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Temporal and Spatial Regulation of Chemotaxis

Review

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The ability to sense and respond to shallow gradients of extracellular signals is remarkably similar in *Dictyostelium discoideum* amoebae and mammalian leukocytes. Chemoattractant receptors and G proteins are fairly evenly distributed along the cell surface. Receptor occupancy generates local excitatory and global inhibitory processes that balance to control the chemotactic response. Uniform stimuli transiently recruit PI3Ks to, and release PTEN from, the plasma membrane, while gradients of chemoattractant cause the two enzymes to bind to the membrane at the front and back of the cell, respectively. Interference with PI3Ks alters chemotaxis, and disruption of PTEN broadens PI localization and actin polymerization in parallel. Thus, counteracting signals from the upstream elements of the pathway converge to regulate the key enzymes of PI metabolism, localize these lipids, and direct pseudopod formation.

Directional Sensing Plays a Key Role in Cell Migration

Cells have a fascinating ability to detect shallow gradients of extracellular molecules and to link that sensing to changes in cell morphology and motility. These capabilities are central to cell polarization and chemotaxis, the guided movement of cells toward attractants or away from repellents. These fundamental cellular responses play a major role in innate immunity and in many aspects of development and tissue maintenance (Jones, 2000; Carlos, 2001; Condeelis et al., 2001; Fernandis and Ganju, 2001; Moore, 2001; Murphy, 2001; Patel and Haynes, 2001; Thelen, 2001; Worthley et al., 2001; Biber et al., 2002; Gangur et al., 2002; Lalor et al., 2002; Rubel and Cramer, 2002). In development, the interplay between adhesion, polarization, and chemotaxis brings shape and organization to the embryo and guides axons in the formation of the nervous system. In adults, these processes play a role in tissue remodeling events, such as wound healing and metastasis. From studies of model chemotactic systems, investigators are elucidating the general mechanisms of gradient detection.

This review focuses on gradient sensing in the social amoebae *Dictyostelium discoideum*, with comparison to mammalian leukocytes. *D. discoideum* has emerged as a powerful model system for the investigation of chemotactic signaling and motility (Parent and Devreotes, 1996; Parent and Devreotes, 1999; van Es and Devreotes, 1999; Chung et al., 2001). As shown in Figure 1, these cells can be attracted over sizeable distances by slight gradients of 3′, 5′-cyclic adenosine monophosphate (cAMP). Chemotaxis to cAMP is part of a program of differentiation where free-living amoebae aggregate to form a multicellular organism. During aggregation the cells orient and migrate directionally toward self-organizing gradients of extracellular cAMP. Studies of these events in *D. discoideum* have led to the identification and localization of key molecules in chemotactic signaling pathways and to the basic mechanisms involved in chemotaxis. For instance, the discovery of a family of receptors, designated cAR1–cAR4, for the chemoattractant cAMP provided the first evidence that chemotactic signaling occurs through seven helix receptors linked to heterotrimeric G proteins. The cARs couple to a specific G protein consisting of $\alpha 2$, one of eleven α subunits, and a unique $\beta\gamma$ complex. A similar system operates in mammalian leukocytes, where twenty chemokine receptors couple principally to the inhibitory G protein, G_i (Murphy, 1994; Klinker et al., 1996; Maghazachi, 2000; Rickert et al., 2000). Additional elements of the pathway are also conserved: exposure of amoebae or leukocytes to chemoattractants results in increases in multiple second messengers, including PIs (phosphoinositides), cAMP, cGMP, IP_3, and Ca^{2+}, and subsequent rearrangements in the cytoskeleton.

Chemotaxis Involves Motility, Polarity, and "Directional Sensing"

The directed movement of cells in response to chemoattractants involves several complex, interrelated processes, including motility, polarity, and directional sensing (Figure 2). A great deal of evidence suggests that the regulated polymerization of actin provides the major driving force required for cell motility (Schmidt and Hall, 1998; Borisy and Svitkina, 2000; Pollard, 2000; Higgs and Pollard, 2001). Migrating cells extend the F-actin-filled filopodia and lamellipodia at the leading edge, which adhere to the substrate and move the cell forward. In amoebae, the extensions occur rhythmically, with a period of about 30 s. Amoebae and neutrophils move at about 20 μm/min. The efficiency and speed of migration depends on the optimal coordination of actin cytoskeletal rearrangements. When the activity of the cytoskeleton is too low, cells cannot generate lamellipodia effectively and cannot generate the force to move. On the other hand, motility is also impaired when the activity of the cytoskeleton is too high to be restricted to a single region of the cell. These activities become highly organized during chemotactic migration.

Polarization refers to the ability of a migrating cell to maintain a stable asymmetric shape with a defined anterior and posterior (Figure 2). The dynamic localization of the cytoskeleton within the lamellipodia of migrating cells contributes to polarization. Actin and actin binding proteins, such as a talin homolog, filopodin, cofilin, and coronin, are localized on the leading edge (Gerisch et al., 1993; Aizawa et al., 1995; Mishima and Nishida, 1999). Other molecules, such as conventional myosin, accumulate at the trailing edge (Clow and

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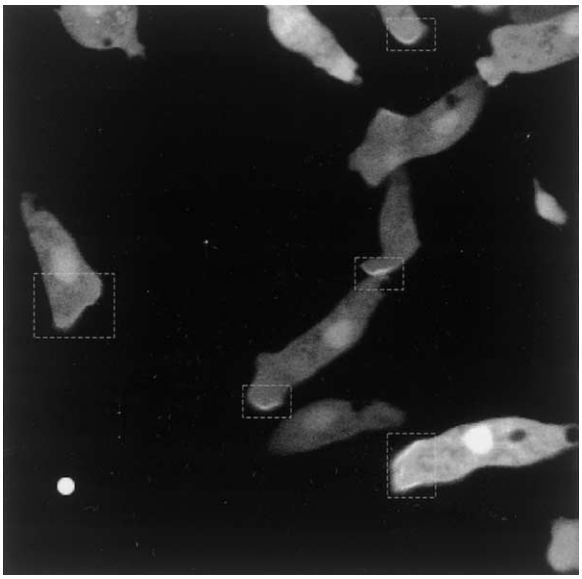

Figure 1. Confocal Microscopic Image of Chemotaxing Cells Expressing PH_{Crac}-GFP

D. discoideum amoebae were exposed to a shallow gradient formed by a micropipette filled with 1 μM cAMP. The external cAMP concentration was determined to vary about 20% along the length of the 15 μm cells. Within 5 min, cells polarized and moved toward a tip of the micropipette (white spot). The PH_{Crac}-GFP is localized sharply at the leading edges of the chemotaxing cells (boxes).

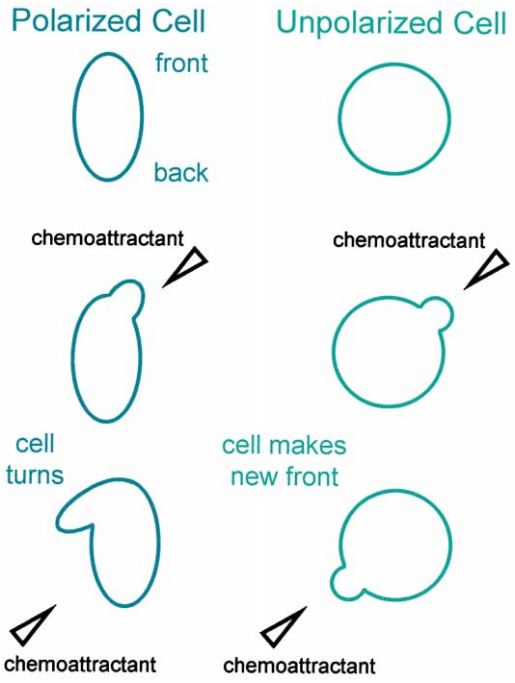

Figure 2. Polarization versus Directional Sensing

As shown at left, polarization refers not only to the asymmetric cellular shape, but also to an asymmetric sensitivity toward chemotactic signals. Polarized cells are more sensitive at the anterior than at the posterior. When the source of the gradient is moved, the cell turns and the original front and back are maintained. Even when the gradient is replaced by a uniform concentration, a highly polarized cell maintains its axis, and signaling molecules remain at their original localized distribution. As shown at right, unpolarized cells are able to sense direction; all points around the perimeter of the cell are equally sensitive. When the source of the gradient is moved, the cell makes a new front, and the former front becomes the back. The axis rapidly disappears when the gradient is removed.

McNally, 1999; Weber et al., 2000). In polarized amoebae and leukocytes, the anterior of the cell is more sensitive to chemoattractants than are other regions. When the direction of the chemoattractant gradient is changed, polarized cells turn toward the high concentration. The polarity is only relatively stable; a sufficiently steep gradient in an opposing orientation, within a minute or so, can override the existing asymmetry and generate a new axis in the new direction. An advantage of the localized sensitivity afforded by polarization is that it focuses the activity of the actin cytoskeleton, resulting in faster movement toward a source. However, it also reduces the portion of the cell participating in sensing to a small zone at the front. In contrast, unpolarized cells maintain equal sensitivity around their perimeters, and the region involved in gradient detection is larger. Each switch in the direction of the gradient quickly extinguishes existing pseudopodia and elicits fresh projections in the new direction. In general, neutrophils are immobile until they are exposed to chemoattractant, whereupon they begin to move about, maintaining a distinct leading edge and uropod (Zigmond, 1978; Devreotes and Zigmond, 1988; Niggli, 2000; Weiner et al., 2002). *D. discoideum* amoebae move without exogenous chemoattractant but become increasingly elongated and motile as they differentiate in response to repeated stimulation (Gerisch, 1987). The extent of polarization of either cell type can also be enhanced by a period of directed movement in a gradient. Importantly, in both amoebae and neutrophils, inhibitors of actin polymerization can eliminate polarized morphology and render cells equally sensitive around their perimeters, suggesting that an interaction of signaling molecules with the cytoskeleton stabilizes the polarized state (Parent et al., 1998; Servant et al., 2000).

Directional sensing refers to the ability of a cell, whether polarized or not, to detect an extracellular gradient, accumulate signaling molecules, and initiate downstream responses asymmetrically. When a chemotactically sensitive cell is exposed to a shallow gradient, slight differences in receptor occupancy lead, at some point within the signaling pathway, to sharply localized responses toward or away from the higher concentration. This localized activation signifies directional sensing and, as described below, it can be visualized with a variety of signaling proteins fused to GFP (green fluorescent protein) as markers (Parent et al., 1998; Meile et al., 1999; Servant et al., 2000; Funamoto et al., 2001). For example, in chemotaxing cells, PH (pleckstrin homology) domain-containing proteins specifically localize at the cell's leading edge, and visualization of these proteins can replace cell movement as the indicator of gradient sensing (Figure 1). These breakthroughs have allowed the temporal and spatial sensing mechanisms involved in chemotaxis to be studied free from the confounding effects of cell polarization and motility.

Directional Sensing Requires Adaptation to Chemoattractant

Consideration of the features of a chemotactic response, such as that illustrated in Figure 1, presents

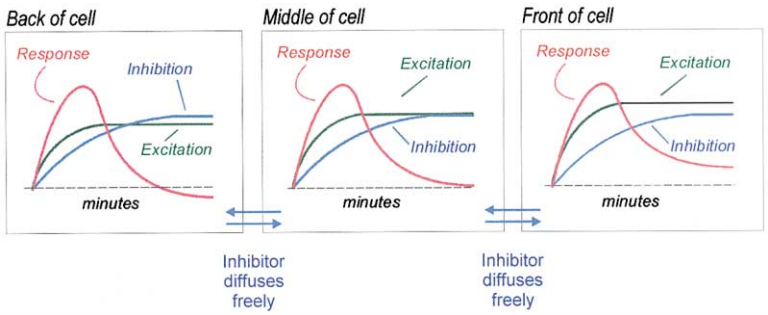

Figure 3. Excitation-Inhibition Model for Temporal and Spatial Sensing

A change in receptor occupancy triggers changes in the steady-state levels of excitation (green) and inhibition (blue). The transient response (red) is proportional to the difference between excitation and inhibition. Excitation rapidly rises, while inhibition increases more slowly, such that the response peaks and then eventually subsides when the value of inhibition reaches that of the excitation. To account for directional sensing by this same response system, we propose that excitation is local and that inhibition is global. In the model, inhibition is global because the inhibitor diffuses freely throughout the cell. At steady state, inhibition reflects the average level of receptor occupancy and is uniform along the cell. The diagram illustrates a "thought experiment" in which a cell is placed into a stable gradient of chemoattractant. When the cell is initially exposed to the gradient, there are responses at the both ends of the cell that peak and reach a steady state. Thereafter, at the front of the cell, excitation exceeds inhibition, while, at the back, excitation is less than inhibition. Thus, the response remains persistently above the baseline (dashed line) at the front of the cell and falls below it at the back of the cell.

several intriguing and unique challenges (Devreotes and Zigmond, 1988; Caterina and Devreotes, 1991; Parent and Devreotes, 1999). First, the shallow external gradients of chemoattractant must generate sharply localized internal biochemical responses, which cause pseudopod formation only at the leading edges of the cells. Second, since cells at different points in the gradient experience different average chemoattractant concentrations yet sense equally well, there must be a powerful mechanism for background subtraction or adaptation. To elucidate how the cells read small differences in receptor occupancy along their lengths, we need an appreciation of how biochemical responses triggered by temporal stimuli relate to localized responses to spatial gradients. Studies in *D. discoideum* and mammalian neutrophils indicate that cells respond to changes in chemoattractant receptor occupancy and adapt when occupancy is held constant. For example, if occupancy is increased from 0% to 50%, levels of second messengers rise rapidly but then subside within a few minutes. If occupancy is then increased further, from 50% to 100%, another set of transient responses ensues. The magnitudes of the responses are generally proportional to the changes in receptor occupancy. When occupancy is reduced, the cells recover sensitivity, adjusting to the lowered level.

These features of adaptation can be modeled in terms of a rapid "excitation" and a slower "inhibition" process, as shown in Figure 3. The difference between these two processes leads to the response. While their kinetics differs, the final steady-state levels of excitation and inhibition are each linked to receptor occupancy. When occupancy is suddenly increased, excitation rises rapidly and, for a time, exceeds inhibition. As a result, the response rises. As the slower inhibitory process then approaches the new steady-state level and reestablishes the balance, the response declines toward the prestimulus level. The adapted cells can respond further if receptor occupancy is increased again. This model conveniently predicts the observed responses of cells to temporal stimuli outlined above. As illustrated in Figure 3, the two-process concept can also account for the sensing of spatial gradients if excitation is "local" while inhibition is "global". That is, if inhibition depends on average receptor occupancy, it will be less than excitation at the front of the cell and exceed excitation at the back of the cell. At steady state in a spatial gradient, these differences would lead to a persistent directional response. In this scheme, the time profile of the response to a stimulus increment is related to its spatial distribution in a gradient: responses that transiently increase in response to an increment, like those in Figure 3, are localized toward the high side of the gradient. Similarly, responses that transiently decrease in response to an increment are localized away from the high side of the gradient. In either case, briefer responses are more sharply localized, while responses that do not subside are not localized and mirror the external gradient. Quantitative, mathematical representations of an extension of this model successfully predict the kinetics and distribution of the observed responses to temporal and spatial stimuli, although the predicted output is less amplified than it is in real cells (Levchenko and Iglesias, 2002; see http://www.ece.jhu.edu/~pi/applets/cell/model2.html). Other recent models for directional sensing share some of these concepts, such as the need for a global inhibitor. Each has specific virtues and difficulties predicting key aspects of the response (Meinhardt, 1999; Postma and Van Haastert, 2001; Narang et al., 2001; Rappel et al., 2002).

The Response Becomes Localized at a Discrete Step in the Signaling Pathway

An extensive series of studies in the last several years have indicated that the upstream components and biochemical reactions in the signaling pathway are uniformly localized in an unpolarized cell exposed to a chemoattractant gradient, as shown in Figure 4. First, studies in *D. discoideum* and mammalian leukocytes have shown that the chemoattractant receptors are distributed uniformly on the cell perimeter (Xiao et al., 1997; Servant et al., 1999). Second, in *D. discoideum*, direct visualization of the occupancy of surface receptors by Cy3-cAMP by single-molecule imaging has shown that it reflects the shallow external gradient of chemoattractant (Ueda et al., 2001). Third, G protein $\alpha 2$ and $\beta\gamma$ subunits are distributed uniformly in unpolarized *D. discoideum* cells (Jin et al., 2000; C. Janetopoulos and N. Zhang, personal communication). Fourth, while G protein activation has not been directly imaged, monitoring of its kinetics in living cells during chemoattractant stimulation suggests that it mirrors receptor occupancy (Janetopoulos et al., 2001). Like in the excitation process

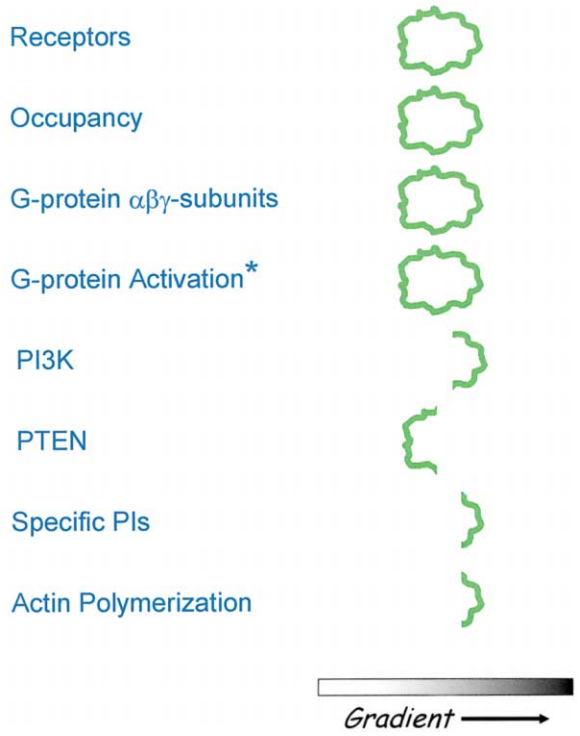

Figure 4. Subcellular Localizations of Signaling Molecules in Directional Sensing

The diagram shows the distribution of chemoattractant receptors, receptor occupancy, G protein αβγ subunits, G protein activation, PI3Ks, PTEN, PH domains representing specific PIs, and polymerized F-actin in *D. discoideum* cells exposed to a gradient of chemoattractant. Information on receptor, G protein subunits, PI3K, PTEN, and PH domain localization come from studies of GFP fusion proteins. Receptor occupancy was determined by single-molecule imaging of Cy3-cAMP. The profile of G protein activation is inferred from its kinetics, not directly imaged (*). F-actin localization was imaged by staining with TRITC-phalloidin. The direction of the chemoattractant gradient is indicated.

in Figure 3, the G protein α2 and βγ subunits remain disassociated as long as chemoattractant is bound to the receptors. Thus, a separate inhibitory process must act downstream of the G protein cycle to terminate physiological responses and cause adaptation. As outlined in Figure 3, in a spatial gradient, this global inhibitory process could also offset G protein activation at the back of a cell and thereby localize responses to the front.

Cell polarization does not greatly alter these upstream components, although the distribution and properties of some become slightly asymmetric. For example, G protein β subunits are depleted at the rear of the cell, and slightly higher amounts are found on the membrane at the anterior (Jin et al., 2000). The association and dissociation rates of cAMP binding are also slightly faster on the anterior of the cell (Ueda et al., 2001). The asymmetric distribution of the G proteins may account for these differences in binding kinetics. Treatment of cells with inhibitors of actin polymerization causes the G protein subunits to redistribute uniformly and likely reverses the effects on cAMP binding kinetics.

The excitatory and inhibitory processes converge within the signaling pathway to achieve selective activation of the cytoskeleton at the cell anterior. As first shown in *D. discoideum*, activation of the chemotactic signaling pathways can be visualized by monitoring the binding of specific PH domains to the membrane (Parent et al., 1998; Meile et al., 1999). Chemoattractants trigger transient membrane association of the PH domain-containing proteins, such as the Crac (cytosolic regulator of adenylyl cyclase) and PKB (protein kinase B). In chemotaxing amoebae and neutrophils, these proteins bind selectively to the membrane at the leading edge of the cell and invariably mark the regions at which new pseudopods extend (see Figure 1). As expected, chemoattractants do not generate binding sites for PH domains in *D. discoideum* cells lacking G protein α2 or β subunits or in neutrophils treated with pertussus toxin to inactivate G_i (Lilly and Devreotes, 1995; Servant et al., 2000). Taken together, these observations suggest that gradient detection by uniformly distributed receptors and G proteins becomes sharply localized at a step downstream of G protein activation and upstream of the generation of binding sites for PH domains. Other responses, such as increases in cAMP, cGMP, IP_3, and Ca^{+2}, might also be localized at the anterior. However, since changes in these second messengers are not critical for the chemotactic response, we will focus on the phosphoinositides (Pitt et al., 1992; Traynor et al., 2000; Roelofs et al., 2001a, 2001b).

The localization of the directional response, as indicated by the recruitment of PH domains to the anterior, does not require polarization, the actin cytoskeleton, or movement. Although amoebae and neutrophils treated with latrunculin, an inhibitor of actin polymerization, become completely immobilized and lose polarity, they can still detect gradients (Parent et al., 1998; Jin et al., 2000; Servant et al., 2000). These cells show crescents of PH domain binding to the plasma membrane toward the higher concentration of chemoattractant. When the direction of the gradient is changed, the regions of the PH domain binding quickly readjust. In unpolarized cells, the localized response requires an external gradient and, if the stimulus becomes uniform, the response will quickly subside because of adaptation. In polarized cells, especially moving neutrophils, the PH domains bind persistently to the membrane at the leading edge in uniform chemoattractant (Wang et al., 2002). We suppose that, in such highly polarized cells, upstream components of the pathway become asymmetrically distributed, causing persistent excitation at the leading edge, even under uniform stimulation. If this supposition were correct, perturbations that interfere with polarity, such as actin polymerization inhibitors, would be expected to abrogate the localized signaling response.

The discovery of membrane binding sites for PH domain-containing proteins as a critical event in directional sensing has turned attention toward chemoattractant regulation of PI metabolism. While PH domains share limited amino acid sequence homology, the structures are highly conserved, and the proteins bind to several types of phosphoinositides with a broad range of specificity (Kavran et al., 1998; Dormann et al., 2002; Lemmon et al., 2002). For example, the PH domain of PLCδ (phospholipase Cδ) binds to $PI(4,5)P_2$, and Crac and PKB bind

to $PI(3,4)P_2$ and $PI(3,4,5)P_3$, whereas Btk (Brunton tyrosine kinase) and Grp (general receptor for phosphoinositides) bind selectively to $PI(3,4,5)P_3$. When GFP fusions of these PH domains are used to visualize subcellular PI accumulation in living amoebae or neutrophils, Crac and PKB most effectively mark the membrane at the leading edges of the chemotaxing cells (Parent et al., 1998; Meile et al., 1999; Servant et al., 2000; C. Parent, personal communication). These observations may indicate that the elevated PIs are a combination of $PI(3,4,5)P_3$ and $PI(3,4)P_2$. PI3Ks (PI 3-kinases), the enzymes that convert PI(4)P and $PI(4,5)P_2$ to $PI(3,4)P_2$ and $PI(3,4,5)P_3$, and PTEN (phosphatase and tensin homolog), which reverses these reactions, have been implicated in many cellular processes, such as vesicle trafficking, growth, DNA synthesis, regulation of apoptosis, and cytoskeletal changes (Maehama et al., 2001; Vanhaesebroeck et al., 2001). The recent studies suggest a role of these enzymes in chemotaxis and raise the question of how their regulation leads to the spatially restricted accumulation of their products (Sotsios and Ward, 2000; Wymann et al., 2000; Chung et al., 2001; Curnock et al., 2002; Stephens et al., 2002).

Is Phosphoinositide Production Essential for Chemotaxis?

Chemoattractant-mediated production of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ suggests that the PI3Ks or PTEN receive input from surface receptors. In neutrophils, the PI3K that is activated by heterotrimeric G protein is PI3Kγ, although recent evidence suggests that other isoforms, traditionally linked to receptor tyrosine kinases, can also be coupled to G proteins (Stoyanov et al., 1995; Hazeki et al., 1998; Yart et al., 2002). Disruption of many of the PI3Ks results in embryonic lethality in mice, but mice lacking PI3Kγ are viable and have differentiated neutrophils (Sasaki et al., 2000; Hirsch et al., 2000; Hannigan et al., 2002; Ueki et al., 2002). However, these neutrophils have decreased accumulation of $PI(3,4,5)P_3$ in response to chemoattractants fMLP and IL-8, subsequent activation of PKB is absent, and chemotaxis is significantly impaired. In *D. discoideum*, there are three genes, closely related to the mammalian PI3Ks (Zhou et al., 1995). These three PI3Ks contain a unique N-terminal hydrophilic region of about 600 residues, in addition to conserved Ras binding (RBD), C2, and PI3K kinase catalytic domains. Cells with individual disruptions of DdPI3K1, 2, or 3 show no strong growth or developmental defects, but cells with double disruptions of DdPI3K1 and DdPI3K2 (*pi3k1*$^-$/*pi3k2*$^-$) display conditional defects during the aggregation stage. In *pi3k1*$^-$/*pi3k2*$^-$ cells, the basal levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ are significantly reduced, as measured by HPLC (Zhou et al., 1998). Chemoattractant-induced PH domain translocation, reflecting the endogenous levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$, is significantly inhibited in *pi3k1*$^-$/*pi3k2*$^-$ null cells, suggesting that these two PI3Ks play an important role in chemotactic regulation of these lipids (Funamoto et al., 2001; unpublished data).

In spite of these defects, clear evidence for the role of PI3K in chemotaxis has been surprisingly difficult to obtain. The reported defects in the chemotactic response of the *pi3k1*$^-$/*pi3k2*$^-$ cells are partial (Funamoto et al., 2001; Funamoto et al., 2002). Compared with wild-type cells, the *pi3k1*$^-$/*pi3k2*$^-$ cells move 50%–60% more slowly and appear to be less polarized. In assays with stable external gradients, directionality, the straight line distance between the start and end of a track divided by the total path length, is about 60% reduced in the *pi3k1*$^-$/*pi3k2*$^-$ versus wild-type cells. Furthermore, in the presence of reasonable concentrations of inhibitors of PI3K, such as LY294002 and wortmannin, directionality is impaired by 30%. In our experience with inhibitors of PI3K and with *pi3k1*$^-$/*pi3k2*$^-$ cells (kindly provided by Dr. R. Firtel), the defects in similar chemotactic parameters are even less pronounced (L. Chen and P. Devreotes, unpublished data). In addition, PI3K inhibitors produce only partial defects in speed and directionality toward chemoattractants in mammalian neutrophils (Wang et al., 2002). Furthermore mammalian lymphocytes lacking PI3Kγ display few or no chemotactic defects. Further studies are needed to determine whether these perturbations fail to completely block changes in $PI(3,4,5)P_3$ or $PI(3,4)P_2$ or whether there is a PI-independent pathway for chemotaxis.

Interventions that elevate $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ have been more successful in implicating PIs in the regulation of motility. Previous studies have shown that PTEN-deficient mammalian cell lines, which have high levels of $PI(3,4,5)P_3$, are more mobile or invasive and display enhanced activity of the actin cytoskeleton (Haas-Kogan et al., 1998; Tamura et al., 1998; Sun et al., 1999; Liliental et al., 2000; Shan et al., 2000). Furthermore, delivery of $PI(3,4,5)P_3$ to neutrophils can trigger actin polymerization and initiate migration (Derman et al., 1997; Niggli and Keller, 1997; Niggli 2000; Insall and Weiner, 2001; Weiner et al., 2002). In *D. discoideum* there is a PTEN that, like its mammalian homologs, contains an N-terminal $PI(4,5)P_2$ binding motif, a protein tyrosine phosphatase domain, and a loosely defined C-terminal C2 domain. Cells lacking this gene have provided a clear link between PIs and chemotaxis. In chemotaxis assays where cells are exposed to a micropipette releasing chemoattractant, *pten*$^-$ cells move 80% more slowly, and the chemotactic index, a measure of the alignment of the cell track with the gradient, is reduced by 25% compared with that of wild-type cells. With uniform stimulation, the *pten*$^-$ cells display a greatly prolonged time course of PH domain association with the membrane, indicating that $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ degradation is severely impaired. When these cells are exposed to a spatial gradient, the protracted response corresponds to a broadened region where PH_{Crac}-GFP associates with the membrane. The cells elaborate multiple pseudopodia from this expanded region, whereas the wild-type cells typically extend only a single projection from the restricted region of PH_{Crac}-GFP binding. The inability of the *pten*$^-$ cells to confine responsiveness also compromises their cellular morphology and polarity. The cells display jagged circular profiles because of the extension of multiple filopodia and pseudopodia in random directions. These observations, together with those in mammalian cells, suggest that elevated levels of $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ are associated with local increases in the activity of the cytoskeleton.

Analysis of the *pten*$^-$ cells in *D. discoideum* has provided further insight into the function of localized

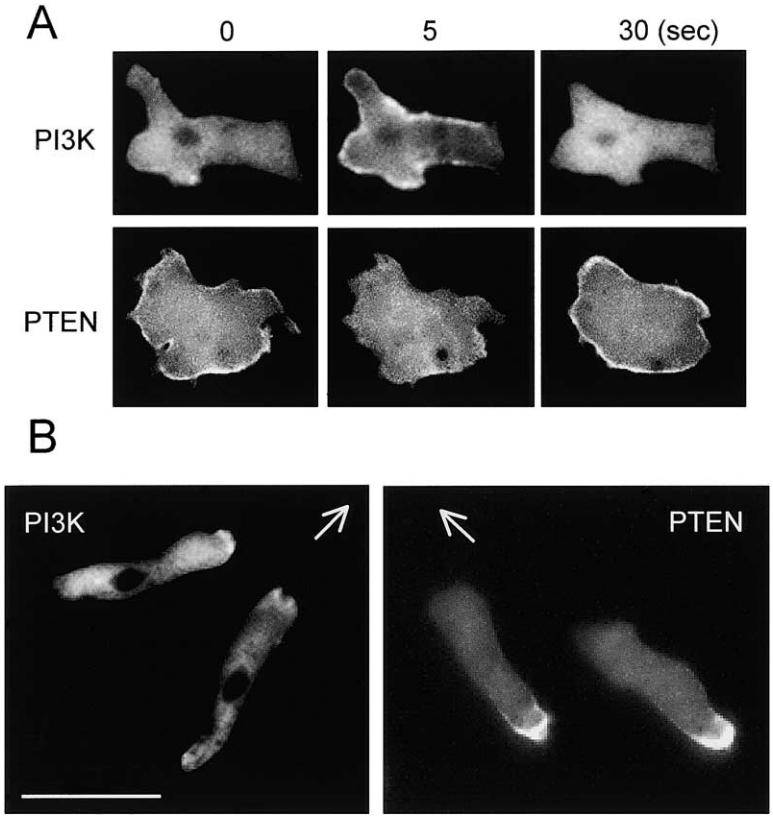

Figure 5. Changes in the Distribution of PI3K and PTEN in Response to Temporal Increases and Spatial Gradients of Chemoattractant

(A) Images show cells expressing PI3K-GFP or PTEN-GFP stimulated with a spatially uniform increase in chemoattractant. In resting cells, the majority of PI3K-GFP is located in cytosol, while PTEN-GFP is associated with plasma membrane. Upon stimulation, PI3K transiently translocates to the plasma membrane, while PTEN-GFP dissociates from the membrane. Within 20–60 s PI3K returns to cytosol, while PTEN reassociates with the membrane.

(B) Images show the distribution of PI3K-GFP or PTEN-GFP in cells moving toward a cAMP-filled micropipette. PI3K-GFP is associated with the membrane at the leading edge of cells, while PTEN-GFP is bound to the membrane at the back. The arrow indicates the direction of the micropipette. The calibration bar indicates 15 μm.

$PI(3,4,5)P_3$ and $PI(3,4)P_2$ accumulation (Iijima and Devreotes, 2002). In wild-type cells, stimulation typically triggers a biphasic actin polymerization response. An initial large peak occurs at 6 s, and then there is a rapid decrease and a subsequent broad, low peak at around 2 min. In *pten*$^-$ cells, the basal level of actin polymerization is higher, and chemoattractants elicit larger and longer increases. The difference is most evident in the second peak, which is 6-fold higher. These observation suggest that $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ plays an instructive role, determining when and where pseudopodia form, by promoting the activity of the cytoskeleton. On the other hand, attempts to reduce $PI(3,4,5)P_3$ and block actin polymerization have been less informative (Funamoto et al., 2001). Inhibitors of PI3Ks and gene disruptions of PI3Ks, which reduce $PI(3,4,5)P_3$ and $PI(3,4)P_2$ levels significantly, reduce actin polymerization less than 50%. The first peak of actin polymerization in both wild-type and *pten*$^-$ cells appears to be insensitive to physiologically relevant doses of LY 294002 (L. Chen and P.D., unpublished data). In PI3Kγ null mice, actin polymerization is unaffected, even though $PI(3,4,5)P_3$ changes are quite reduced (Li et al., 2000). Thus, it appears that a portion of the actin polymerization response requires only slight increases in $PI(3,4,5)P_3$ and $PI(3,4)P_2$. As noted above, further studies are needed to determine whether the rapid response is truly independent of PIs. The effects of PIs on actin polymerization may be mediated through a small G protein guanine nucleotide exchange factor that is recruited to the membrane at the front of the cell. Recently, there has been a report of the exchange factor P-Rex1, which is activated synergistically by G protein βγ subunits and $PI(3,4,5)P_3$ (Welch et al., 2002).

Chemoattractants Regulate the Binding of PI3Ks and PTEN to the Plasma Membrane

Further evidence of the key role of PI metabolism in chemotaxis has come from studies of the distribution of PI3Ks and PTEN during chemotactic stimulation. As shown in Figure 5, PI3Ks and PTEN are reciprocally regulated in response to chemotactic stimulation in *D. discoideum* (Funamoto et al., 2002; Iijima and Devreotes, 2002). In resting cells, PI3K1 and PI3K2 are cytosolic, while a fraction of PTEN is bound to the plasma membrane. When receptor occupancy is increased and held constant, the PI3Ks rapidly bind to, and PTEN dissociates from, the membrane. As stimulation continues for about 60 s, the PI3Ks return to the cytosol, while PTEN returns to the membrane at higher than prestimulus levels. In a chemoattractant gradient, PI3Ks are recruited to the membrane at the front of the cell, while PTEN dissociates at the front and accumulates on the membrane at the back. These coordinated binding events are likely to play an important role in enzyme regulation.

There are some clues as to the regulation of membrane association and activity of these enzymes (Funamoto et al., 2002; Iijima and Devreotes, 2002). For the PI3Ks, the N-terminal hydrophilic regions are sufficient for chemoattractant-induced membrane targeting, and proteins lacking these regions cannot support activation of PKB. The transient membrane recruitment of PI3Ks

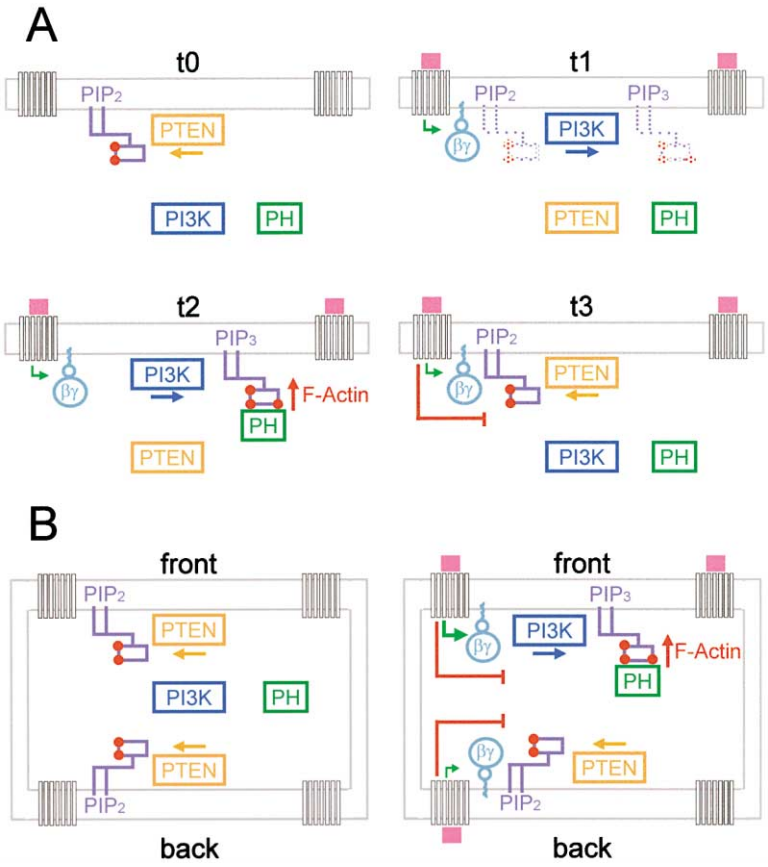

Figure 6. Cellular Responses to Temporal and Spatial Signals

(A) The diagram illustrates temporal responses of a cell before and at times during stimulation with a uniform increase in chemoattractant. In resting cells, PTEN is bound to the membrane, and PI3Ks are in the cytosol (t0). An increase in receptor occupancy (purple squares) triggers, through the heterotrimeric G protein, a rapid increase in excitation (bent green arrow), which leads to the binding of PI3Ks to the membrane and causes PTEN to dissociate from the membrane (t1). The combined effect causes a large increase in $PI(3,4,5)P_3$. The concomitant loss in $PI(4,5)P_2$ is exaggerated for illustration; levels of $PI(4,5)P_2$ have not yet been measured (t2). At longer times, inhibition (bent red bar) increases and eventually balances excitation. As a result, the PI3Ks return to the cytosol, PTEN returns to the membrane, and $PI(3,4,5)P_3$ returns to prestimulus levels (t3).
(B) The diagram shows the spatial response before (left) and at steady state (right) in a gradient. The global inhibitor (bent red bars) is equal at the two ends of the cell. Local excitation (bent green arrows) is slightly higher at the front, causing PI3K binding to, and PTEN loss from, the membrane at the front. This leads to a large increase of $PI(3,4,5)P_3$ selectively at the front. The decrease of $PI(4,5)P_2$ at the front is exaggerated for illustration.

is not affected by the treatment of cells with LY294002, suggesting that changes in $PI(3,4,5)P_3$ are not required. In contrast, it has been suggested that $PI(3,4,5)P_3$ activates its own production in neutrophils, since exogenously added $PI(3,4,5)P_3$ induces PH_{PKB}-GFP membrane binding and this recruitment is blocked by LY294002 (Weiner et al., 2002). Like their mammalian counterparts, the PI3Ks in *D. discoideum* contain a Ras binding domain. Mutation in this domain blocks PKB activation, but the enzymes still relocate normally to the membrane during chemoattractant stimulation. For PTEN, deletion of the putative $PI(4,5)P_2$ binding domain located at the N terminus completely redistributes the protein from the membrane to the cytosol and blocks its ability to rescue the aggregation defects of the *pten*$^-$ cells. These studies show that PI3Ks and PTEN must be associated with the membrane containing their substrates to be functional. It is intriguing that PTEN appears to bind to its product, suggesting that it may participate in a positive-feedback loop.

The movements of PI3K and PTEN display a remarkably degree of coordination. Transient increases in $PI(3,4,5)P_3$ and $PI(3,4)P_2$, inferred from changes in PH-GFP binding to the membrane and PKB activation, could be due to activation of PI3K, inhibition of PTEN, or both. The increase in PIs in the *pten*$^-$ cells suggests that PI3K is activated. In fact we have recently made the direct measurement of the state of PI3K activation during chemoattractant stimulation. We found that PI3K is activated 4-fold within five seconds of stimulation (Y.E.H. and P.D., unpublished data). Whether the regulatory control is exerted on PI3K, PTEN, or both, these observations further localize the steps in the signaling pathway that bring about directional sensing and focus attention on the binding sites and activators of these enzymes. These binding sites may be the earliest point where the asymmetry in signaling that underlies directional sensing is apparent.

A Model for Directional Sensing and Polarization Is Emerging

Figure 6 brings together many of these observations into a working model. In the model the same biochemical regulatory circuits that bring about the transient responses to temporal increases in receptor occupancy lead to directional sensing of spatial gradients. One can think of the temporal and spatial movements of PI3Ks and PTEN in terms of the local excitation-global inhibition scheme presented in Figure 3. A uniform stimulus leads to increases in excitation and inhibition and a transient difference in their levels. We envision that this transient difference generates membrane binding sites for PI3Ks and destroys those for PTEN. In a spatial gradient at steady state, the binding sites for PI3K would be generated selectively toward the higher concentration, while those for PTEN would persist toward the lower concentration. The activities of the enzymes are presumed to mirror their association with the membrane. The reciprocal regulation of the two key enzymes of PI metabolism would provide a powerful regulatory

mechanism, which could amplify small changes in the balance between excitation and inhibition and produce very large temporal and spatial accumulation of $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Feedback control of the enzymes may further amplify the response. This reciprocal system is likely to be quite resistant to perturbation. Since both increases in PI3K activity and decreases in PTEN activity contribute to elevation of PIs, inhibition of either activity alone may have a smaller than expected effect. Indeed, this robustness may, in part, explain the incomplete defects resulting from interference with PI3K and PTEN.

While this working hypothesis corresponds to the directional sensing response by an unpolarized cell, we can speculate on how the same components could bring about spontaneous or gradient-induced polarization. In particular, since the actin cytoskeleton appears to be critical for polarization, its role may be to stabilize the asymmetrically distributed components. For instance, if, in a polarized cell, the association of PI3K and PTEN with the membrane were stabilized, the cell would continue to display polarized morphology and sensitivity in the presence of a uniform concentration of chemoattractant. This would lead to persistent generation of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ at the leading edge, which, in turn, would maintain the asymmetry in the cytoskeleton in the absence of a gradient. Similarly, we have noted the asymmetric distribution of G proteins in polarized cells. Treatment with inhibitors of the actin cytoskeleton would revert the cell to the unpolarized state and shut off the signaling.

This recent series of observations provide an unprecedented opportunity to understand the fundamental mechanisms of gradient sensing. The evidence so far with PH-GFP markers indicates that local accumulations of PIs are a central part of the directional sensing mechanism. Quantitative measurements of the temporal and spatial changes in these lipids are needed. The studies also indicate the significance of the pathways regulating the activities and localization of PI3Ks and PTEN. It is at this step in the pathway that the excitatory and inhibitory processes triggered by chemoattractants converge to localize the signal. Future research must focus on the identification of the membrane binding sites for these enzymes, determine how these sites are turned on and off by chemoattractants, and discover the components that link elevated PIs to cytoskeletal rearrangements.

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