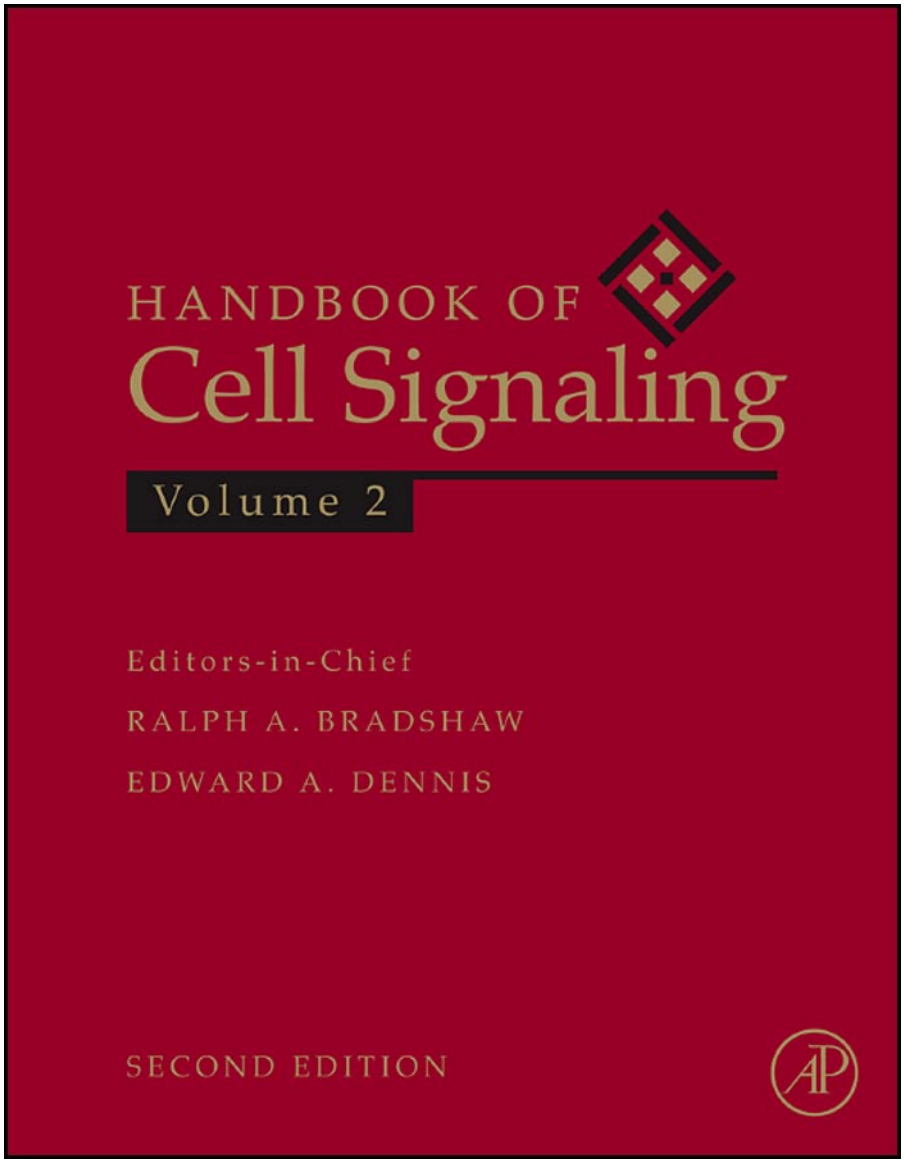

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Chapter 207

G-Protein Signaling in Chemotaxis

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INTRODUCTION

Chemotaxis is the directed migration of cells in response to concentration gradients of extracellular signals. In unicellular organisms, such as bacteria and amoebae, chemotaxis is frequently used as a foraging mechanism [1]. In multicellular organisms, it ensures that the right cells get to the right place at the right time during development, and plays an essential role in processes such as wound healing and inflammation [2, 3]. Chemotaxis is also a contributing factor to many diseases. For example, metastatic cancer cells migrate toward stereotypic regions of the body that promote further growth, and the unregulated chemotaxis of immune cells can lead to inflammatory diseases such as asthma and arthritis.

Much of our current understanding of chemotaxis-signaling pathways through G-protein-coupled receptors (GPCRs) is derived from studies on the social amoeba, *Dictyostelium discoideum*, and mammalian neutrophils (this term will be used to refer to both primary neutrophils and HL60s, a neutrophil-like cell line). *Dictyostelium* cells feed on microorganisms that they track down by chemotaxis towards secreted metabolites such as folic acid. More dramatic, however, is the response of this organism to starvation. The individual amoebae aggregate and, through a series of morphogenetic changes and cell-fate choices, form multicellular structures containing spores that can survive starvation. The process of aggregation is directed by gradients of cAMP, and can easily be studied under physiologically relevant conditions using combined genetic, biochemical, and cell biological analyses [1]. Neutrophils are important cells of the immune system, and are most frequently studied in the context of chemotaxis to either formyl-Met-Leu-Phe (fMLP) or chemokines – chemoattractants that regulate inflammation *in vivo*. Neutrophils from knockout mice and cell lines that can be manipulated with retroviruses are available. As studies in these two systems have revealed many similarities, distinctions will only be made when differences have been observed.

CHEMOTAXIS: MEMBRANE EXTENSIONS, DIRECTIONAL SENSING, AND POLARIZATION

Chemotaxis can be thought of as the result of three separate processes: membrane extensions, directional sensing, and polarization [2, 4]. Membrane extensions are the periodic pseudopods and blebs that cells make at regular intervals, and drive cell motility [5–7]. In *Dictyostelium*, membrane extensions can occur in cells lacking functional heterotrimeric G proteins [8]. Neutrophils, though, are relatively quiescent in the absence of ligand. Directional sensing refers to the capacity of chemotactic cells to sense the direction of external gradients and localize proteins or reactions towards or away from the high concentration. This process obviously requires receptor/G-protein signaling, but can occur when cell movement is inhibited. Polarization refers to the elongated cell morphology and the stable localization of molecules to the anterior and posterior poles that is acquired by neutrophils and starved *Dictyostelium* cells during chemotaxis. Polarization depends on the cytoskeleton as well as chemoattractant receptor/G-protein signaling, but does not require a gradient.

CHEMOATTRACTANT SIGNALING REGULATES MULTIPLE DOWNSTREAM PATHWAYS

Recent advances in our understanding of the molecular mechanisms that regulate chemotaxis have revealed the important and diverse roles played by G proteins [9, 10].

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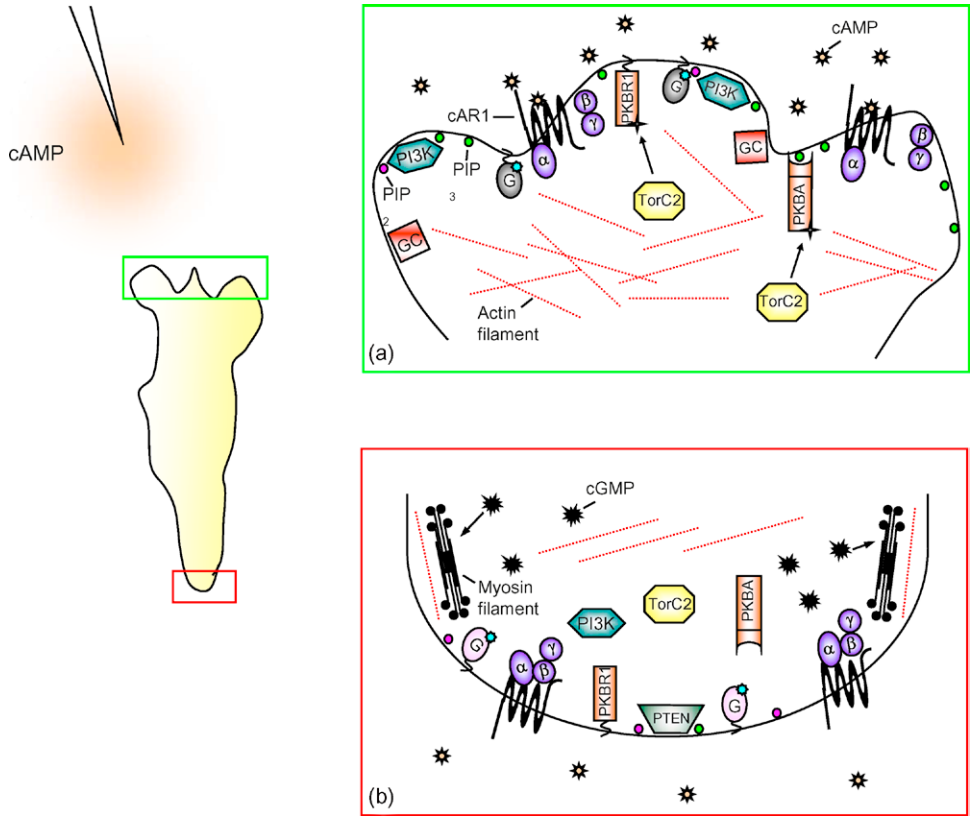

FIGURE 207.1 Signaling at the front and back of chemotaxing amoebae.
Panels (a) and (b) illustrate some key signaling components that are localized to the front and back of migrating cells in a gradient of cAMP. At the front (a), cAMP binding to cAR1 results in PI3K recruitment, production of PIP3, PKBA translocation to the membrane, GTPase (such as Ras, Rap and Rac; gray ovals) activation, PKB phosphorylation (white stars) by TorC2, sGC activation and F-actin polymerization. These signaling events, as well as others (see text for an expanded list), are required for efficient chemotaxis. At the back (b), PI3K is cytosolic and PTEN is localized to the membrane where it degrades PIP3 to PIP2. In addition, Rho is activated (light gray oval) and cGMP regulates myosin II filament formation.

These studies not only highlight the critical function of G proteins as "molecular switches," but also show how their signaling in the context of chemotactic signaling networks allows cells to translate the directional information of external concentration gradients into directional movement.

Downstream of GPCRs, many signal transduction events are initiated via heterotrimeric G proteins. *In vivo*, chemoattractant binding triggers a rapid dissociation or rearrangement of Gα and Gβγ subunits. Within seconds, this leads to activation of the small G proteins Ras, Rho, Rac, Cdc42 and Rap; the increase or decrease of the second messengers phosphatidylinositol (3′,4′,5′) trisphosphate (PIP3), arachidonic acid, diacylglycerol (DAG), inositol trisphosphate (IP3), cAMP, cGMP, Ca^{2+} and H^{+} ions; and stimulation of the kinases protein kinase A (PKA), protein kinase C (PKC), target of rapamycin (Tor), mitogen activated protein kinase (MAPK), protein kinase B (PKB), and a PKB-related kinase (PKB-R1). Interestingly, although the heterotrimeric G-protein complex is thought to remain dissociated as long as receptors are occupied, most of the downstream pathways are only transiently activated in response to a uniform stimulus and return to basal levels within a few minutes (see below) [11, 12]. A key breakthrough in understanding how this signaling network controls chemotactic migration was the finding that in a gradient, many responses are persistently activated and become asymmetrically localized and oriented according to the direction of the gradient (Figure 207.1) [13].

FRONT AND BACK SIGNALING

PIP3 was the first molecule found to have an asymmetric localization in a gradient, and has served as a model for understanding the temporal and spatial activation of chemotactic signal transduction pathways [14]. In *Dictyostelium*, the correct orientation of PIP3 in a gradient is achieved by the coordinated regulation of phosphatidylinositol-3′-kinase (PI3K) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [15, 16]. PI3K produces PIP3 by phosphorylating the 3′-hydroxl group of phosphatidylinositol (4′,5′) bisphosphate (PIP2) and PTEN catalyzes the reverse reaction. In response to chemoattractant, PI3K is rapidly recruited from the cytosol to the plasma membrane,

where it is likely activated by binding to Ras-GTP [17, 18]. Conversely, PTEN is bound to the plasma membrane of resting cells and with stimulation it dissociates. In a uniform stimulus, the response is transient, as PI3K and PTEN return to their original locations after a few minutes. In a gradient, however, PI3K is persistently bound to the front and PTEN is restricted to the back, resulting in steady-state PIP3 accumulation at the front (Figure 207.1). Previous studies have demonstrated that PIP3 can recruit proteins to the plasma membrane via pleckstrin homology (PH) domains, indicating that this may be a mechanism to localize downstream effectors [18–20].

Recent work has provided some insight into the mechanisms of PI3K and PTEN localization in *Dictyostelium*. The N-terminal domains from PI3K isoforms 1 and 2 are necessary and sufficient for cAMP-dependent membrane translocation [15, 17]. Furthermore, this work has shown that PI3K also appears to localize to the membrane and in a narrow band adjacent to the membrane. Treating cells with latrunculin A, an inhibitor of actin polymerization, impairs localization, suggesting that PI3K recruitment to the cell cortex may depend on the cytoskeleton [21]. For PTEN, it has been shown that the N-terminus contains an amphipathic "PIP2 binding motif," and that this stretch of about 15 amino acids is essential for membrane binding [22]. A recent study suggests that signaling through phospholipase C (PLC), which degrades PIP2, may play a role in controlling PTEN localization. In plc^- cells, PTEN does not dissociate from the membrane during stimulation, whereas in cells overexpressing PLC, PTEN is not associated with the membrane [23]. Interestingly, some cAMP analogs, by coupling the receptor to different Gα proteins, can inhibit PLC and thereby act as repellents [24].

PIP3 also marks the front of neutrophils, suggesting that chemoattractant regulation of PIP3 metabolizing enzymes occurs in these cells. The recruitment of the PI3Kγ catalytic subunit is dependent on the interaction with the p101 regulatory subunit and is regulated by G$\beta\gamma$ (coupled to Gα_i) and Ras [25, 26]. In migrating neutrophils, PI3Kγ is found in a broad region at the leading edge. The requirement for binding to G$\beta\gamma$ and Ras-GTP may further confine PI3K activity to an even narrower region. Compared with the *Dictyostelium* enzyme, less mammalian PTEN is associated with the plasma membrane, but its binding can be detected at the single molecule level by Total Internal Reflection Fluorescence (TIRF) microscopy [27]. Membrane association is essential for activity and depends on the conserved "PIP2 binding motif." The C2 domain has also been implicated in membrane binding as mutations in this domain have been found to inhibit lipid binding *in vitro* [28]. Other evidence suggests that phosphorylation and interactions with binding proteins may be important for localization. Mutating phosphorylated residues on the C-terminus to alanine is thought to favor an "open" conformation and strongly enhances membrane recruitment. The interaction of PTEN with several membrane proteins via its PDZ domain may also play a role [29]. However, it is somewhat controversial whether membrane binding occurs preferentially at the back and sides of migrating neutrophils [25, 30].

Although many studies have highlighted the deleterious effects of elevated PIP3, there is now general agreement that chemotaxis is less severely impaired when PIP3 production is inhibited. *Dictyostelium* amoebae lacking PTEN are defective in their ability to degrade PIP3 and chemotax poorly due to the production of numerous lateral pseudopods [16]. Chemotaxis defects due to high PIP3 levels are also seen in neutrophils, although the role of PTEN is less clear in this system. One study found that chemotaxis, PIP3 levels, and actin polymerization are normal in $pten^{-/-}$ cells, and instead suggest that SHIP1, which removes the 5′ phosphate from PIP3, is the key regulator of PIP3 in neutrophils [31]. $Ship1^{-/-}$ neutrophils were found to have a prolonged PIP3 response and a chemotaxis defect similar to that of $pten^-$ amoebae. A second study reported that $pten^{-/-}$ neutrophils have marginally elevated levels of PKB phosphorylation and actin polymerization, but do not have a strong chemotaxis defect [32]. Several groups have also looked at chemotaxis in conditions where PIP3 production is inhibited. Most recently, *Dictyostelium* cells lacking all type I PI3Ks and PI3K$\gamma^{-/-}$ neutrophils were found still to perform chemotaxis relatively well [33, 34]. Similar results have also been obtained in cells where PI3K activity was inhibited pharmacologically [35–37].

The limited effects of inhibiting PIP3 production clearly suggest that other pathways may act in parallel, and this has been substantiated by recent results. In *Dictyostelium*, loss of phospholipase A2 (PLA2) activity, either through inhibitors or genetic manipulation, does not have a significant effect [35, 38]. However, when combined with a loss of PI3K function, chemotaxis is severely impaired. PLA2 cleaves phospholipids to produce free fatty acids (such as arachidonic acid) and lyso-phospholipids. Additionally, it appears that the activity of this enzyme is regulated by chemoattractant [38]. It remains unclear what the downstream effects of this pathway are, and whether PLA2 enzymes play a similar role in neutrophils. There is also increasing evidence for the role of the TorC2 complex in regulating chemotaxis. *Ras interacting protein 3* (*Rip3*) and *Pianissimo* (*PiaA*), were originally isolated as chemotactic mutants in *Dictyostelium* [39, 40]. The homologs of these proteins, Sin1/Avo1 and Rictor/Avo3, respectively, were subsequently found to be part of the highly conserved TorC2 complex that is thought to play a critical role in regulating PKB activity [41–43]. This function appears to be conserved in *Dictyostelium*, which has two PKB homologs: PKB-A and PKB-R1. Furthermore, in chemotaxing cells, activation of TorC2 is localized to the leading edge (Yoichiro Kamimura, personal communication).

Small GTPases play important roles in regulating actin polymerization and myosin II function in neutrophils and

Dictyostelium [10]. For example, activated Rac and Cdc42 localize to the front of neutrophils and are thought to play a key role in initiating actin polymerization [44]. Consistent with this hypothesis, expression of dominant-negative Rac1 inhibits neutrophil migration and actin polymerization, while dominant-negative Cdc42 prevents neutrophils from maintaining a persistent leading edge [45]. *Dictyostelium* has 17 Rac isoforms which, at the sequence level, cannot be divided into specific Rac, Rho, or Cdc42 homologs, and for simplicity have been named RacA–Q. Many have been knocked out, and RacB, C, and G are reported to have defects in chemotaxis and actin polymerization [46, 47]. Additionally, cells overexpressing dominant-negative RacB have reduced pseudopod extension and migration, as do cells lacking RacGEF1 [46]. The small G proteins Rho and Rap have been implicated as regulators of myosin II function. Myosin II (a hexameric enzyme composed of two myosin heavy chains (MHC), two essential light chains (ELC), and two regulatory light chains (RLC)) is a key regulator of chemotaxis which is thought to both facilitate the retraction of the cell rear and to suppress lateral pseudopods through its actin crosslinking and motor protein functions [48]. Both of these functions depend on multiple myosin II molecules assembling into bipolar filaments that can then associate with cortical actin cytoskeleton. Rap is activated at the front of *Dictyostelium* cells, and expressing constitutively active Rap inhibits myosin II filament formation, possibly by promoting the phosphorylation of MHC [49, 50]. This may be mediated either directly or indirectly by Phg2, a Rap effector kinase that is required for chemoattractant-stimulated MHC phosphorylation [51]. In contrast, Rho is localized to the back and sides of neutrophils and is thought to promote myosin II motor activity by phosphorylating the RLC through p120 ROCK [25]. Inhibiting this kinase impairs RLC phosphorylation and leads to increased lateral pseudopod production, an indicator of reduced myosin II activity. A similar result is seen when dominant-negative Rho is expressed. In neutrophils, this is probably regulated by $G\alpha_{12/13}$ as pertussis toxin (PT), which inhibits $G\alpha_i$ but not $G\alpha_{12/13}$, does not inhibit Rho activation. Consistent with the important role of Rho at the back and not the front, PT-treated cells fail to generate pseudopods but extend uropods at the back when a chemoattractant gradient is applied. An analogous pathway may exist in *Dictyostelium* involving another Rac isoform and p21 activated kinase A (PakA), a Rac effector. This protein is reported to co-localize with myosin II at the back, and cells lacking PakA appear to have a defect in myosin II filament assembly [52, 53].

Other proteins and reactions have also been found to localize to the front of migrating amoebae, including myosin II heavy chain kinase (MHCK-A), a Na^+–H^+ exchanger (NHE), and soluble Guanylate Cyclase (sGC). MHCK-A is concentrated at the front by associating with newly polymerized F-actin, and can inhibit myosin II filament assembly by phosphorylating the heavy chain [54]. *NHE* mutants make increased numbers of lateral pseudopods and, given that myosin filament assembly is enhanced by an acidic pH *in vitro*, the concentration of this protein at the leading edge may inhibit filament assembly in this region by making the cytosol more alkaline [55]. The binding of sGC to the membrane is essential for guanylate cyclase activity, and although this protein is recruited to the leading edge, cGMP diffuses throughout the cell and has mainly been implicated in promoting myosin II filament assembly through cGMP binding protein C (GbpC) at the back [56, 57]. Cells lacking either sGC or GbpC have strong chemotaxis defects that can be attributed to dramatic reduction in the levels of myosin II at the actin cortex. Amoebae lacking $G\alpha_9$ have decreased cGMP levels, suggesting a regulatory role for this protein.

MECHANISMS OF DIRECTIONAL SENSING

The asymmetric localization of the molecules discussed above raises the question, How do cells orient these events based on a chemoattractant gradient? Models based on Local Excitation–Global Inhibition (LEGI) have proved very successful at explaining many features of directional sensing [4, 58]. In these models, it is proposed that receptor–ligand binding triggers at least two signals: an excitatatory signal that is turned on rapidly and diffuses slowly (local excitation), and an inhibitory signal that is turned on slowly and diffuses rapidly (global inhibition). These models can explain responses to both uniform and gradient stimuli. In LEGI models, cells respond transiently to a uniform stimulus because excitation occurs more rapidly than inhibition. Thus the initial activation of downstream pathways is attenuated over time as the slower forming global inhibitor builds up (Figure 207.2a). In a gradient, the LEGI model accurately predicts that downstream signaling will be persistently activated at the front. Since diffusion of the excitatory signals is slow, the level of excitation at each point along the cell membrane reflects the receptor occupancy at that site, and is higher at the front than at the back. In contrast, since the inhibitor is freely diffusible, inhibition will be averaged across the cell. Consequently, at steady state, excitation will exceed inhibition at the front but not at the back (Figure 207.2d). In this way the cell translates the directional information of the gradient into differences between front and back, and can readily adjust to changes in the temporal–spatial pattern of stimulation.

At what point in the pathway does this asymmetry occur? By expressing CFP and YFP fusions of $G\alpha$ and $G\beta$, the dissociation state of the heterotrimeric G proteins can be monitored by FRET [12, 59]. In immobilized amoebae, the dissociation of heterotrimeric G proteins matches the

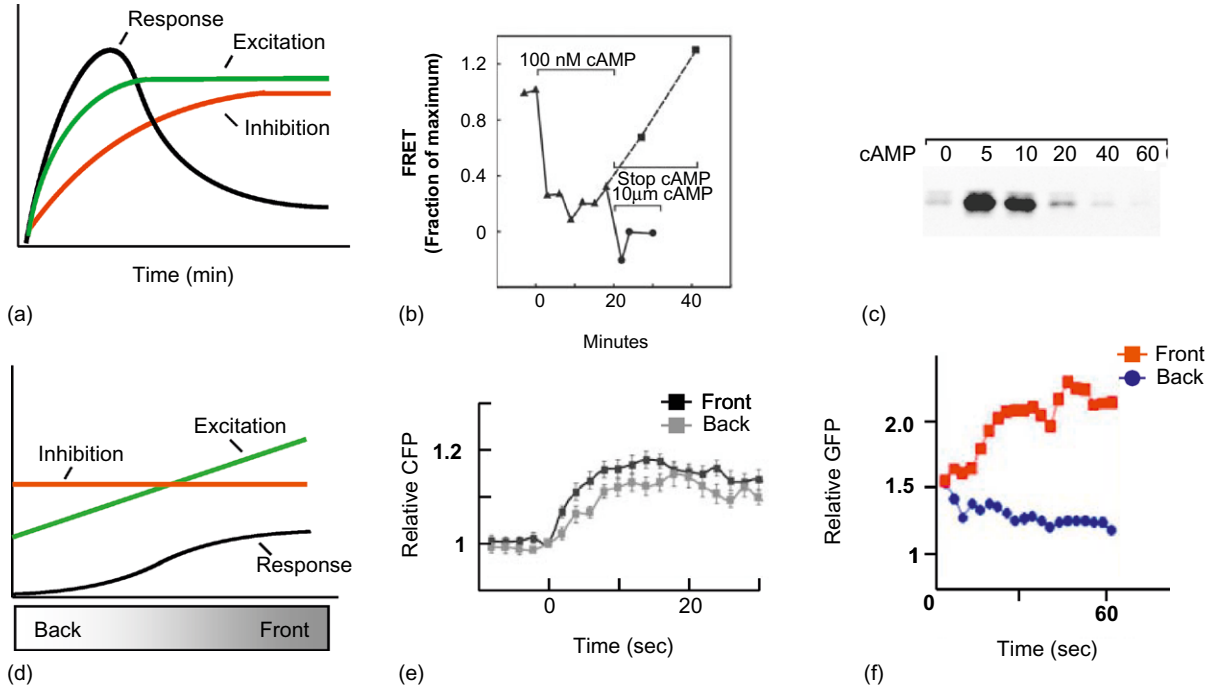

FIGURE 207.2 Temporal and spatial dynamics of chemotactic signaling.
Panels (a)–(c) illustrate the temporal dynamics of signals according to the LEGI model. In (a), the graphic representation of the LEGI model shows that many signaling responses ("response") are transient after a uniform stimulus, since build-up of the diffusible inhibitor ("inhibition") eventually dampens the quickly diffusing excitation signal ("excitation"). Not all responses are transient, however. In (b), FRET studies with fluorescently labeled Gα and Gβγ show that once the Gβγ complex dissociates, it remains dissociated as long as steady-state levels of the stimulus are present. When the stimulus is removed, the FRET response returns and additionally, increased concentrations of cAMP elicit further Gβγ dissociation (adapted from [12]). In contrast, in (c), precipitation of activated Ras shows that the GTPase remains active only transiently following cAMP stimulation (adapted from [8]). Panels (d)–(f) refer to spatial regulation of signaling. The LEGI model predicts that in a gradient of chemoattractant, responses (black line in (d)) will only be seen at the front of the cell, since excitation is greater than inhibition here. Panel (e) displays G-protein dissociation in a gradient of cAMP. An increase in Gα-CFP signal indicates Gβγ dissociation in this experiment, which can be seen at higher levels at the front of the cell than at the back (taken from [11]). Note that this response reflects the shallow gradient outside the cell. In (f), Ras binding domain-GFP localizes very strongly to the front of the cell; the intensity of this response indicates that amplification of this signal has occurred downstream of G proteins (adapted from [11]).

steepness of the gradient across the cells (Figure 207.2b, d). The earliest localized events are activation of Ras (Figure 207.2c, f) and loss of PTEN at the front [16, 21]. Thus, directional sensing must occur in between the G proteins and these downstream events, possibly by regulating the localization or activity of a RasGEF.

While LEGI models accurately describe the behaviors of proteins within immobilized cells, they cannot account for certain features of chemotaxis displayed by polarized cells. In particular, LEGI predicts that when challenged with a change in the direction of the gradient, cells should respond by establishing a new anterior–posterior axis. Polarized cells such as starved *Dictyostelium* cells and neutrophils, however, typically respond by turning, and thus maintain the same front and back regions [6, 36]. Careful analysis of pseudopod extensions has also revealed other interesting points. First, cells tend to form pseudopods by splitting existing pseudopods, indicating that one outcome of polarization is to restrict the regions in the cell that can produce pseudopod extensions. Second, once a pseudopod splits, the cell appears to make a choice to maintain one pseudopod or the other based on which one is closer to the chemoattractant source. The mechanism cells use to make the "right" choice could be similar to the LEGI model we have described for directional sensing. These observations indicate that the interplay between directional sensing and polarization mechanisms must be accounted for in a complete description of chemotaxis.

POLARIZATION

Feedback loops are the key to establishing and maintaining polarization. Positive feedback loops amplify the absolute level of front or back signaling respectively, while negative feedback loops serve to increase the separation of these two pathways in space. In neutrophils, a positive feedback loop has been identified in the PIP3–Rac–actin polymerization pathway. First, introducing PIP3 lipid directly into neutrophils is sufficient to make neutrophils polarize and

migrate [60, 61]. As this lipid is degraded over time, a positive feedback loop is required to account for the persistence of these effects. Second, inhibiting actin polymerization attenuates both PIP3 production and Rac activation, even in the presence of constant stimulus. As actin is thought to be downstream of both PIP3 and Rac, this indicates that persistent and robust activation requires an actin-dependent positive feedback loop. The ability of several molecules to localize to the leading edge by associating with newly polymerized F-actin provides a possible mechanism for this feedback loop. As F-actin is polymerized, proteins that promote PIP3 production and Rac activation are recruited to the leading edge and thereby initiate more actin polymerization. PI3K, Ras and RacGEF1 in *Dictyostelium* and PI3Kγ in neutrophils are possible candidates. There is also evidence for the existence of negative feedback pathways. Expressing constitutively active Rho in neutrophils inhibits actin polymerization and Rac activation, while expressing activated Rac inhibits GTP exchange of Rho and the assembly of contractile myosin II filaments in the rear [25]. The effect of Rho is probably mediated by myosin II filament assembly, as expressing activated myosin II RLC inhibits actin and Rac. How Rac inhibits Rho and myosin II in neutrophils is less clear. One possibility is that, as in *Dictyostelium*, actin polymerization may recruit MHCKs to the leading edge. Whatever the mechanism, two recent experiments highlight the importance of this negative feedback loop in neutrophils. First, whereas untreated cells turn when the gradient is reversed, cells treated with an inhibitor of p120 ROCK retract the original pseudopod and extend a new one towards the chemoattractant source [25]. Second, neutrophils that are treated with latrunculin B, to inhibit actin polymerization, have a reversed localization of activated Rho in a gradient [62]. These data indicate that this feedback loop is critical for maintaining polarization and for restricting Rho and myosin II activity to the rear.

CONCLUSION

Chemotaxis can be viewed as a modular process composed of membrane extensions, directional sensing, and polarization. G proteins play a central role in regulating each of these modules, and we are beginning to understand how the signal transduction pathways they regulate are controlled in space and time. Future work will need to examine how these signaling networks interact, and new models need to be developed that can account for both directional sensing and polarization.

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