

Signaling pathways mediating chemotaxis in the social amoeba, *Dictyostelium discoideum*

Stacey S. Willard, Peter N. Devreotes*

Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205, USA

Abstract

Chemotaxis, or cell migration guided by chemical cues, is critical for a multitude of biological processes in a diverse array of organisms. *Dictyostelium discoideum* amoebae rely on chemotaxis to find food and to survive starvation conditions, and we have taken advantage of this system to study the molecular regulation of this vital cell behavior. Previous work has identified phosphoinositide signaling as one mechanism which may contribute to directional sensing and actin polymerization during chemotaxis; a mechanism which is conserved in mammalian neutrophils. In this review, we will discuss recent data on genes and pathways governing directional sensing and actin polymerization, with a particular emphasis on contributions from our laboratory.

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Introduction

Chemotaxis, or the directed migration of cells in response to external chemical cues, is essential for development and homeostasis. For example, without proper directional migration, tissues do not form properly and embryonic development is severely impaired (Bottcher and Niehrs, 2005). In adult mammals, chemokine-guided migration is critical for wound healing and for the migration of lymphocytes during immune response (Eccles, 2004; Martin and Parkhurst, 2004). Chemotaxis also plays an important role in the development and progression of many diseases including asthma, arthritis, atherosclerosis, and cancers (Charo and Taubman, 2004; Eccles, 2005; Trusolino and Comoglio, 2002). Further insight into the molecular

mechanism of directional sensing and cell migration is crucial for the development of treatments for these disorders, as well as in understanding normal biological processes. We have used the free-living soil amoeba, *Dictyostelium discoideum*, as a model for the regulation of chemotaxis in eukaryotic cells. *D. discoideum* is an ideal model for genetic and biochemical studies: Its genome sequence is available (Eichinger et al., 2005), which facilitates forward and reverse genetic approaches; it exists as an easily transformed haploid, making it accessible for many genetic techniques; and living cells are easily imaged using standard microscopy techniques. Most importantly, amoebae rely on chemotaxis to find food and to survive nutrient-poor conditions.

During development under starvation conditions, *D. discoideum* cells sense and migrate in response to waves of the chemoattractant, cAMP. These waves, generated by aggregation centers, are propagated in a population of amoebae by cAMP release from individual cells

*Corresponding author. Tel.: +1 410 955 3225;
fax: +1 410 614 9461.

E-mail address: pnd@jhmi.edu (P.N. Devreotes).

following sequential chemoattractant stimulations. Polarized migrating cells arrange in an end-to-end formation creating streams that cluster and move up the concentration gradient towards the source of chemoattractant. Chemotaxing cells aggregate to form mounds consisting of thousands of cells. Differentiation and morphogenesis culminate in the formation of a fruiting body, or a stalk of vacuolated cells with a spore head on top.

Chemotaxis consists of three distinct and separable cellular processes: polarization, directional sensing and migration (Fig. 1A). Under starvation conditions, cells become polarized, a process characterized not only by elongation, but also by localization of specific proteins to either pole. Polarity only enhances the ability of a cell to move, however, since polar and non-polar cells are able to migrate randomly. Similarly, neither polarity nor migration are required for a cell to sense direction, since cells that are round and immobile due to pharmacological blockage of actin polymerization are still able to localize intracellular markers reflecting the external chemoattractant gradient (Parent et al., 1998). Our work in recent years has focused on signals required for directional sensing and the role of actin polymerization

in chemotaxis. We have proposed a model based on this work, termed local excitation, global inhibition (LEGI), to describe the dynamic signaling during directional sensing (Fig. 1B) (Parent and Devreotes, 1999).

G-protein signaling regulates directional sensing and migration during chemotaxis

Previous work has shown that chemotaxis to cAMP during development is regulated by an adaptive G-protein-coupled receptor signal transduction cascade (Fig. 2). Despite possible redundant signaling mechanisms downstream of the cAMP receptors (cARs) (please refer to Table 1 for *D. discoideum* gene names and database accession numbers for proteins discussed here) and hetero-trimeric G-proteins (discussed below), null phenotypes in knockout strains indicate that the cARs and the α_2 and β G-protein subunits are critical and their functions non-redundant during chemotaxis. Strains deleted in *cARI* and the G-protein α_2 -encoding gene display a complete inability to aggregate and do not respond to cAMP stimulation; However, they retain

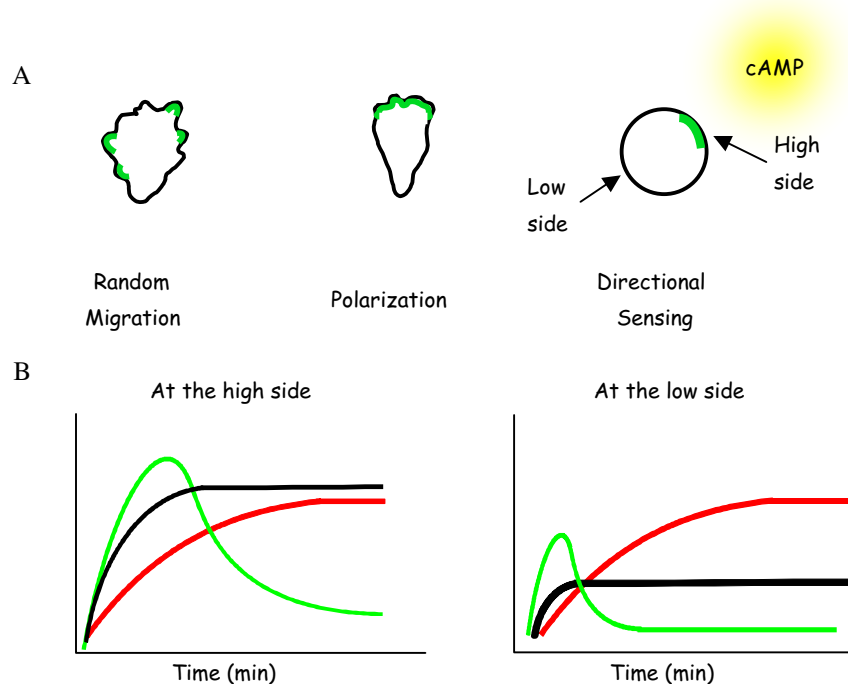


Fig. 1. Cellular responses and signaling during chemotaxis. (A) This panel illustrates from left to right three typical cellular responses during which PI(3,4,5)P₃ (green) marks specific areas of the plasma membrane. First, during random migration, which occurs regardless of nutritional status, PI(3,4,5)P₃ is a marker for pseudopod formation. Second, starved amoebae exhibit polarity, and during chemotaxis towards cAMP, PI(3,4,5)P₃ is a marker for the high side of the cell, or the side experiencing the highest concentration of cAMP. Third, in round immobile cells exposed to a gradient of chemoattractant, PI(3,4,5)P₃ labels the high side of the cell. (B) The LEGI model at the high and low sides of the cell. In these graphs, excitation is depicted in black, response in green and inhibition in red. According to this model, a robust response occurs when excitation is greater than inhibition (at the high side of the cell), while when inhibition is greater than excitation (at the low side), responses are minimal.

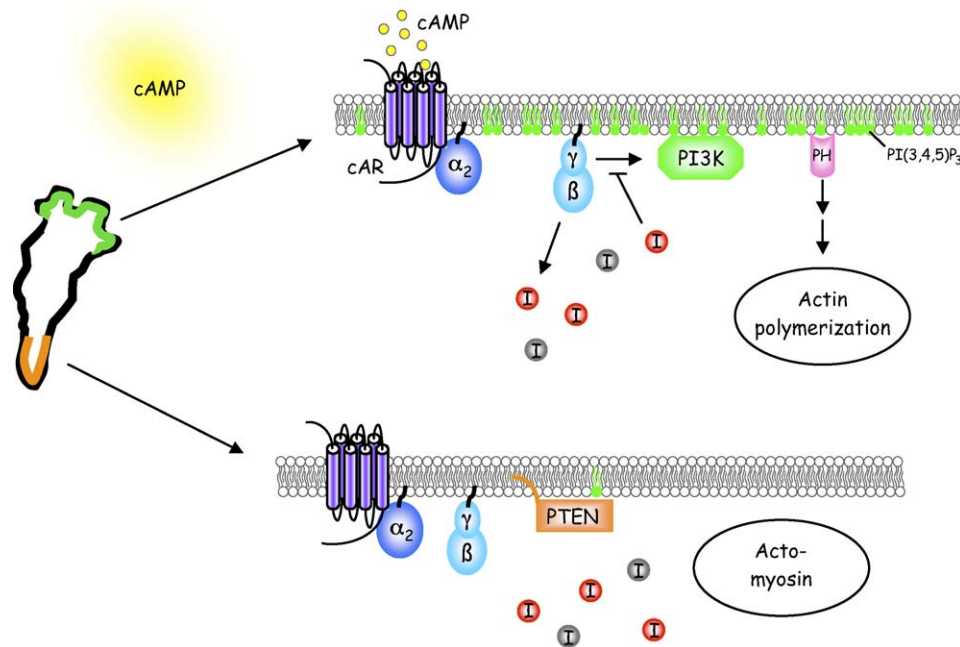


Fig. 2. Major signaling events at the high and low sides of the cell during chemotaxis up a concentration gradient. Red and gray “I” circles depict proposed diffusible inhibitors. Abbreviations and a full explanation of signaling components are found in the text.

Table 1. Signaling proteins involved in chemotaxis regulation in *D. discoideum*

Protein	Gene	Dictybase ID
PI3K-1	<i>pikA</i>	DDB0214949
PI3K-2	<i>pikB</i>	DDB0191474
PTEN	<i>ptenA</i>	DDB0191093
cAR1	<i>carA</i>	DDB0185024
G protein α_2	<i>gpaB</i>	DDB0191327
G protein β	<i>gpbA</i>	DDB0185046
G protein γ	<i>gpgA</i>	DDB0185201
CRAC	<i>dagA</i>	DDB0191434
ACA	<i>acaA</i>	DDB0214814
Pianissimo	<i>piaA</i>	DDB0185055
Tsunami	<i>tsuA</i>	DDB0229382
Tortoise	<i>torA</i>	DDB0185067
ERK2	<i>mekA</i>	DDB0191164
YakA	<i>dagB</i>	DDB0191191
Protein kinase B-A	<i>pkbA</i>	DDB0191195
Protein kinase B-R1	<i>pkgB</i>	DDB0191365
Ras-interacting protein 3	<i>ripA</i>	DDB0201626

the ability to sense the chemoattractant folic acid (Kim et al., 1998; Kumagai et al., 1989; Wu et al., 1995). Similarly, disruption of G-protein β - and γ -encoding genes results in no aggregation and a failure to sense chemoattractants, although the cells retain random motility, reinforcing the idea that directional sensing can be uncoupled from general cell motility (Wu et al., 1995; Zhang et al., 2001). An analysis of the

localization and dissociation rates of these proteins in vivo has revealed that membrane polarity during chemotaxis is established downstream of G-proteins. cAR1 is uniformly expressed on the plasma membrane (Xiao et al., 1997), as are the G-protein subunits α_2 , β and γ (Janetopoulos et al., 2001; Jin et al., 2000), while receptor occupancy mirrors the external concentration of cAMP in a gradient (Janetopoulos et al., 2001).

The activation of the G-protein and the dissociation of $\beta\gamma$ triggers a number of transient responses (reviewed in detail in Manahan et al., 2004) which possess the ability to reversibly adapt to a persistent stimulus: Ca^{2+} influx (Caterina et al., 1994), adenylyl and guanylyl cyclase (ACA and GCA, respectively) activation, subsequent cAMP and cGMP production and release (Kuwayama et al., 1993; Pitt et al., 1992), the recruitment and activation of the phosphoinositide 3-kinase (PI3K) (Huang et al., 2003), myosin heavy and light chain phosphorylation (de la Roche and Cote, 2001; Rubin and Ravid, 2002), actin polymerization (Chen et al., 2003) and actomyosin bundle formation.

Sensing direction: spatial and temporal regulation of phosphoinositides

In a gradient of chemoattractant, cells must translate cAMP levels outside the cell into directional information

inside the cell. A number of proteins are spatially restricted to either the high (the side of the cell experiencing the highest concentration of chemoattractant) or low side of the cell in a gradient. Myosin II and acto-myosin bundles are found at the low side of the cell (Laevsky and Knecht, 2003) and are thought to participate in contraction, while the actin-binding protein, coronin, is one of many proteins targeted to the high side of the cell (Chen et al., 2003; Fukui et al., 1997). Additionally, intracellular phosphoinositol (3,4,5)-trisphosphate [PI(3,4,5)P₃] production at the high side serves as a robust marker for directional sensing and the region of actin polymerization during chemotaxis (Figs. 1A and 2) (Chen et al., 2003; Parent et al., 1998). Spatial restriction of phosphoinositide production is accomplished by reciprocal temporal and spatial regulation of PI3K (Funamoto et al., 2002) and the 3-phosphoinositide phosphatase, PTEN. In resting cells, PTEN is localized uniformly at the plasma membrane where it prevents PI(3,4,5)P₃ accumulation by dephosphorylating PI(3,4,5)P₃ to produce phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P₂]. In a gradient, PTEN is restricted from the plasma membrane at the high side, but remains at the low side (Iijima and Devreotes, 2002). In contrast and simultaneously, PI3K is recruited from the cytosol to the membrane at the high side and activated via a mechanism involving the Ras protein (Huang et al., 2003; Sasaki et al., 2004). As a result, PI(3,4,5)P₃ levels rise and pleckstrin homology (PH) domain-containing proteins such as cytosolic regulator of adenylyl cyclase (CRAC) are recruited (Parent et al., 1998). The 120-amino-acid PH domain binds specifically to the head group of 3,4,5-phosphorylated phosphoinositides. The fluorescently tagged CRAC PH domain is routinely used to monitor PI(3,4,5)P₃ dynamics and therefore directional sensing in vivo, allowing a unique look at how cells adapt to changing cAMP levels in their environment (reviewed in Devreotes and Janetopoulos, 2003; Iijima et al., 2002; Van Haastert and Devreotes, 2004). In fact, PI(3,4,5)P₃ is a marker for many actin-driven events, such as pseudopod formation during migration and membrane invagination during phagocytosis (reviewed in Devreotes and Janetopoulos, 2003).

The regulation of the membrane translocation of PI3K and PTEN occurs by an unknown mechanism; however, progress has been made in elucidating the protein regions that are necessary for targeting. The N-terminus of PI3K is necessary and sufficient for membrane targeting, since removal of the 600 amino terminal residues results in an inability of the remaining C-terminal portion to translocate to the membrane upon cAMP stimulation. In addition, the N-terminal 600 residues alone are sufficient to target heterologous proteins to the plasma membrane with a normal temporal and spatial profile (Huang et al., 2003). This

region, although critical in three *D. discoideum* PI3Ks (PI3K-1, -2 and -3), does not contain any recognizable motifs or consensus sequences. Sufficient targeting information for PTEN has been more elusive. Removal of the PI(4,5)P₂-binding motif of PTEN abolishes its membrane localization but this domain is not enough to target fusion proteins to the membrane (Iijima et al., 2004). Single molecule studies of *D. discoideum* and human PTEN have indicated that the protein associates and dissociates on a sub-second timescale from the plasma membrane in vivo (Vazquez et al., 2006). This dynamic membrane interaction, coupled with spatial restriction, may allow PTEN to maintain low levels of PI(3,4,5)P₃ during resting state while also permitting PI(3,4,5)P₃ levels to rise quickly after stimulation. For example, perhaps the fast dissociation rate of PTEN allows for binding sites to change rapidly under changing chemoattractant environments. Consequently, the localization of PTEN can be rapidly changed, allowing PI(3,4,5)P₃ to accumulate at new sites in the plasma membrane.

Disruption of genes encoding phosphoinositide signaling components have reinforced the model that properly regulated PI(3,4,5)P₃ production plays a role in directional sensing. The removal of PTEN function causes an inability of cells to degrade PI(3,4,5)P₃, resulting in defects during chemotaxis and failure of aggregation under starvation conditions (Iijima and Devreotes, 2002). Uniform cAMP stimulation of *pten*⁻ cells results in the uniform translocation of PH_{CRAC}-GFP to the plasma membrane at 5 s post-stimulus, but unlike normal cells in which PI(3,4,5)P₃ levels return to baseline within 60 s, elevated PI(3,4,5)P₃ levels persist for over 3 min. In a gradient, *pten*⁻ cells create broad PI(3,4,5)P₃-labeled projections, and although the cells do move toward the source of cAMP, they do so at a severely reduced speed and efficiency (Iijima and Devreotes, 2002). Disruption of PI3K function, however, indicates that PI(3,4,5)P₃ signaling may be one mechanism of multiple redundant signaling mechanisms governing directional sensing. The combined removal of PI3K-1 and PI3K-2 function from cells impairs chemotactic efficiency and speed, albeit to a significantly lesser extent than removal of PTEN (Funamoto et al., 2001). Even treatment of *pi3k*¹⁻²⁻ cells with the PI3K inhibitor, LY294002, does not significantly abrogate chemotaxis. Our studies indicate that only 5% of the normal level of PI(3,4,5)P₃ is produced in these cells, and no PH_{CRAC}-GFP is detected at the plasma membrane in a gradient. Since the removal of 95% of PI(3,4,5)P₃ production is not sufficient to appreciably disrupt chemotaxis, we propose that while PI(3,4,5)P₃ production may be required for maximally efficient directional sensing, redundant pathways may contribute to this regulation. New data from our lab suggest that the phospholipase, PLA₂A, may participate in PI(3,4,5)P₃-independent

directional sensing and migration (L. Chen, unpublished observation).

Adaptation to cAMP and the LEGI model

A number of features of cAR-mediated phosphoinositide signaling have indicated that directional sensing is highly adaptive. During short timeframes, multiple stimulations with the same cAMP dose do not elicit further reactions after the initial transient response, but even a slight increase in cAMP triggers a PI(3,4,5)P₃-mediated response. Therefore, cells respond only to changes in cAMP concentration. This adaptation mechanism allows cells to ignore ambient cAMP and only respond to gradients, either locally or long-range. To reset the signaling system after a response, a proposed diffusible inhibitor of cAMP signaling which is also activated by receptor occupancy acts globally within the cell. Our data suggest that this inhibitor acts downstream of G-proteins, since fluorescence resonance energy transfer (FRET) analysis using fluorescently tagged α_2 and β G-protein subunits revealed that the subunits remain dissociated as long as cAMP is present (Janetopoulos et al., 2001). The identity of this inhibitor(s) is still under investigation.

The LEGI model describes the ability of a cell to sense direction and adapt to stimuli, and depends on a balance between excitation and inhibition (Fig. 1B) (Iglesias and Levchenko, 2002; Kutscher et al., 2004). According to this model, receptor occupancy regulates rapid excitation and a slower accumulation of a global inhibitor. Therefore, at the high side of the cell in a gradient, receptor occupancy is high, excitation levels are higher than inhibitor and a persistent response occurs. At the low side of the cell, the accumulation of the diffusible inhibitor coupled with lower excitation levels prevents a response from occurring. In this way, PI(3,4,5)P₃-mediated responses occur at the high side of the cell and not at the low side, allowing a cell to sense direction in a gradient of chemoattractant. In fact, because the dependence of PI3K and PTEN membrane translocations on the gradient are different, two LEGI modules acting in parallel are required to adequately model phosphoinositide-mediated directional sensing (Ma et al., 2004). According to this dual LEGI model, receptor occupancy regulates the creation and destruction of both PI3K- and PTEN-binding sites at the plasma membrane. One LEGI unit controls the transient production of PI3K-binding sites and the other the transient loss of PTEN-binding sites, with PI(3,4,5)P₃ production as the output or response. In a gradient, ligand binding results in the creation of PI3K-binding sites, and destruction of PTEN-binding sites at the high side of the cell. Since inhibition is greater than excitation at the low side of the

cell, PTEN-binding sites remain intact and PI3K-binding sites are not created.

Additional signals required for chemotaxis

Stimulation of cARs at the high side of the cell triggers multiple signaling cascades (listed above and in Table 1) such as ACA activation and subsequent cAMP production. Upon activation, ACA is spatially restricted to the back of the cell, where cAMP is produced and a small amount is secreted to maintain the signal relay loop required for streaming during aggregation. Proper regulation of ACA requires PI3K and downstream CRAC recruitment to the plasma membrane: amoebae deficient for ACA or CRAC are unable to propagate waves of cAMP and therefore do not aggregate in starvation conditions (Kriebel et al., 2003; Lilly and Devreotes, 1995). Similarly, *pi3k*¹⁻² cells do not recruit CRAC and do not properly activate ACA (Comer et al., 2005; Comer and Parent, 2006). In addition to PI3K and CRAC, proper activation of ACA requires the pianissimo (Pia) protein. In *pia*⁻ cells, cAMP production is completely absent, but expression of cAR1, ACA and G-proteins is relatively normal. Additionally, *pia*-deficient cells display impaired aggregation, which may result in part from an inability to properly regulate cAMP production (Chen et al., 1997). Pia is a known component of the TOR2 complex in many organisms (reviewed in Dann and Thomas, 2006), and this complex has been shown to act downstream of PI3K for activation of ACA in *D. discoideum*.

We and others have characterized a number of additional genes that participate in the regulation of chemotaxis. For example, mutations in *tortoise* (*torA*), *tsunami* (*tsuA*) and the gene encoding mitogen-activated protein kinase ERK2 (*mekA*) display mutant phenotypes that are remarkably similar to one another. Knockout strains of all three of these genes exhibit impaired ability to polarize and therefore respond very slowly to cAMP (Manahan et al., 2004; Segall et al., 1995; van Es et al., 2001). Under starvation conditions on non-nutrient media, this phenotype manifests as a longer period of cAMP wave propagation followed by the formation of small fruiting bodies. In *torA*⁻, *tsuA*⁻ and *mekA*⁻ cells, cAR1 expression levels are normal and cAMP is produced at normal levels following stimulation, indicating that these cells are competent to respond to cAMP and to propagate cAMP waves (Manahan et al., 2004; Segall et al., 1995; van Es et al., 2001). TorA expression is detected in mitochondria, and this may indicate that it regulates mitochondrial function during chemotaxis (van Es et al., 2001). ERK2 appears to function in a cAR1- and G-protein-independent mechanism in order to activate cAMP phosphodiesterases, a regulatory step which may act to reset cAMP levels.

The motor: actin dynamics in chemotaxing cells

In a gradient, actin polymerization occurs at the high side of the cell downstream of receptor binding (Chen et al., 2003). Proper regulation of actin polymerization in *D. discoideum* involves a concert of proteins including Rho family GTPases and Wasp activation of Arp2/3 (reviewed in Franca-Koh and Devreotes, 2004). We have observed two phases or peaks of actin polymerization after chemoattractant stimulation, each displaying different temporal dynamics, morphological consequences and magnitude. Similar to the PI(3,4,5)P₃ response after cAMP stimulation, the first phase of polymerization occurs rapidly, i.e. within 5 s and comprises approximately a two-fold increase in total F-actin in the cell. Concomitant with this first peak, cells round up and are immobile. The second peak (a modest but reproducible 1.25-fold increase) crests approximately 2 min after stimulation and is temporally associated with pseudopod extension (Chen et al., 2003). Actin dynamics and chemotaxis ability in PI3K and PTEN mutants has refined our understanding of the importance of these separate actin peaks. For instance, *pi3k*¹⁻²⁻ double mutants display a relatively normal first peak, but no second peak, indicating that PI3K activity is crucial for the second peak, but may be redundant for the first peak. Since *pi3k*¹⁻²⁻ cells can clearly respond directionally to chemoattractant, albeit at a slightly impaired efficiency, the second actin polymerization peak is not sufficient for directional sensing (Chen et al., 2003). Removal of PTEN function results in an elevated basal level of F-actin and upon stimulation, the second peak is sustained longer than in wild-type cells, supporting the model that phosphoinositides are required for the second peak (Iijima and Devreotes, 2002). Removing PI3K-1, PI3K-2 and PTEN results in a similar actin profile as the double PI3K mutant, however chemotaxis is much more impaired in these cells than the double PI3K mutant alone (Chen et al., 2003). The results from these mutant strains indicate that although redundant mechanisms may regulate the first phase of actin polymerization, the second peak appears to require regulated PI(3,4,5)P₃ signaling. Moreover, the second peak may be responsible for refining and improving chemotactic efficiency since abrogating it results in inefficient chemotaxis.

Membrane polarity created by phosphoinositides is also critical for cytokinesis

We have recently shown that, as in chemotaxis, PI3K and PTEN are spatially and temporally regulated during cytokinesis (Janetopoulos et al., 2005). In migrating cells, PH_{CRAC}-GFP is located in membrane ruffles, but

when cells round up to begin cytokinesis, PH_{CRAC}-GFP remains cytosolic. As the spindle elongates, PI(3,4,5)P₃ is once again detected at the plasma membrane and is produced specifically in membrane ruffles at the poles throughout the remaining stages of cytokinesis. PI3K and PTEN membrane dynamics follow a predicted pattern that accounts for PI(3,4,5)P₃ localization. PTEN is found uniformly at the membrane when cells round up, and later at the furrow and cytoplasmic bridge, whereas PI3K is exclusively at the poles in actin-rich membrane projections. The stimulus which initiates this polarized phosphoinositide response is unknown, and is clearly not cAR1-dependent, because the receptor is not expressed in vegetative dividing cells. Disruption of this membrane polarity results in impaired cytokinesis, as demonstrated by cells of a triple mutant for PI3K-1, PI3K-2 and PTEN (*pi3k*¹⁻²⁻ *pten*⁻). These triple knock-out cells are completely unable to divide without a substrate to adhere to (in shaking liquid culture), and even when provided with a plastic substrate, large multinucleate cells are numerous, indicating an inability to complete cytokinesis. PH_{CRAC}-GFP localization in *pi3k*¹⁻²⁻ *pten*⁻ cells shows that PI(3,4,5)P₃ is inappropriately produced throughout the membrane in cells which attempt to divide (Janetopoulos et al., 2005).

Similar to chemotaxing cells, in dividing cells, PI(3,4,5)P₃-rich membrane domains are associated with F-actin and actin-binding proteins such as coronin, while PTEN-rich domains are correlated with concentrated myosin II and acto-myosin (Janetopoulos et al., 2005). It is possible that phosphoinositide signaling allows the dividing cell to restrict actin polymerization from the furrow to allow for myosin-based contractions. This model is substantiated in *pi3k*¹⁻²⁻ *pten*⁻ cells. In addition to their inability to complete cytokinesis in many cases, these cells are unable to maintain myosin-II expression at the ingressing furrow (Janetopoulos et al., 2005), an effect which may be due to the presence of PI(3,4,5)P₃ at the furrow.

Phosphoinositide control of membrane polarity during chemotaxis is conserved in mammalian neutrophils

In mammals, neutrophils are key defenders against bacterial infection. These immune cells chemotax in response to chemokines released by invading bacteria and phagocytose the offending cells. Similar to *D. discoideum* cells, phosphoinositide signaling is crucial for directional sensing and actin polymerization in this system (reviewed in Hannigan et al., 2004). The G-protein-coupled formyl peptide (fMLP) receptor interacts with heterotrimeric G-proteins in a similar manner as cAR1, i.e., ligand binding induces the dissociation of

α and $\beta\gamma$ subunits at a greater frequency at the high side of the cell. The $\beta\gamma$ subunits lead to activation of PI3K and PI(3,4,5)P₃ production occurs up the concentration gradient. In agreement with this, treatment of neutrophils with PI3K inhibitors such as wortmannin or LY294002 impairs chemotaxis and polarization. The downstream signaling effectors that are directly responsible for actin polymerization in these cells are an active topic of research. However, neutrophils are not easily transfected or microinjected, and it is possible that simple models like *D. discoideum* where genetic tools are abundant will allow us to further understand this signaling mechanism.

Conclusions

Since chemotaxis in starvation conditions is of utmost importance to the survival of the organism, it would be favorable to create layers of redundant regulation to ensure that aggregation and spore formation occurs. Although previous models postulated that directional migration requires properly regulated phosphoinositide signaling, recent data may indicate the existence of redundant regulatory mechanisms that are also able to drive directional migration. Future work will continue to investigate alternative pathway(s) which are necessary for directional sensing and migration, as well as to further characterize the role of phosphoinositide signaling. *D. discoideum* represents an established simple model in which to expand our understanding of the regulation of chemotaxis.

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