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Signaling Mechanisms for Chemotaxis

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Abstract

Cells recognize external chemical gradients and translate these environmental cues into amplified intracellular signaling that results in elongated cell shape, actin polymerization toward the leading edge, and movement along the gradient. Mechanisms underlying chemotaxis are conserved evolutionarily from *Dictyostelium* amoeba to mammalian neutrophils. Recent studies have uncovered several parallel intracellular signaling pathways that crosstalk in chemotaxing cells. Here, we review these signaling mechanisms in *Dictyostelium discoideum*.

Introduction

Chemotaxis plays important roles in a variety of physiological events, such as axon guidance, wound healing, and tissue morphogenesis. Moreover, immune cells use chemotaxis to circulate between the vascular and lymphatic systems, as well as to migrate from the blood towards sites of infection. In addition to these roles in normal physiology, chemotaxis also plays a role in many pathological conditions. For example, chemotaxis of tumor cells results in cancer metastasis, while unwanted immune cell chemotaxis causes chronic inflammatory diseases such as asthma and arthritis (Moser and Loetscher, 2001; Murphy, 2001; Brauersreuther and Mach, 2006; Mrass and Weninger, 2006; Kedrin et al., 2007). Previous studies have established that mechanisms underlying chemotaxis, including chemical sensing, intracellular signaling, and cytoskeleton rearrangement, are similar in mammalian neutrophils and the cellular slime mold *Dictyostelium discoideum* (Insall, 2010; Swaney et al., 2010; Chisholm and Firtel, 2004; Parent, 2004; Van Haastert and Veltman, 2007; Janetopoulos and Firtel, 2008; for comparison between neutrophil and *Dictyostelium* chemotaxis, see Stephens et al., 2008; Iglesias et al., 2009; Wang 2009; Charest and Firtel, 2010).

In *Dictyostelium*, chemotaxis is observed during growth and development. During growth, cells use chemotaxis to migrate toward bacteria and engulf them. Folic acid secreted by the bacteria acts as a chemoattractant. Upon nutrient deprivation, *Dictyostelium* cells undergo a developmental program in which spores are formed for survival until the environment becomes favorable for growth. During this process, individual cells crawl toward aggregation centers in response to cyclic AMP and form multicellular structures that eventually transform into fruiting bodies carrying spore cells (Kimmel and Firtel, 2004; Van Haastert and Devreotes, 2004; Williams et al., 2006).

Dictyostelium mutants defective in chemotaxis are incapable of aggregating and therefore cannot develop normally. Mutant development phenotypes, including lack of aggregation and fruiting bodies, are readily observed on bacterial lawns and have been used successfully to enrich for potential chemotaxis mutants in mutagenesis studies. A more direct test for

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chemotaxis involves analysis of migration toward cAMP using time-lapse microscopy with a micropipette that releases the chemoattractant. Genetic isolation of developmentally-defective mutants, combined with subsequent cell biological and biochemical analyses, has revealed many proteins involved in chemotaxis (Manahan *et al.*, 2004; Willard and Devreotes, 2006).

During chemotaxis, *Dictyostelium* cells become elongated and achieve an asymmetric shape to form pseudopods at the leading edge and uropods at the trailing edge. In polarized cells, different components involved in signaling and cytoskeletal rearrangement are selectively localized to the leading or trailing edge. With this, the leading edge can easily sense the direction to migrate towards, while the trailing edge follows without being affected by subtle changes in the extracellular environment. Such polarization also works to translate changes in extracellular chemical gradients into amplified intracellular signaling events. Mutants that are defective in morphological polarization fail to maintain robust directional persistence and efficiency in moving along the gradient.

Actin polymerization is the major driving force of pseudopod extension at the leading edge. Chemotactic signaling pathways control actin polymerization through different actin binding proteins. However, it remains largely unknown how intracellular signaling is coupled to the actin cytoskeleton in chemotaxis (Franca-Koh *et al.*, 2006). In contrast, actin polymerization appears to be suppressed at the trailing edge of moving cells. In fact, contraction of the actin meshwork is required to push the bulk of the cytoplasm forward and retract uropods, which also involves actin binding proteins such as myosin II (Bosgraaf and van Haastert, 2006; Vicente-Manzanares *et al.*, 2009).

Recognition of chemoattractants: cAMP receptors and heterotrimeric G-proteins

The first step in chemotaxis involves recognition of cAMP by its seven transmembrane domain-containing receptors (Saxe *et al.*, 1991; Insall *et al.*, 1994b; Kim *et al.*, 1998). Four such cAMP receptors (cAR1-4) have been identified in *Dictyostelium*. cAR1 exhibits high affinity for cAMP binding and is expressed during early development to mediate chemotactic aggregation of single amoeba. This function of cAR1 overlaps partially with that of cAR3, which also shows high affinity binding to cAMP. cAR2 and cAR4 are important for later developmental events, such as mound formation and culmination. cAR1 is coupled to heterotrimeric G-proteins, which consist of alpha, beta, and gamma subunits, and activates them upon association with cAMP (Chen *et al.*, 1996; Milne *et al.*, 1997). G-protein activation leads to local actin polymerization at the leading edge and drives pseudopod extension. How cAR1 activates heterotrimeric G-proteins remains to be determined; however, several models have been proposed. For example, one model hypothesizes that, prior to stimulation, G-proteins are associated with cAR1 and that cAMP binding releases the heterotrimeric G-proteins and activates them. Another model posits that G-proteins are transiently recruited to cAR1 upon cAMP binding and become activated (Xu *et al.* 2010).

Although cAR1 is uniformly distributed along the plasma membrane, interactions between cAMP and cAR1 are prolonged at the leading edge compared to the trailing edge during chemotaxis (Xiao *et al.*, 1997; Ueda *et al.*, 2001). These observations suggest that asymmetric activation might occur at the level of the receptor. However, this may not lead to robust amplification of downstream signaling as the extent of activation of the receptor-coupled G-protein shows only a modest bias toward the higher concentration of the gradient (Xiao *et al.*, 1997; Jin *et al.*, 2000). Below, we summarize four major signaling pathways that are downstream of cAR1 and the G-proteins.

Four signaling pathways that control chemotaxis

1. Production and degradation of PtdInsP3 by PI3Ks and PTEN

One mechanism that amplifies intracellular signaling is mediated by the generation of a second messenger called PtdIns(3, 4, 5)P₃ (PIP₃), which is a lipid signaling molecule that promotes actin polymerization (Parent *et al.*, 1998; Comer and Parent, 2002; Funamoto *et al.*, 2002; Iijima and Devreotes, 2002). PIP₃ is present as a steep gradient within chemotaxing cells and increases about several times more steeply than receptor occupancy. Therefore, the extracellular cAMP gradient is translated into an enhanced PIP₃ gradient that produces different responses along chemotaxing cells, including local actin polymerization at the leading edge.

The *Dictyostelium* genome contains five class I PI3Ks, PI3K1-5, which phosphorylate PtdIns(4,5)P₂ (PIP₂) to produce PIP₃. These PI3Ks are activated by the cAR1-coupled heterotrimeric G-proteins upon cAMP stimulation and are transiently recruited to the leading edge of chemotaxing cells. This recruitment of PI3Ks to the plasma membrane promotes PIP₃ production and involves the actin cytoskeleton, but not the activity of PI3Ks or PIP₃ since mutations in its catalytic domain does not affect the recruitment (Zhang *et al.*, 2010; Parent *et al.*, 1998; Meili *et al.*, 1999; Haugh *et al.*, 2000; Funamoto *et al.*, 2001). The N-terminal region without the catalytic domain is necessary and sufficient for this translocation. PI3K activity is also regulated as these enzymes interact with Ras GTPases, which stimulate PI3K kinase activity at the plasma membrane (Sasaki *et al.*, 2004; Sasaki and Firtel, 2006; Sasaki *et al.*, 2007). We will discuss how Ras GTPases are activated in Section 4 below.

PTEN, a known tumor suppressor that is mutated in many types of cancers, converts PIP₃ to PIP₂. *pten*-null cells exhibit PIP₃ overproduction, hyperactivation of the actin cytoskeleton, and failure to restrict pseudopodia extension to the leading edge in a chemoattractant gradient (Funamoto *et al.*, 2002; Iijima and Devreotes, 2002; Iijima *et al.*, 2002). Like PI3Ks, PTEN localization is also regulated by cAMP, but in an opposite manner. When cells are exposed to a cAMP gradient, PTEN accumulates towards the rear. The interaction of PTEN with the membrane is regulated by its PIP₂ binding domain and independent of PIP₃. The actin cytoskeleton is also dispensable. Since membrane association of PTEN is dependent on PIP₂, phosphorylation of this lipid by PI3K may decrease the amount of PIP₂ at the leading edge and therefore binding sites for PTEN (Iijima *et al.*, 2004). It is possible that this decrease in PTEN binding leads to further accumulation of PIP₃ at the leading edge and amplifies its asymmetric distribution. Thus, reciprocal localization of PI3K and PTEN combined with this potential positive feedback mechanism results in spatial regulation of PIP₃ production, with synthesis occurring at the leading edge and degradation at the rear. In addition to PTEN, the PI5-phosphatase SHIP dephosphorylates PIP₃ during mammalian chemotaxis (Li *et al.*, 2003; Nishio *et al.*, 2007; Subramanian *et al.*, 2007). In contrast to PTEN, which is located at the trailing edge in both *Dictyostelium* and mammalian cells, SHIP is distributed evenly within the cytoplasm of mammalian cells. *Dictyostelium* express four PI5-phosphatases and the degree to which PI5-phosphatases contribute to PIP₃ dephosphorylation remains to be determined (Loovers *et al.*, 2003).

Downstream effectors of PIP₃: PH domain-containing proteins, including PKB, PhdA, PhdB, PhdG, and GEFs for small GTPases

PKBA, a *Dictyostelium* homolog to mammalian PKB/AKTs, contains a PIP₃-responsive pleckstrin homology (PH) domain and is recruited to the leading edge upon cAMP-stimulated PIP₃ production (Meili *et al.*, 1999; Meili *et al.*, 2000; Chung *et al.*, 2001; Lim *et al.*, 2001; Kamimura *et al.*, 2008; Kamimura *et al.*, 2009). As a clear demonstration of

PKBA as the major mediator of PIP3 signaling, chemotaxis defects in *pten*-null cells can be partially rescued by additional loss of PKBA (Tang et al., 2011). This result also suggests the presence of additional downstream effectors for PIP3 in the absence of PKBA. Upon membrane recruitment, PKBA phosphorylates several substrates, including p21-activated kinase (PAK) (Chung and Firtel, 1999; Chung et al., 2001; Muller-Taubenberger et al., 2002; Li et al., 2003; Lee et al., 2004). Like PKBA, additional loss of PAK restores the chemotactic deficiency observed in *pten*-null cells, suggesting that PAK is a critical downstream component of the PIP3 pathway. PAK activity is stimulated by PKBA-dependent phosphorylation and has been proposed to regulate myosin II phosphorylation. Although its function is still controversial, PAK may regulate the lateral pseudopod extension through myosin II phosphorylation to maintain cell polarity. Another PKB homolog, PKBR, is tethered to the plasma membrane through myristoylation (Cai et al., 2010; Charest et al., 2010; Lee et al., 2010; Liao et al., 2010; Meili et al., 2000; Lee et al., 2005; Kamimura et al., 2008; Kamimura et al., 2009). This enzyme is not regulated by PIP3, but its phosphorylation is mediated by target of rapamycin complex 2 (TORC2), which is discussed in Section 4 below.

Another PH domain-containing protein, PhdA, also binds to the plasma membrane at the leading edge in a PI3K-dependent manner and is important for cAMP-stimulated actin polymerization (Funamoto et al., 2001; Sun and Firtel, 2003). Mutants lacking PhdA exhibit defective chemotaxis and loss of cell polarization. PhdA is not required for PKB activation. Therefore, pathways downstream of PhdA and PKBA may differ even though both eventually stimulate actin polymerization.

A recent proteomic study identified novel PIP3-binding PH domain-containing proteins, PhdB, PhdG, and PhdI (Zhang et al., 2010; Jeon et al., 2009). Although all three proteins bind to PIP3 *in vitro*, PIP3-mediated regulation is relatively weak compared to that of PKBA, indicating that there may be additional mechanisms that control them. Supporting the importance of PhdB and PhdG in chemotaxis, loss of PhdB or PhdG blocks efficient chemotaxis in response to cAMP. How these proteins mediate PIP3 signaling remains to be determined.

Cytosolic regulator of adenylyl cyclase (CRAC) is required for activation of adenylyl cyclase, which converts ATP to cAMP at the trailing edge (Insall et al., 1994a; Lilly and Devreotes, 1994, 1995). Interestingly, CRAC also contains a PIP3-specific PH domain and was the first protein identified to be recruited to the plasma membrane of the leading edge upon cAMP stimulation (Parent et al., 1998; Comer et al., 2005). Translocation of this enzyme to the plasma membrane is required for chemotaxis, cAMP-stimulated actin polymerization and adenylyl cyclase activation (Comer et al., 2005). Therefore, CRAC appears to stimulate both actin polymerization and adenylyl cyclase activity at the leading edge. It has been proposed that CRAC initiates a series of events that ultimately lead to the activation of adenylyl cyclase (Comer et al., 2005).

Tsunami: a possible upstream regulator of PIP3 signaling

Although microtubules play important roles in the polarization of many cell types, the role of these structures in *Dicyostelium* chemotaxis is relatively less characterized. Recent studies using forward genetics identified a Fused-related kinase called Tsunami that is associated with microtubules (Tang et al., 2008). A fraction of Tsunami also forms puncta in the cell periphery. Cells lacking Tsunami showed compromised chemotaxis in response to cAMP and extended many elongated filopodia in many different directions. Consistent with this increase in filopodia production, levels of actin filaments are increased in *tsunami*-null cells. Furthermore, cAMP-stimulated actin polymerization is dramatically increased in the

mutant. How Tsunami affects actin polymerization is currently unknown. Nevertheless, because PIP3 production is increased in *tsunami*-null cells and its kinase domain is essential for function, Tsunami may regulate the PIP3 pathway by phosphorylating upstream components.

2. PLA2: another lipid signaling pathway parallel to the PIP3 pathway

PIP3 is not the only mediator of intracellular signaling in chemotaxis and actin polymerization at the leading edge. *Dictyostelium* mutant cells lacking all class I PI3Ks still demonstrate actin polarization and migrate toward cAMP though their chemotactic responses are slightly reduced (Hoeller and Kay, 2007). Recent studies have revealed additional signaling pathways that functionally overlap with the PIP3 pathway.

For example, phospholipase A2 (PLA2A) controls actin polymerization during chemotaxis (Liao *et al.*, 2010; Chen *et al.*, 2007; van Haastert *et al.*, 2007; Kolsch *et al.*, 2008; Rericha and Parent, 2008). PLA2A is a Ca^{2+} -sensitive cytosolic phospholipase and converts phosphatidylcholine to arachidonic acid *in vitro*. While individual loss of PIP3 signaling or the PLA pathway affected chemotaxis only slightly, cells defective in both PI3Ks and PLA2A showed significant impairment in chemotaxis, cAMP-stimulated actin polymerization, and morphological polarity. It is not clear whether PLA2 is activated at the leading edge since PLA2A is distributed evenly throughout the cytosol and does not localize to the plasma membrane in the presence or absence of cAMP gradients. Interestingly, the amount of arachidonic acid-derived lipid increases upon cAMP stimulation in a PLA2A-dependent fashion. Moreover, addition of arachidonic acid restores the ability of *pla2a*-null cells to form chemotactic aggregates in the presence of LY294002, an inhibitor of PI3Ks. Such arachidonic acid-related lipids may therefore function to stimulate actin polymerization and promote cell polarization during chemotaxis. Interestingly, arachidonic acid can also serve as a chemoattractant in *Dictyostelium*, and it would be interesting to investigate whether PLA2A is involved in this process (Schaloske *et al.*, 2007).

3. Soluble guanylyl cyclase: regulation at the leading and trailing edges

Analysis of chemotaxis in cells that underwent development for 7 hours instead of the usual 5 hours commonly studied revealed the involvement of additional pathways involving soluble guanylyl cyclase (sGC), which produces cGMP upon cAMP stimulation (Veltman *et al.*, 2008). This pathway controls both extension and suppression of pseudopods (Bosgraaf *et al.*, 2002; Goldberg *et al.*, 2002; Veltman *et al.*, 2005; Veltman and Van Haastert, 2006; Veltman and van Haastert, 2008). At the trailing edge, cGMP and its target protein GbpC, which is a member of the Roco protein family containing a kinase domain, suppresses pseudopod extension by regulating myosin II filaments in this region (van Egmond and van Haastert, ; Goldberg *et al.*, 2002; Bosgraaf *et al.*, 2005). This allows cells to restrict pseudopod extension to the leading edge, resulting in efficient chemotaxis.

Myosin II is an actin-based motor protein that assembles into bipolar thick filaments, which are then concentrated in the lateral and trailing cortices of chemotaxing cells. Myosin II has been proposed to facilitate posterior contraction of cells and suppression of lateral pseudopodia (Yumura and Uyeda, 2003; Bosgraaf and van Haastert, 2006). However, recent evidence also suggests the involvement of myosin II inhibition of leading edge signaling (Lee *et al.* 2010). Regulation of myosin II is mediated by phosphorylation of regulatory light chains and heavy chains (Bosgraaf and van Haastert, 2006; Vicente-Manzanares *et al.*, 2009). Phosphorylation of regulatory light chains controls ATPase activity of myosin II, while heavy chain phosphorylation controls its assembly and localization. Specific kinases mediate myosin II phosphorylation, namely myosin light chain kinases and heavy chain kinases. GbpC is important for phosphorylation of both the light and heavy chains. In *gbpc*-

null cells, basal level phosphorylation, as well as cAMP-stimulated light and heavy chain phosphorylation, was decreased. GbpC may directly or indirectly phosphorylate myosin light and heavy chain kinases to control their activities.

Interestingly, sGC is also important for the extension of pseudopods at the leading edge, and this function is independent of its catalytic activity and therefore cGMP. The N-terminal region of sGC, which lacks the catalytic domain, can be localized to the leading edge and sufficient to regulate pseudopod extension. Since GbpC has a RasGEF domain, this protein may crosstalk with the Ras GTPases-TORC2 pathway as described below.

4. Ras GTPases and TorC2: crosstalk with the PIP3 pathway

Another pathway parallel to PIP3 signaling involves Ras GTPases, TORC2, and PKBR (Cai *et al.*, 2010; Charest *et al.*, 2010; Liao *et al.*, 2010; Kamimura *et al.*, 2008; Kortholt and van Haastert, 2008; Zhang *et al.*, 2008; Sasaki and Firtel, 2009). The *Dictyostelium* genome contains 15 Ras GTPases, and Ras C and G have been shown to mediate chemotactic signaling (Khosla *et al.*, 2000; Lim *et al.*, 2001). Ras activation occurs downstream of heterotrimeric G-proteins as mutants lacking subunits of G-proteins display defective Ras activation. Proteins that directly activate Ras GTPases are guanine nucleotide exchange factors (GEFs), which work by replacing GDP with GTP within Ras GTPases. Two GEFs, Aimless and RasGEFH, have been suggested to activate Ras C (Charest *et al.*, 2010; Insall *et al.*, 1996). Importantly, these GEFs form a protein complex together with the scaffolding protein Sca1 and the protein phosphatase PP2A. This complex, called the Sca1 complex, is located at the leading edge of chemotaxing cells where Ras GTPase activation occurs.

Ras C in turn activates TORC2, a multi-subunit protein kinase (Cai *et al.*, 2010; Charest *et al.* 2010). One subunit, Rip3, contains a Ras binding domain, suggesting that Ras C regulates TORC2 directly (Lee *et al.*, 2005). As described above, Ras GTPases also stimulate PI3Ks. Therefore, Ras GTPases likely regulate both the TORC2-PKBR and PI3K-PKBA pathways. Upon activation, TORC2 phosphorylates and activates PKBA, a PIP3-responsive PH domain-containing PKB, and PKBR, a membrane-tethered form of PKB (Kamimura *et al.*, 2008).

Phosphorylation sites are located within their C-terminal hydrophobic motif. After phosphorylation by TORC2, PKBA and PKBR are further phosphorylated by two phosphoinositide-dependent kinases (PDKs) within their activation loops, which are different from their hydrophobic motifs. This second phosphorylation event is required for PKB activation. Activation of PKBR is suggested to occur at the leading edge since PKBR substrates are mainly phosphorylated in this region (Kamimura *et al.*, 2008). PKBA is also activated at the leading edge as PKBA is recruited to this region by PIP3-PH-domain interactions (Cai *et al.*, 2010; Charest *et al.* 2010). Therefore, Ras C-TORC2 signaling most likely converges and participates in crosstalk with the PIP3 pathway through PKBA phosphorylation.

Interestingly, PKBA and PKBR phosphorylate Sca1, which results in inhibition of Ras C activation and provides negative feedback for the Ras C/TORC2/PKB pathway (Charest *et al.*). In support of this idea, *pkba*- or *pkbr*-null cells exhibit prolonged Ras C activation. This feedback allows cells to activate this pathway transiently in response to cAMP. The role of PP2A in the Sca1 complex is less well understood. Since Sca1 mutants lacking the PP2A binding region are unable to activate Ras C and PKBs, PP2A is functionally important. Understanding the exact function of PP2A in the Sca1 complex would be important and awaits further study.

Conclusion and Perspective

The study of *Dictyostelium* chemotaxis has deepened our understanding of functionally overlapping pathways that mediate chemotactic signaling. These discoveries provide a framework for dissecting the complex signaling network involved in this biological process and also reveal additional important questions. How does G-protein activation lead to stimulation of RasGEFs? What recruits PI3K to the plasma membrane? What is the exact lipid product that mediates PLA2 signaling? What are additional downstream effectors for PIP3? What are other functional substrates for TORC2 and PKBs? How does the chemotactic signaling network stimulate actin polymerization? Understanding these key questions will be the next exciting challenges in the field of chemotaxis.

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Figure. Chemotactic signaling pathways

During chemotaxis, cAMP stimulates intracellular signaling through the cAR1 receptor and coupled heterotrimeric G-proteins (G). These signaling pathways control actin polymerization as well as the assembly and activity of myosin II. In the PtdIns(3,4,5)P₃ (PIP₃) pathway, the amount and localization of PIP₃ are regulated by PI3Ks and PTEN. This signaling cascade participates in crosstalk with the Ras GTPase-TORC2 pathway by interacting with PI3K-Ras GTPase and activating PKBs. In the Ras GTPase-TORC2 pathway, PKBs generate a negative feedback loop by phosphorylating and inactivating the Sca1 complex (Sca1C). Soluble guanylyl cyclase (sGC) also modulates actin polymerization and myosin II. PLA2A generates a yet-to-be-determined arachidonic acid-related lipid (AARL) as a second messenger. Tsu: Tsunami, PDK: phosphoinositide dependent kinase, GbpC: cGMP binding protein C, MHCK: myosin heavy chain kinase, MLCK: myosin light chain kinase.