enhancer) (Anai et al., 2005; Enomoto et al., 2005). Binding of Girdin/APE increases PI3K-mediated phosphorylation of two critical sites of Akt (T308 and S478) (Anai et al., 2005). Girdin/APE binds to F-actin and localizes at the leading edge. Interestingly, the C-terminus of Girdin/APE contains an Akt phosphorylation site and the region is responsible for localizing to the plasma membrane and F-actin stress fibers. Girdin/APE phosphorylated by Akt preferentially localizes at the leading edge. Strikingly, siRNA-mediated Girdin/APE knockout abolishes cell motility completely. Expression of wild-type Girdin/APE in the knockout cells restores the cell motility, whereas Girdin/APE with a mutated Akt phosphorylation site fails to complement
the phenotype. Although the molecular mechanisms by which Akt and Girdin/APE regulate chemotaxis remain to be uncovered, the findings are particularly interesting because Girdin/APE represents the molecule missing between Akt and F-actin polymerization, and it may participate in the suggested feedback activation loop between Ras/PI3K and F-actin (Sasaki et al., 2004; Sasaki and Firtel, 2005; Weiner et al., 2002). From the other evidence, Akt seems to be a part of this feedback process. Expression of constitutively active Akt can induce activation of Rac and Cdc42, while dominant negative Akt suppresses cell motility driven by Rac and Cdc42 or a growth factor (Higuchi et al., 2001). Rac1- and Rac2-deficient neutrophils, B cells, and hematopoietic cells have lower levels of Akt activation in response to a chemoattractant (Gu et al., 2003; Sun et al., 2004; Walmsley et al., 2003). Girdin/APE may play an important role in the feedback circuit as a part of Akt signaling. Besides Akt, genetic studies identified other PI3K effector molecules that are involved in regulating chemotaxis, of which PhdA and CRAC are crucial for chemotaxis (Funamoto et al., 2001; Comer et al., 2005). Their molecular mechanisms are currently being investigated.

Directed accumulation of PI3K and its product PI(3,4,5)P₃

PI(3,4,5)P₃ is strongly accumulated at the leading edge in motile cells (Fig. 2), by which intracellular signaling molecules (including WAVE, RhoGEFs, Akt, CRAC, and PhdA) locate and activate cellular events. In contrast, the chemoattractant receptors distribute uniformly along the whole of the plasma membrane (Servant et al., 1999; Xiao et al., 1997). The G-protein β subunit exhibits a very shallow anterior–posterior gradient, which mirrors receptor occupancy, but this cannot explain the steep generation of PI(3,4,5)P₃ (Jin et al., 2000). Furthermore, FRET analyses have shown that the G-protein α and βγ subunits remain dissociated as long as receptors are occupied by a chemoattractant (Janetopoulos et al., 2001), whereas PI(3,4,5)P₃ generation as well as PI3K activation are transient (Funamoto et al., 2002; Huang et al., 2003; Janetopoulos et al., 2001). A central question is how cells create a strong, restricted PI(3,4,5)P₃ accumulation at the leading edge. The first insight into the mechanisms of locally accumulating PI(3,4,5)P₃ has come from studies of the subcellular distribution of PI3Ks and PI 3-phosphatases (PTEN) in Dictyostelium (Funamoto et al., 2002; Iijima and Devreotes, 2002). Dictyostelium PI3K transiently translocates to the plasma membrane in response to chemoattractant stimulation and to the leading edge in chemotaxing cells. This PI3K translocation does not require its kinase activity, C2 domain, or Ras-binding domain, but requires the N-terminal 100 amino acids that are not homologous to any known functional domains (K. Takeda and R.A. Firtel, unpublished data). PTEN exhibits spatial localization reciprocal to that of PI3K. PTEN is localized at the plasma membrane and transiently delocalizes from the plasma membrane to the cytosol after chemoattractant stimulation. During chemotaxis, PTEN is excluded from the leading edge and becomes localized to the lateral sides and back. It has been shown recently that ROCK, a RhoA-GTP regulated Ser/Thr kinase, phosphorylates PTEN, which causes PTEN translocation to the back (Li et al., 2005; Meili et al., 2005). The molecular mechanisms of PTEN localization have yet to be clarified. The loss of PTEN results in extended and non-spatially restricted PI3K activity. pten null cells exhibit expansion of PI(3,4,5)P₃ as well as F-actin accumulation at the membrane, demonstrating that PTEN is required for temporally and spatially restricting both PI3K activity and F-actin synthesis at the leading edge. Subsequent studies in neutrophils have confirmed that recruitment of PI3K to the leading edge and the loss of PTEN from the leading edge are conserved in at least some amoeboid chemotaxing mammalian cell types (Gomez-Mouton et al., 2004; Li et al., 2003, 2005; Wang et al., 2002). These regulatory events elicit the strong asymmetry in PI(3,4,5)P₃ signaling that leads to directional movement.

Cellular machinery that sets the direction of cell movement

PI3K and PTEN are very important for directed cell movement, although not essential for directional sensing. Dictyostelium cells lacking PI3K1/2 or neutrophils lacking PI3Kγ, which have no detectable Akt/PKB activation, have highly reduced cell polarization and chemotaxis ability, but are still able to move toward a chemoattractant. N-terminal PI3K still localizes at the leading edge in p3k1/2 null cells (Funamoto et al., 2002; Iijima and Devreotes, 2002). These findings suggest the existence of pathways upstream of PI3K that sense the chemoattractant gradient. Strikingly, translocation of PI3K does not occur in LatA-treated cells in GFP-based and cell fractionation assays (Sasaki et al., 2004; Sasaki and Firtel, 2005). LatA is a toxin that inhibits F-actin polymerization by sequestering monomeric actin; therefore, PI3K localization requires actin polymerization. Nonetheless, directed PI(3,4,5)P₃ accumulation occurs in LatA-treated pten null cells, in which PI3K distributes uniformly (Sasaki et al., 2004; Sasaki and Firtel, 2005). This finding demonstrates that an intracellular PI(3,4,5)P₃ gradient or directional sensing event can be achieved without PI3K translocation and PTEN...
activity. Although the linkage between F-actin-mediated PI3K localization and a membrane raft has been suggested, the details of the mechanism underlying the localization of PI3K are still unknown (Gomez-Mouton et al., 2004). As the localization pattern of PI3K partially overlaps with that of coronin, which binds newly synthesized actin, one plausible model is that PI3K preferentially binds to newly synthesized, ATP-bound F-actin.

**Ras is a proximal regulator of PI3K**

Ras proteins are well-characterized small GTPases that promote cellular growth and are involved in tumorigenesis (Ehrhardt et al., 2002; Hancock, 2003). The mammalian Ras family comprises H-Ras, N-Ras, and K-Ras, which are frequently mutated in human cancer and leukemia (Giehl, 2005; Malumbres and Barbacid, 2003; Repasky et al., 2004). The Ras subfamily comprises R-Ras, TC21/R-Ras2, M-Ras/R-Ras3, and E-Ras. Various Ras effector molecules have been identified, among which the Raf family kinase, PI3K, and RafGDS are well-studied downstream effectors and important mediators of the tumorigenic function of Ras. Tumor cells carrying an activated Ras mutation tend to have higher motility. Targeted deletion of oncogenic K-Ras from the colon cancer cell line HCT-116 reduces cellular motility that is mediated through PI3K signaling (Pollock et al., 2005). Treatment with a farnesyltransferase inhibitor, which is believed to inactivate Ras by inhibiting Ras farnesylation, abolishes the motility of certain lung carcinoma cells (Okudela et al., 2004). Overexpression of oncogenic Ras induces rearrangement of the actin cytoskeleton (Rodriguez-Viciana et al., 1997; Heo and Meyer, 2003; Ridley et al., 1995). H-, N-, and K-Ras have the same effector domain and can activate the same set of downstream effectors when they are overexpressed. However, in vivo imaging studies of the dynamic activation and localization of Ras in mammalian cells reveals a previously unexpected complexity of Ras signaling in vivo. H-Ras and N-Ras are activated not only at the plasma membrane but also at internal membranes such as the ER and the Golgi in COS-1 fibroblast cells and Jurkat T cells, while K-Ras is activated exclusively at the plasma membrane (Bivona et al., 2003; Chiu et al., 2002; Mochizuki et al., 2001; Perez de Castro et al., 2004). The differential activation sites of each Ras are attributed to their C-terminal motifs; in addition to being prenylated, H-Ras and N-Ras undergo palmitoylation in the Golgi and reside at the plasma membrane, the ER and the Golgi, while K-Ras4B lacks palmitoylation but has a polybasic domain by which it can exclusively locate to the plasma membrane (Chiu et al., 2002; Hancock, 2003). Translocation of K-Ras to the Golgi and endosomes in response to neurotransmitter-induced calmodulin activation was reported recently (Fivaz and Meyer, 2005). The intriguing question is what portion and which Ras isoforms are activated in mammalian cells in response to different stimuli. A study that used a high-affinity probe for Ras-GTP shows that pan-endogenous Ras activation in response to growth factors occurs exclusively at the plasma membrane (Augsten et al., 2006). These studies examined Ras signaling in response to a uniform application, but not to growth factor gradients.

**Ras regulates directed cell motility and cell polarization**

To date, an incredible amount of analyses related to Ras have been performed (probably >10,000 published reports). However, only a small number of studies have investigated the role of Ras in regulating amoeboid-type chemotaxis, cell polarization, and directional sensing. We do not understand where in the cell Ras is activated during cellular movement, and how Ras signaling pathways are integrated into the activation of a downstream effector to mediate directional movement. Studies from *Dictostelium* have shed light on these questions. *Dictostelium* has five Ras proteins that are related to mammalian H-Ras and K-Ras. Among these, RasB, RasD, and RasG are most closely related to their mammalian counterparts and have a conserved effector domain sequence (Khosla et al., 2000). Analyses of several Ras mutants have provided evidence that Ras regulates directional movement and cell polarity (Tuxworth et al., 1997; Wilkins et al., 2000). One of the RasGEF mutants, Aimless, is a clue that Ras-mediated directional sensing operates independently from Ras-mediated cell polarization, because *aimless* null cells exhibit a reduced directionality without any loss of cell polarity or chemotaxis speed (Insall et al., 1996; Sasaki et al., 2004). There are >20 putative RasGEFs in the *Dictostelium* genome. We speculate that RasGEFs, in addition to Aimless, regulate directional movement, as further depletion of Ras function, through the expression of dominant negative Ras in *aimless* null cells, results in severe defects in directionality and cell polarity (Sasaki et al., 2004). RasGEFs may activate certain sets of Ras to regulate them in a spatially and temporally restricted manner.

**Dynamic Ras activation during chemotaxis**

A biochemical assay in which the GST-fused Ras-binding domain (RBD) from human Raf1 kinase is used as an indicator for Ras activation has demonstrated that
Ras is rapidly and transiently activated upon global stimulation with a chemoattractant in *Dictyostelium* cells. The rapid kinetics of Ras activation are similar to those of PI3K. Ras activation does not require PI3K activation nor PTEN, demonstrating Ras is upstream of PI3K (Kae et al., 2004; Sasaki et al., 2004). Importantly, a chemoattractant cannot induce Ras activation in Gβ or Gz2 mutant cells (Kae et al., 2004; A.T. Sasaki and R.A. Firtel, unpublished observation), and Ras activation level is reduced in *aimless* null cells, one of the Ras GEFs in *Dictyostelium* (Sasaki et al., 2004).

All forms of *Dictyostelium* Ras identified lack a palmitoylation site. In response to global chemoattractant stimulation, Ras activation, as determined through the use of an RBD-GFP reporter, occurs rapidly and transiently at the plasma membrane, which is similar to K-Ras activation at the plasma membrane. The initial Ras activation occurs as early as, or slightly faster than, PI3K translocation, and PI(3,4,5)P3 accumulation follows. In chemoattractant gradients, activated Ras is restricted to the leading edge of the cell whereas total Ras protein is uniformly distributed along the plasma membrane (Fig. 2; Sasaki et al., 2004). When the position of the chemoattractant is changed to the opposite side of the cell, the activated Ras signal is rapidly lost from the initial site and concomitantly re-accumulates at the new site of the membrane closest to the micropipette. Directed, spatially restricted Ras activation occurs without PI3K activation, PI3K translocation, and PTEN activity (Figs. 2 and 3). This demonstrates that localized Ras is able to drive the formation of a steep intracellular PI(3,4,5)P3 gradient at the leading edge.

In vivo, PI(3,4,5)P3 production is greatly influenced by the localization of PI3K and directed activation of

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**Fig. 3.** A model for feedback loop-mediated directional sensing and cell polarization. The models show intracellular signaling to local PIP3 production (left), and chemoattractant-induced F-actin polymerization (right). The chemoattractant locally activates Ras at the presumptive leading edge (site of the membrane closest to the chemoattractant source) where Ras locally activates PI3K. There is a local polymerization of F-actin at the presumptive leading edge which is partially independent of the Ras/PI3K pathway. F-actin mediates the translocation of PI3K. Locally produced PI(3,4,5)P3 and F-actin-mediated Rac activation induces further F-actin polymerization, which enhances the recruitment of PI3K to the membrane.
Ras. PI3K localization is regulated through machinery driving F-actin polymerization. Therefore, robust PI3K activation, which sets a direction to move via a positive feedback loop between F-actin, PI3K, and Ras, starts when directed Ras activation meets F-actin polymerization sites under certain physiological conditions (Fig. 3). We suggest this feedback event is crucial for nascent leading edge formation under physiological conditions. 

pten null cells accumulate high PI(3,4,5)P_3 levels at the plasma membrane without chemotactic stimulation. It has been considered that elevated levels of PI(3,4,5)P_3 in pten null cells may be the result of basal PI3K activity. However, spontaneous multiple pseudopodia that form in pten null cells are exclusively labeled with the GFP-RBD and PH domain reporters, which means that Ras and PI3K are activated progressively yet concomitantly at specific sites on the plasma membrane. This spontaneous Ras activation is from a feedback loop comprised of F-actin and PI(3,4,5)P_3, as it is abolished after treatment with either PI3K or an F-actin polymerization inhibitor (Sasaki et al., 2004).

**Downstream pathways of Ras that control cell polarity**

In addition to PI3K, cells must have PI3K-independent F-actin polymerization pathways as well as a PI3K-independent polarization system. PI3K is indispensable for proper chemotaxis of cells placed in physiological levels of chemotactant, and for cells that are intrinsically unable to become highly polarized. PI3K seems dispensable for certain cells that utilize intermediumated motility, or cells containing a well-developed and polarized actin cytoskeleton, presumably because the cells have a compensatory system for cell polarization in addition to the autonomous feedback regulation of F-actin and myosin. Recently, the TOR pathway was found to control cell polarity, and the involvement of Ras in the TOR pathway was suggested (Lee et al., 2005).

**TOR complex 1 and 2**

TOR is a PI3K-related Ser/Thr-kinase that forms two evolutionarily conserved protein complexes, TOR complex 1 (TORC1) and TORC2. TORC1 is a rapamycin-sensitive complex composed of TOR, Lst8/GbL, and Raptor/KOG (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). TORC1 positively regulates cellular growth, size, metabolism, autophagy, and longevity (Holz et al., 2005; Kaeberlein et al., 2005; Manning and Cantley, 2003; reviewed in Martin and Hall, 2005; Sarbassov et al., 2005a, b). Deregulated up-regulation of the TOR pathway is observed in many tumor types (Guertin and Sabatini, 2005; Hay, 2005). The role of TORC1 in actin rearrangement and chemotaxis is not yet clear (Jacinto et al., 2004; Sarbassov et al., 2004; Segarra et al., 2006; Zhang et al., 2005). The upstream negative regulator of TORC1, the TSC1/2 complex, can induce actin rearrangement via Rho GTPases, and this ability seems distinct from its negative role towards TORC1. The monomeric state of TSC1 activates RhoA and induces stress fibers, while a TSC1/TSC2 heterodimer induces Rac activation. Cells lacking TSC2 have constitutive stress fiber formation presumably due to elevated activation of RhoA. The tsc2 null cells diminish stress fiber disassembly in response to PDGF stimulation, and fail to produce lamellipodial formation (Goncharova et al., 2004). As the TSC1/2 heterodimer dissociates on TSC2 phosphorylation by either Akt, ERK, RSK (Ma et al., 2005), TSC1/2 may control actin remodeling with the cooperation of Akt, RSK, and ERK, but this possibility has not yet been tested. Akt-mediated TSC1/2 down-regulation seems to be a mammalian or cell-type-specific event, as a non-phosphorylateable form of *Drosophila* TSC2 completely complements the viability of TSC2 null flies and *Dictyostelium* TSC2 does not have well-conserved phosphorylation sites for Akt (Dong and Pan, 2004).

Accumulating evidence indicates that one of the primary functions of TORC2 is actin cytoskeleton rearrangement. TORC2 is a rapamycin-insensitive complex and consists of AVO1/RIP3/SIN1, AVO2, AVO3/ Pianissimo/Rictor, and Lst8/GbL (Jacinto et al., 2004; Sarbassov et al., 2005a). In serum-starved NIH3T3 fibroblast cells, knockdown of mAVO3/Rictor and mLst8/GbL results in defective stress fiber formation in response to serum, whereas knockdown of mKOG/ Raptor that is a component of TORC1 does not cause a detectable defect of the actin cytoskeleton (Jacinto et al., 2004). In non-starved HeLa cells, lentivirus-mediated delivery of mAVO3/Rictor but not mKOG/Raptor siRNA leads to increased F-actin bundles concomitant with increased paxillin foci (Sarbassov et al., 2004). Interestingly, mammalian TORC2 induces phosphorylation of PKCζ. This is consistent with the studies from yeast that demonstrate TORC2 controls cell polarity through a PKC-Rho GTPase pathway. Similar to the knockdown analyses done in mammalian cells, the yeast mutants of AVO1, AVO3, TOR2, and Lst8 display depolarized actin patches (Ho et al., 2005; Loewith et al., 2002; Schmidt et al., 1996, 1997).

The molecular mechanism by which TORC2 mediates actin reorganization is not understood. Recent reports indicate that, in yeast, the PH-domain-containing protein family members Slm1 and Slm2 bind to PI(4,5)P_2 and TORC2. Slm1 and Slm2 are phosphorylated by TORC2. A temperature-sensitive mutant analysis determined that inactivation of Slm1 and
Slm2 diminishes polarized cortical actin distribution and incubation of the TS cells at a non-permissive temperature, leading to cell lysis (Audhya et al., 2004; Fadri et al., 2005).

Intriguingly, Akt appears to be one of the TORC2 substrates (Hresko and Mueckler, 2005; Sarbassov et al., 2005b). To be activated, Akt requires three ordered processes: (1) translocation to the plasma membrane through PH domain binding to PI(3,4,5)P3; (2) the phosphorylation of Ser473 in the C-terminal hydrophobic motif, which increases kinase activity 10-fold and is a prerequisite for Thr308 phosphorylation; and (3) PDK1, recruited and activated by binding to PI(3,4,5)P3 via its PH domain, phosphorylates Thr308 in an activation loop, which further induces a 100-fold kinase activation (a total of 1000-fold) (Alessi et al., 1996; Scheid et al., 2002; Vanhaesebroeck and Alessi, 2000).

PDK1 has been implicated as the kinase responsible for Thr308 phosphorylation. Several kinases, such as integrin-linked kinase (ILK), PKC, double-stranded DNA-dependent protein kinase (DNA-PK), and ataxia telangiectasia mutated (ATM) kinase, have been proposed to phosphorylate Ser473 (Dong and Liu, 2005). However, the identity of the kinase responsible for S473 has been controversial. TORC2 appears to be a bona fide molecule that phosphorylates Ser473 in vivo and in vitro (Hresko and Mueckler, 2005; Sarbassov et al., 2005b). RNAi-mediated knockdown of AVO3/Rictor completely blocks Ser473 phosphorylation of Akt, whereas knockdown of Raptor/mKOG enhances the Ser473 phosphorylation, which could be explained by depletion of TORC1 increasing the number of TORC2 complexes.

Studies from Dictyostelium reveal an essential role of TOR complexes in chemotaxis. TORC2 is well-conserved in Dictyostelium cells. Interestingly, a couple of mutants of TORC2 components, named Ras-interacting protein-3 (RIP3, a homolog of AVO1/hSIN1) and Pianissimo (a homolog of Rictor/mAVO3), were identified and reported to be required for cell polarization and chemotaxis (Chen et al., 1997; Lee et al., 1999). Recently, an Lst8/Gbl homolog in Dictyostelium was knocked out (Lee et al., 2005). A null mutation of Lst8/Gbl results in defective cell polarity and severe reduction of chemotactic speed, similar to the RIP3 (AVO1) mutant and the Pianissimo (AVO3/Rictor) mutant. Intriguingly, the phenotypes of TORC2 mutant Dictyostelium cells are highly similar to those of Akt mutants (Meili et al., 1999, 2000). Activation of Akt is severely reduced in the RIP3, Pianissimo, and Lst8 null cells, indicating an evolutionarily conserved role of TORC2 in regulating Akt through phosphorylation at the site equivalent to Ser473 in mammalian Akt. RIP3 was identified as a human H-Ras-binding molecule and shown to bind Dictyostelium RasG-GTP but not RasG-GDP. As seen in AVO1, RIP3 contains a Ras-binding domain. Expression of a RIP3 mutant that harbors a mutation in the Ras-binding domain that abrogates Ras binding does not effectively complement the chemotactic and developmental defects of rip3 null strains, whereas expression of the wild type does (Lee et al., 1999, 2005). These results imply that Ras is integrated into TORC2 regulation via RIP3. Supporting this notion, the mutation of AVO1 suppresses the effects of constitutively active Ras2, showing the coupling between Ras and TORC2 in yeast. The phenotypes of pip3k1/2 and rip3, pianissimo, and lst8 null cells suggest that at least two mechanisms controlled by proteins that bind Ras-GTP contribute to cell polarity through the PI3K and RIP3 pathways. We speculate that Ras regulates chemotaxis through PI3K, TOR, and most likely other effectors. The activation of adenyl cyclase is tightly integrated with chemotaxis during aggregation as production of cAMP relays the chemoattractant cAMP signal during aggregation. Interestingly, mutations in TORC2 components and the RasGEF Aimless are unable to activate adenyl cyclase in response to chemoattractant stimulation (Chen et al., 1997; Lee et al., 1999, 2005). Furthermore, PI3K, a Ras effector, is required for the down-regulation of adenyl cyclase (Comer et al., 2005). Therefore, Ras integrates both the activation and inhibition of adenyl cyclase through distinct pathways, TORC2 and PI3K, respectively. Future analyses may reveal a molecular linkage among signaling pathways and the components of TORC.

Chemotaxis arising from signaling interplay and feedback activation

Several models have been proposed to explain how initial asymmetry in cells is generated or how cells accomplish directional sensing. The various models ultimately need or must utilize signaling interplay and a feedback loop to explain these processes (Iijima et al., 2002; Janetopoulos et al., 2004; Postma and Van Haastert, 2001). We will now focus on the signaling interplay and feedback loop-mediated cell polarization and directional sensing.

A signaling pathway is a cascade of amplification, and the phenomenon of signaling molecule activation is a consequence of positive and negative regulation. Each step has one or more positive and negative regulators. At each step containing enzymes such as GEFs and kinases, signaling is amplified stupendously. Importantly, at each step, almost every enzyme requires several processes to be activated (Fig. 1). For example, translocation takes place via a cognate domain, such as a PH domain or an SH2 domain, and sequential modification such as phosphorylation, which is seen in the VAV activation process, or binding to additional...
molecules such as activated Ras, which is seen in TIAM1. WAVE2 is recruited to the plasma membrane by itself, although it requires activated Rac and phosphorylation. The DOCK/ELMO complex requires two functional PI(3,4,5)P3-binding domains for translocation. Akt is required for PI3K activation and TORC2 activation that may be under the influence of Ras. Dictyostelium PI3K requires membrane translocation via F-actin polymerization and Gβ and Ras interaction. Knockout or disturbance of each molecule causes defective cell motility, and many phenotypes are similar or significantly overlap. We think that the similar phenotypes are due partially if not completely to the disturbance of the signaling interplay, and this is a good indication of the importance of signaling interplay in vivo.

The initial amplification step may lay in GPCR-mediated heterotrimeric G-protein activation. However, this activation does not create a steep gradient, and FRET analysis shows that a small portion of the G-protein is activated (Janetopoulos et al., 2001). Although it has not yet been probed, we assume that single molecules of activated Gz2 or Gβγ might not activate vast numbers of downstream effectors, as a G-protein basically binds to activate an effector by inducing an allosteric change and/or by changing the localization of an effector. Therefore, RasGEF-mediated Ras activation could be the first enzymatic amplification step after GPCR activation, which is strong and directed. Ras activation process must involve GAP activation, as Ras down-regulation also occurs rapidly. Interestingly, single-molecule FRET analysis on Ras activation in living cells reveals that single Ras protein activation lasts only ~1 s (Murakoshi et al., 2004). We suggest that the rapid turnover of activated Ras, presumably applicable to many signaling proteins, is the fundamental property of the cell by which signaling interplay and the feedback system can immediately adapt in response to the change of extracellular cues. A second amplification event is PI3K activation. Once PI3K is activated, it produces PI(3,4,5)P3 expansion as long as Ras and Gβγ are activated. A third important amplification system consists of feedback loops between Ras activation and F-actin polymerization-induced PI3K translocation (Fig. 3), which leads functional activation of PI3K effectors, such as Akt, RhoGEFs, DOCK, and the WAVE complex (Wang et al., 2002; Weiner et al., 2002). If RacGEF translocates to the membrane in an F-actin-dependent manner, this would provide a positive feedback loop to amplify F-actin (Park et al., 2004). We presume that we have yet to discover a large number of feedback loops and interplays that amplify and modulate the signal. These initial amplification and modulating events cause cells to become polarized according to the extracellular environment. In addition, antagonistic mechanisms such as the reciprocal localization of PI3K and PTEN, and reciprocal inhibition from the leading edge to back and lateral sides through Rac/F-actin and Rho/Myosin, play essential roles to establish a stable leading edge and posterior. When Ras activation meets PI3K and TOR is activated, cells start moving forward.

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