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Oscillatory Signaling and Network Responses during the Development of *Dictyostelium discoideum*

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Abstract

Periodic biological variations reflect interactions among molecules and cells, or even organisms. The *Dictyostelium* cAMP oscillatory circuit is a highly robust example. cAMP oscillations in *Dictyostelium* arise intracellularly by a complex interplay of activating and inhibiting pathways, are transmitted extracellularly, and synchronize an entire local population. Once established, cAMP signal-relay persists stably for hours. On a two-dimensional surface, >100,000 cells may form a single coordinated territory. In suspension culture, >10¹⁰ cells can oscillate in harmony. This review focuses on molecular mechanisms that cyclically activate and attenuate signal propagation and on chemotactic responses to oscillatory wave progression.

Keywords

cAMP; adaptation; chemotaxis; protein kinases; PI3K; PTEN; PLA₂; cGMP

1. Oscillatory signals in *Dictyostelium discoideum*

Dictyostelium discoideum are social amoebae that grow as individual cells in the presence of a nutrient-rich food source. During conditions of starvation, *Dictyostelium discoideum* cells spontaneously form signaling centers that secrete cAMP. Surrounding cells chemotax inwardly toward the cAMP source and synthesize and relay cAMP to more outlying, neighboring cells. These latter cells, in turn, follow the response-relay path, thus defining a territory and directing streams of chemotaxing cells toward the centers of signal-initiation. Eventually, these aggregate into groups of ~100,000 cells and develop as a multicellular organism. Related species follow similar aggregation events, but utilize different chemoattractants.

Synthesis and secretion of cAMP during aggregation is not continuous. Outward propagation of cAMP from the signaling centers occurs with a “wave-pattern” and an

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oscillation period of ~6 min. This ensures a directional movement of the chemoattractant signal with a defined positive temporal and spatial gradient. Cellular response during aggregation can be monitored in real-time by dark-field, video microscopy (see Figure 1A). The observed optical density waves reflect the different light scattering patterns of cells that are alternatively responsive and moving or adapted (see below), as they encounter and respond to the progressing waves (Devreotes et al., 1983; Rietdorf et al., 1996). Synchronized cAMP oscillations can also be measured directly in suspension cultures of *Dictyostelium* (Figure 1B).

cAMP signaling is critical for many aspects of *Dictyostelium* development (Kimmel and Firtel, 2004; Kimmel, 2005). In addition to directing chemotaxis, oscillatory signaling promotes an intracellular positive feedback loop necessary for the maximal expression of many of the components within the cAMP regulatory pathways. Following aggregation, cAMP signaling coordinates cell sorting, pattern formation, and morphogenetic changes. Wave-type oscillations within the multicellular organism can organize cell movement (Dormann and Weijer, 2001), but a more continuous response is required for post-aggregation and cell-type specific gene expression (Kimmel and Firtel, 2004; Kimmel, 2005).

Dictyostelium has proven an excellent and unique system to study the dynamics of signal oscillation and natural wave progression. During early development, prior to the formation of multicellular structures, cAMP propagation and chemotactic response can be separately analyzed. cAMP relay involves interactions among activators and inhibitors that cyclically balance the actions of the synthetic pathway and the degradative machinery. Response to the extracellular cAMP signal elicits multiple intracellular second messenger pathways with varying kinetics of activation (van Haastert et al., 2007). cAMP and cGMP accumulate as the adenylyl and guanylyl cyclases (AC, GC) are respectively activated. Ion fluxes are observed for H⁺, K⁺, and Ca²⁺. Other activated pathways involve Phosphoinositide 3-kinase (PI3K), Phospholipase C (PLC), and Phospholipase A₂ (PLA₂). Their biochemical associations ultimately organize chemotactic response.

2. cAMP Oscillations and signal relay

cAMP oscillations require receptor stimulation, activation and adaptation of adenylyl cyclase, and the cyclical degradation of both the intracellular and extracellular pools of cAMP. In this section we primarily focus on the components that regulate cAMP synthesis and signal-relay. The response pathways that direct chemotactic movement to oscillating cAMP waves are discussed in the section following. Ultimately, it was not possible to include every regulatory input.

2.1. Cell surface cAMP receptors, CARs

The cell surface receptors that mediate extracellular cAMP signaling are members of the 7-transmembrane family that couple to heterotrimeric G proteins. There are four closely related cAMP receptors in *Dictyostelium*, CAR1, CAR2, CAR3, and CAR4, with distinct temporal and spatial patterns of expression during development (Saxe et al., 1991; Johnson

et al., 1993; Saxe et al., 1993; Louis et al., 1994) and affinities for cAMP (Johnson et al., 1992; J.M. Louis and A.R. Kimmel, unpublished observations).

CAR1 has the highest affinity for cAMP, with 2 affinity classes (K_d ~30nM and ~300nM), depending on G protein coupling status (see below). CAR3 affinity for cAMP is slightly lower than is CAR1, while CAR2 and CAR4 affinities are an order of magnitude lower (K_d >5 μ M). As would be expected, their lower affinities reflect their relative exposure to increasing cAMP during development.

CAR1 is expressed earliest during development. It is not detected during logarithmic growth in rich nutrients, but is induced as cells starve and expression increases ~10-fold during aggregation (Saxe et al., 1991). Gene induction during development follows in the order of *CAR3*, *CAR2*, and then *CAR4* (Johnson et al., 1993; Saxe et al., 1993; Louis et al., 1994). CAR3 is first expressed during late aggregation as cAMP levels rise, while CAR2 and CAR4 are not detected until multicellular structures form.

CAR1 is the predominant cAMP receptor during aggregation, and cAMP production and aggregation are primarily mediated by CAR1 (Figure 2). CAR1 is distributed uniformly around the cell periphery (Figure 3), allowing all regions of the cell to be equally responsive to a progressing cAMP wave (Xiao et al., 1997). Cells lacking CAR1 fail to initiate cAMP signaling, respond chemotactically, or form multicellular aggregates (Klein et al., 1988). Although CAR3, CAR2, and CAR4 function largely as morphogen receptors for cAMP and regulate cell-type specific gene expression and later developmental events (Kimmel and Firtel, 2004; Kimmel, 2005), they may also help organize pattern formation and cell motility during multicellular development (Dormann and Weijer, 2001).

CAR3 has a minimal role during wild-type (WT) aggregation, but developmental defects of *car1*-nulls can be rescued by prematurely inducing CAR3 expression. Under these conditions, CAR3 can substitute fully for CAR1. While *car1/car3*-nulls are unresponsive to exogenous cAMP, their use for expression of WT and mutant variants of the various CARs has been invaluable. Data indicate that receptor number and relative affinity for cAMP can influence wave dynamics and geometry through changes in frequency and amplitude of cAMP oscillations (Dormann et al., 2001).

The different cAMP affinity classes of CAR1 impact separate regulatory pathways. While most (~80%) CAR1 binding sites are of the lower affinity (~300nM) class, the half-maximal response, EC_{50} ~10nM, for activation of AC and production of cAMP approximates that of the higher affinity class (van Haastert et al., 1986; J. Brzostowski and A.R. Kimmel, unpublished observations); no further AC activation can be elicited beyond 100nM. In contrast, the CAR1-regulated activation curve for Ca^{2+} influx follows precisely that of the lower affinity class, EC_{50} ~250nM (Milne and Devreotes, 1993). Chemotactic response is more complex, has a broader dynamic range, and involves multiple pathways (see below), which are maximally responsive to differing concentrations of cAMP.

Regardless of affinity, many CAR1-activated pathways adapt (desensitize) to a non-varying extracellular cAMP stimulus (Figure 2). If the stimulus is sub-saturating, a further response can only be elicited if the cAMP stimulus is increased. At saturation, however, no additional

response can occur. Adaptive pathways include AC and GC activation, Ca^{2+} influx, Filamentous (F-) actin polymerization, cell shape changes, etc. (Kimmel et al., 2004). Also during early development, cAMP-regulated gene expression requires oscillatory signaling and is inhibited by a continuous cAMP stimulus (Kimmel and Firtel, 2004; Kimmel, 2005). Once adapted, cells remain unresponsive until the extracellular stimulus is removed. Adaptation occurs rapidly, whereas de-adaptation is a slower process.

Still, not all CAR-regulated pathways adapt (Figure 2). Persistent cAMP stimulation promotes the phosphorylation of multiple serine residues in the cytoplasmic carboxyl-terminus of CAR1 and the phosphorylation and activation of ERK2 (Hereld et al., 1994; Brzostowski and Kimmel, 2006). Both proteins remain phosphorylated until the cAMP stimulus is removed. In contrast to early gene expression, post aggregation gene expression requires a continuous exposure to high concentrations of cAMP (Kimmel and Firtel, 2004; Kimmel, 2005).

2.2. G proteins, cAMP affinity, and adaptation

CAR1 signaling during aggregation requires coupling with and signaling via the heterotrimeric G proteins comprised of α , β , and γ subunits. In unstimulated cells, $G\alpha$ is bound to GDP in a complex with $G\beta\gamma$ that interacts directly with CAR1. Upon stimulation with cAMP, the G proteins become activated; GTP is exchanged for GDP and the heterotrimeric complex dissociates from CAR1 as a $G\alpha$ -GTP monomer and a $\beta\gamma$ heterodimer.

Dictyostelium has single genes for $G\beta$ and $G\gamma$, but 12 $G\alpha$ genes (Wu et al., 1995; Zhang et al., 2001; Brzostowski et al., 2002). $G\beta$ is absolutely essential for cAMP signaling, aggregation, and development. $G\alpha_2$ is the principal $G\alpha$ protein that interacts with CAR1 during aggregation, and among the cell lines deleted for a single $G\alpha$, only ga_2 -nulls are developmentally blocked (Kumagai et al., 1989; Kumagai et al., 1991). The primary, but not sole, function of $G\alpha_2$ may be to ensure $\beta\gamma$ signal transduction, rather than to directly activate a downstream effector in its GTP-bound state (Pupillo et al., 1992). For example, ga_2 -nulls are unable to activate early gene expression, but they can be rescued by enhancing $G\beta\gamma$ -signaling by overexpression of $G\alpha_9$ (Brzostowski et al., 2004).

Activation of $G\alpha_2$ by cAMP has been observed with live-cell FRET (fluorescence resonance energy transfer) imaging in cells expressing both a $G\alpha_2$ fused to the CFP fluorescent reporter and a $G\beta$ fused to a YFP reporter (Janetopoulos et al., 2001). When $G\alpha_2$ and $G\beta$ are closely complexed as in quiescent cells, spectral excitation of CFP causes a resonance transfer to and energy emission from YFP. However upon cAMP stimulation and G protein activation and dissociation, there is a loss of FRET, as reflected in a quantifiable decrease in YFP emission and a corresponding increase in CFP fluorescence. Maximal FRET loss is observed in <10 sec. and persists for the length of the cAMP stimulus. Conversely, full FRET recovery is observed within 2 min. of the removal of cAMP, faster than is re-sensitization (de-adaptation). These data indicate that the activation state of $G\alpha_2\beta\gamma$ does not mediate adaptation (Janetopoulos et al., 2001). FRET analyses have also been used to determine the spatial activation of G proteins in *Dictyostelium* chemotaxing in a stable gradient; $G\alpha_2$ is spatially activated in proportion to the cAMP signal-strength experienced

by the various cell regions (Figure 3). This activation pattern is distinctly less polarized than that of more downstream components and indicates pathway complexity beyond simple G protein cycling (Janetopoulos et al., 2001; Figures 2,3; see below).

The two affinity classes for CAR1 (~30nM and ~300nM) may indicate the presence or absence of functional G protein coupling. The EC₅₀ (~10nM) for G protein activation by cAMP approximates that of the high affinity cAMP binding sites (Janetopoulos et al., 2001). In addition, coincident with the activation and dissociation of G proteins in response to cAMP stimulation, the high affinity CAR1 binding sites convert to a lower affinity class (van Haastert et al., 1986). Direct activation of G proteins with GTPγS also induces their uncoupling from CAR1 and the loss of high affinity cAMP binding. By these criteria there are relatively few high affinity sites in *ga2*-nulls (Kumagai et al., 1991), but they can be partially restored by overexpression of another CAR1-binding Gα protein, Gα9 (Brzostowski et al., 2004).

In *Dictyostelium*, adaptation/de-adaptation temporally reflects the phosphorylation state of CAR1 and several experimental approaches have indicated that phosphorylated CAR1 has low affinity for cAMP (Caterina et al., 1995; Xiao et al., 1999). Although in mammalian systems the interaction of phosphorylated G protein-coupled receptors with arrestins causes desensitization, a causal link between CAR1 phosphorylation and adaptation in *Dictyostelium* is less clear. First, low affinity responses (e.g. Ca²⁺ influx) can still adapt (Milne and Devreotes, 1993). Also, receptor phosphorylation may not prevent G protein interaction (Janetopoulos et al., 2001). Cells stimulated with cAMP, but washed free of the ligand at 0°C, undergo G protein re-association in the absence of receptor de-phosphorylation. Furthermore, these cells are fully re-responsive to cAMP-stimulated G protein re-activation, i.e. loss of FRET. The data suggest that low affinity phosphorylated receptors still mediate G protein signaling. However, *car1/car3*-nulls that only express CAR1 variants, with C-terminal truncations or Serine-to-Alanine substitutions, that cannot be phosphorylated may not undergo normal adaptive relay (J. Brzostowski and A.R. Kimmel, unpublished observations).

Finally, several CAR-mediated responses occur in *gβ*-null cells and are presumed to be independent of functional G protein interaction (Brzostowski and Kimmel, 2001). These include CAR1 phosphorylation, Ca²⁺ influx, ERK2 activation, and STAT (signal transducer and activator of transcription) phosphorylation.

2.3. The Adenylyl Cyclases (ACs)

The primary adenylyl cyclase that is activated during aggregation is ACA (Figure 2). ACA is a 12-transmembrane protein, with topological homology to the mammalian G protein-regulated ACs (Pitt et al., 1992). ACA expression is induced by starvation and, although, *aca*-null cells are unable to establish extracellular cAMP signaling and aggregate by themselves, they are fully responsive to cAMP and will synergize (co-develop) within a population of WT cells. Two additional ACs, ACB/R and ACG, exist in *Dictyostelium* (Kim et al., 1998; Soderbom et al., 1999). ACB/R and ACG are structurally distinct from ACA and are insensitive to exogenous cAMP and unresponsive to G protein activation. As the activities of ACB/R and ACG do not appear to undergo oscillatory regulation, they are

unable to support chemotactic aggregation; they do, however, appear to compensate for the loss of ACA for all other cAMP-regulated developmental parameters. ACB/R is expressed in growing cells through terminal differentiation and has a significant function after aggregation. ACG is normally expressed very late during development, but is prematurely induced in cells lacking ACA.

ACA is activated following stimulation with extracellular cAMP. Activity increases within ~30 sec. and then rapidly adapts if the stimulus is constant and/or saturating (Figure 2). ACA activity can also be monitored *in vitro*. The addition of GTP γ S to cell lysates directly activates both heterotrimeric and Ras superfamily G proteins (see below) and thus bypasses the requirement for extracellular cAMP stimulation of CAR1 to activate ACA (Theibert and Devreotes, 1986). Such analyses have allowed the evaluation and placement of potential effectors in the activation pathway of ACA. Although ACA can not be activated by cAMP in cells lacking either G α 2 or G β , *ga2*-nulls are fully responsive to GTP γ S, whereas *g β* -nulls are insensitive (Kumagai et al., 1991; Wu et al., 1995). Data suggest that while ACA activation requires CAR1/G protein signaling, this appears to be mediated via G $\beta\gamma$, and not by G α 2. Other required signaling components include CRAC and TORC2 (Figure 2; see below).

As cells stream within aggregating territories, they align anterior-to-posterior. Significantly, ACA-YFP fusion proteins localize to intracellular vesicles and to the rear of polarized chemotaxing cells (Kriebel et al., 2003; Figure 3). In a mixed population of aggregating WT and *aca*-null cells, the placement of an *aca*-null within a stream terminates signal propagation. The data suggest that cAMP may be directionally secreted from the posterior of cells to orient neighboring cell movement. It is unresolved how ACA becomes spatially localized, how signaling at the cell anterior may be transduced distally to ACA (Figure 2,3; see below), or how cAMP is secreted.

2.4. CRAC

CRAC, cytosolic regulator of adenylyl cyclase, was first recognized during a genetic screen for cells that were unable to aggregate. Eventually the gene and protein were identified (Insall et al., 1994; Lilly and Devreotes, 1994). Cytosolic CRAC is absolutely required in GTP γ S assays for ACA activation. However, structural and biochemical analyses indicate that CRAC also requires functional membrane association. The amino-terminus of CRAC contains a pleckstrin homology (PH) domain, an ~100 amino acid motif that interacts with phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5P; PIP₃) to facilitate recruitment to membrane compartments (Figure 2).

PIP₃ is generated by the 3' phosphorylation of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PI4,5P₂; PIP₂) by PI3K, a CAR1/G protein-activated enzyme. PI3K action is antagonized by the PI 3-phosphatase PTEN (phosphatase and tensin homolog), which re-converts PIP₃ to PIP₂ (Figure 2; see below). Upon PIP₃ accumulation following a cAMP stimulus, CRAC is recruited to the membrane (Parent et al., 1998). Both membrane recruitment of CRAC and CRAC-dependent activation of ACA require a functional PH moiety (Comer et al., 2005).

In chemotaxing cells, CRAC is primarily localized to the leading edge of cells (Parent et al., 1998) and is thus spatially separated from ACA (Figures 2, 3; see below). Beyond, the mechanistic activation of ACA by CRAC is not known. Finally, *crac*-nulls are not only unable to activate ACA in response to cAMP, but they also chemotax and aggregate poorly when co-developed with WT cells. The role of CRAC in chemotaxis is not understood, but it involves sequences separate from those required for ACA activation (Comer et al., 2005).

2.5. TORC2 and Akt/PKBR1

Other screens for regulators of aggregation have identified Pia, RIP3, Akt, and PKBR1, a PKB/Akt-related protein kinase (Chen et al., 1997; Lee et al., 1999; Meili et al., 1999; Meili et al., 2000). *pia*-nulls have an aggregation-minus phenotype and cannot activate ACA when stimulated with exogenous cAMP (Chen et al., 1997). RIP3 was identified in a yeast two-hybrid screen using an activated form of human H-Ras as bait, and cells deficient in RIP3 have a phenotype similar to that of *pia*-nulls (Lee et al., 1999). It is now appreciated that Pia and RIP3, with TOR kinase and Lst8, are components of TOR complex 2 (TORC2; Loewith et al., 2002), and loss of Lst8 also causes a phenotype similar to that of *pia*- and *rip3*-nulls (Lee et al., 2005). *tor*-nulls are lethal due to a growth defect involving a separate TOR protein complex, TORC1 (X. Huang and A.R. Kimmel, unpublished).

Akt and PKBR1 are structurally and functionally related members of the AGC (PKA, PKG, PKC) protein kinase family. Akt and PKBR1 share a similar kinase domain, activation loop, and C-terminal regulatory domain (Meili et al., 1999; Meili et al., 2000). Akt has an N-terminal PH domain that allows membrane recruitment upon PI3K activation; PKBR1 lacks the PH domain but has an amino-terminal myristoylation site, which enables its constitutive localization on the membrane. Loss of either Akt or PKBR1 causes poor development, while the double mutant *akt/pkbr1*-null leads to complete developmental failure.

Activation of both kinases involves phosphorylation at two sites, one within the activation loop and one at a hydrophobic (HM) motif within the C-terminal regulatory domain (Meili et al., 1999; Meili et al., 2000). The activation loop is targeted by PDK1, phosphoinositide-dependent protein kinase-1, while TORC2 mediates phosphorylation of the HM motif. *pia*-, *rip3*-, and *lst8*-nulls all have decreased levels of Akt and PKBR1 kinase activity (Lee et al., 2005).

Similar to *crac*-nulls, lysates from cells lacking Pia, RIP3 or Lst8 are insensitive to GTP γ S-activation of ACA, as are mixed extracts from cells lacking different TORC2 components (Lee et al., 2005). However, ACA activation by GTP γ S can be reconstituted in mixed cell extracts from *crac*-nulls and either *pia*-, *rip3*-, or *lst8*-nulls (Lee et al., 2005). Thus, CRAC and TORC2 appear to function separately to activate ACA (Figure 2). Potentially, TORC2-Akt/PKBR1 may participate as a regulatory module upstream of ACA (Figure 2).

2.6. The PDEs

cAMP wave propagation not only requires regulated oscillations in ACA activity, but also the cyclical degradation of accumulated cAMP (Figure 1A,B). Different cyclic nucleotide phosphodiesterases (PDEs) act on the intracellular and extracellular pools of cAMP to

generate AMP. Disruption or overexpression of the various PDEs interferes with aggregation (Maeda et al., 2004; Sawai et al., 2005).

Extracellular cAMP is degraded by a secreted PDE (Franke et al., 1991), whose activity is regulated by a secreted glycoprotein phosphodiesterase inhibitor (PDI). High extracellular cAMP concentrations promote PDE expression, whereas reduced cAMP levels enhance PDI expression. Extracellular PDE activity is absolutely essential for cAMP oscillatory signaling (Figure 2).

Intracellular cAMP is degraded by a different PDE, RegA, among others (Shaulsky et al., 1998). RegA activity is subject to inhibitory regulation by phosphorylation via ERK2 (Maeda et al., 2004). *regA*-nulls have elevated cAMP, while *erk2*-nulls have very low cAMP levels. *regA/erk2*-nulls have high levels of cAMP, similar to that of *regA*-nulls (Maeda et al., 2004). Biochemical data are also consistent with ERK2 acting negatively upstream of RegA (Brzostowski and Kimmel, 2006). ERK2 is only active when phosphorylated. In unstimulated cells the upstream ERK2 kinase is inactive, while the de-phosphorylating ERK2 PPase is active (Figure 2), causing ERK2 inhibition, RegA activation, and resulting low intracellular cAMP levels. In contrast, ERK2 is phosphorylated and activated in cAMP-stimulated cells, thus inhibiting RegA and allowing the accumulation of intracellular cAMP (Figure 2).

As an intracellular second message, cAMP can activate the cAMP-dependent kinase PKA. PKA is not essential for cell growth, but is required for aggregation and development, and for expression of ACA (Kimmel, 2005). In the inactive state PKA is a heterodimer with a catalytic subunit and a cAMP-binding regulatory subunit (PKA-R). Once cAMP binds to PKA-R, the catalytic subunit is released and activated. Apart from activating a myriad of downstream effectors, PKA may also participate in an inhibitory feedback loop to down-regulate cAMP accumulation. Cells that overexpress the catalytic subunit of PKA or that lack the inhibitory subunit accumulate only low levels of cAMP (Maeda et al., 2004). While several targets for PKA action have been postulated, none are consistent with experimental evidence (Brzostowski and Kimmel, 2006). RegA has a putative PKA site, making it a potential regulatory motif. Perhaps, PKA phosphorylation of RegA can ameliorate the inhibitory effects of ERK2 (Figure 2).

3. Chemotaxis

During early development, the extracellular cAMP oscillations are propagated as waves that direct chemotaxis and aggregation. *Dictyostelium* are able to sense, orient, and move directionally “up” gradients of cAMP that differ by <5% across the length of the cell. Despite these relatively shallow extracellular gradients and the proportional activation of cAMP receptors at the opposite cell margins (Figure 3), chemotaxing cells are highly polarized, with intracellular components spatially compartmentalized to leading edge and posterior locales (Figure 3). F-actin is preferentially polymerized at the anterior of these cells where pseudopodia also arise (Figure 3), as their formation is suppressed laterally. Pseudopodia extending toward the gradient are selectively maintained, reinforcing directional movement. Multiple mechanisms, including adaptive responses and feedback

amplification, are required to integrate a complex series of downstream intracellular signaling pathways that ultimately define cell polarization during chemotaxis to oscillating cAMP waves.

3.1. PIP₃-Signaling

Activated PI3K phosphorylates PIP₂ to produce PIP₃, while PTEN hydrolyzes PIP₃ to PIP₂ (Figure 2). PI3K and PTEN are spatially separated in the cell, reinforcing their reciprocal actions (Funamoto et al., 2002; Iijima and Devreotes, 2002; Figure 3). Further, since PIP₂ and PIP₃ diffuse through the membrane at rates that are ~100-times slower than cytosolic messengers (e.g. cAMP, cGMP, Ca²⁺), strong intracellular phospholipid gradients can be maintained through regulation of the local concentrations of PI3K and PTEN (Postma and Van Haastert, 2001).

PI3K/PTEN response to cAMP follows that of adaptive signaling (Figure 2). In unstimulated cells, PI3K is primarily cytosolic, whereas PTEN, which has high affinity for PIP₂, is distributed at the plasma membrane (Funamoto et al., 2002; Iijima and Devreotes, 2002; Iijima et al., 2004); thus, basal levels of PIP₃ are relatively low. Upon stimulation by cAMP, PI3K is rapidly (~10 sec.) translocated to and activated at the cell periphery, as PTEN is de-localized, leading to the accumulation of PIP₃ (Funamoto et al., 2002; Iijima and Devreotes, 2002; Huang et al., 2003). In the presence of saturating levels of cAMP, this response is transient. PI3K becomes de-localized from the plasma membrane within 30 sec., PTEN translocates back, and PIP₃ levels decline (Funamoto et al., 2002; Huang et al., 2003). However, during chemotaxis in the presence of a persistent, non-saturating cAMP gradient, the reciprocal localizations of PI3K and PTEN establish relatively stable and polarized asymmetric intracellular distributions (Figures 3), with the former restricted to the leading edge of the cell nearest the signaling source, and the latter to the sides and cell posterior (Funamoto et al., 2002; Iijima and Devreotes, 2002; Huang et al., 2003). Accordingly, PIP₃ accumulates at the leading edge, with PIP₂ exhibiting a reciprocal pattern, in parallel to PTEN.

Downstream, PIP₃ acts as a localizing moiety rather than as a regulatory element per se. PH-containing proteins, such as CRAC and Akt, consequently also become restricted to the leading edge of *Dictyostelium* during chemotaxis (Parent et al., 1998; Meili et al., 1999), as visualized in cells expressing a PH domain in fusion with a fluorescent reporter (e.g. GFP; Figure 3). Functional activation of Akt requires membrane translocation via PH domain interaction with PIP₃ (Meili et al., 1999), and cells that only express variants of CRAC that lack PH domains have significant developmental defects (Comer et al., 2005).

The co-association of PIP₃ accumulation at the leading edge has inferred a dependent role during chemotaxis. F-actin and actin-mediated cellular projections are rapidly accumulated at the leading edge in response to cAMP (Rubino et al., 1984; Condeelis et al., 1988; Figure 3), and the pseudopodia, which extend directionally toward the chemoattractant source, arise primarily in this region. *pten*-nulls that artificially expand PIP₃ beyond the cell anterior produce multiple peripheral cellular protrusions (Iijima and Devreotes, 2002; Iijima et al., 2004; Kortholt et al., 2007). Similarly, expression of a constitutively membrane bound, myristoylated-tagged PI3K shows elevated and constitutively unregulated PIP₃ localization

as well as expansion and persistence of lateral pseudopod formation (Funamoto et al., 2002; Iijima and Devreotes, 2002; Iijima et al., 2004). Both cell lines have poor directionality and impaired chemotaxis, implying that localized PIP₃-signaling is capable of activating pathways that promote F-actin polymerization, cell orientation, and associated chemotactic responses (Funamoto et al., 2002; Iijima and Devreotes, 2002; Iijima et al., 2004).

Dictyostelium possess five genes that encode gamma-type PI3Ks involved in chemotactic signaling (Takeda et al., 2007). Until recent technical developments (Faix et al., 2004), this genetic complexity had limited the ability to analyze cells fully compromised for PIP₃-signaling (see below). As an alternative, LY294002 was used as a selective inhibitor of PI3K activity in WT cells or cells lacking *PI3K1* and *PI3K2* (Funamoto et al., 2001). Data clearly indicate that while such cells had severe reductions in PH-domain membrane translocation in response to cAMP stimulation, they were still highly chemotactic (Loovers et al., 2006; Figure 4).

Nonetheless, other aspects of PIP₃ involvement in chemotactic response are apparent through modulation of phosphoinositide levels by PLC (Figure 5), which hydrolyzes PIP₂ into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ receptors on the ER mobilize Ca²⁺ release from intracellular membrane stores and impact receptor-mediated Ca²⁺ influx. In mammalian cells, DAG primarily functions to activate protein kinase C (PKC). While a bona fide PKC is not identified in *Dictyostelium*, they do possess a DAG kinase that catalyzes the conversion of DAG to phosphatidic acid, which can also mediate various intracellular processes. Like PI3K, PLC is transiently activated by cAMP in a CAR1/G protein-dependent manner (Drayer and van Haastert, 1992; Bominaar et al., 1994). Activation requires Gα₂, whereas Gα₁ mediates an inhibitory pathway (Bominaar and Van Haastert, 1994). Despite these defined signaling pathways, *plc*-null cells do not have significantly reduced levels of IP₃. IP₃ levels are maintained by a compensatory pathway through the degradation of IP₆ (Drayer et al., 1994); DAG is also produced from additional stores.

The *plc*-null cells, however, have reduced levels of PIP₃, as measured by a reduction in PH-GFP localization upon cAMP stimulation and a decline in the activation of the PI3K effector Akt/PKB (Kortholt et al., 2007). In addition, cAMP-stimulated *plc*-nulls maintain PTEN at the leading edge as well as the lateral and posterior regions of chemotaxing cells (Kortholt et al., 2007). It may be that an increased level of PIP₂ in *plc*-nulls enhances PTEN binding and, thus, suppresses PI3K action at the leading edge (Figure 5). It should be noted that *plc*-nulls are distinct from *pi3k1/2*-nulls and have minimal chemotactic deficiencies (van Haastert et al., 2007), although both show defects during development at low cell density (Kortholt et al., 2007). Conversely, cells that over-express PLC show an expansion in PH-GFP localization at the anterior and also reduced directionality and migratory speed towards a micropipette emitting cAMP. The PLC over-expressing cells, thus, phenocopy defects of *pten*-nulls (Kortholt et al., 2007). Pharmacological and genetic manipulation of other parts of the inositol/IP pathway may also alter PIP₃-signaling (Williams et al., 2004). While PIP₃ levels can be decreased by PI 5-phosphatases, their actions alone have only a limited effect on their response to cAMP (Loovers et al., 2006). Other issues remain unresolved. Although PI3K/PIP₃-signaling is argued to regulate both the activation and de-activation of ACA

(Comer et al., 2006), aggregation and AC activation and adaptation follows normally in cells lacking PLC and PIP₃ accumulation (Drayer et al., 1994).

Experiments with the cAMP analogue 8-para-chlorophenylthio-cAMP (8CPT-cAMP) underscore the importance of maintaining the specificity of phosphoinositide localization in chemotaxing cells. 8CPT-cAMP can activate many of the downstream targets that cAMP does, such as ACA and GC (Peters et al., 1991; Bominaar and Van Haastert, 1993; Bominaar and Van Haastert, 1994). However, rather than activating PLC, 8CPT-cAMP suppresses PLC, and reduces its activity below that of basal controls (Bominaar and Van Haastert, 1993). While developing WT *Dictyostelium* chemotax directionally toward a cAMP source, they are actively repelled by a similar 8CPT-cAMP gradient (Keizer-Gunnink et al., 2007). Indeed all parameters of chemotactic polarity appear to be reversed, with the leading edge components localizing opposite to that of the 8CPT-cAMP source (Figure 6).

Unlike WT cells, *plc*-, *pi3k1/2*-, and *pten*-null cells migrate directionally toward 8CP-cAMP rather than away (Keizer-Gunnink et al., 2007), demonstrating that these regulatory paths facilitate the repulsive effect. Inhibition of PLC by 8CPT-cAMP enhances the relative levels of PIP₂, thus recruiting PTEN to the site nearest the 8CPT-cAMP source. PIP₃ degradation is increased relative to the “down-gradient” side of the cell causing a reversal of PI3K and actin cell polarity, and, hence, a repulsive movement relative to 8CP-cAMP (Keizer-Gunnink et al., 2007). The inability of *pi3k1/2*- or *plc*-null cells to manifest a robust intracellular gradient of PIP₃ makes these cells non-responsive to the 8CPT-cAMP-induced inhibition of PLC. Here, 8CPT-cAMP acts a bona fide attractant (Keizer-Gunnink et al., 2007).

Potentially, polarity reversal reflects the complexity of endogenous signal-response. When PH-GFP expressing *Dictyostelium* are presented with a global cAMP stimulus, there is a rapid appearance of fluorescence at the cell periphery and an equally rapid disappearance. But with time, cells produce new protrusions marked by the PH-GFP reporter (Postma et al., 2003). Cellular response to a cAMP gradient is somewhat analogous to the two-phases observed in the global PH-GFP translocation response (Xu et al., 2005). Initially, PH-GFP is asymmetrically distributed around the cell perimeter, with the fluorescent intensity proportional to the exposure of cAMP within the gradient. The cells then adapt, followed by the second translocation phase where PH-GFP is highly polarized to the leading edge of the cell. These actions do not simply reflect responses to an increasing concentration of cAMP that occurs as cells move closer to the gradient source. Identical PH-translocation events are also observed in latrunculin-treated cells, which are unable move due to inhibition of actin polymerization, exposed to a stable (non-varying) cAMP gradient (Xu et al., 2005).

When the gradient is rapidly terminated, PH-GFP de-localizes from the plasma membrane. Remarkably, however, if the exact cAMP gradient is reestablished before the cells have had an opportunity to de-adapt, PH-GFP translocation exhibits polarity reversal, with PH-GFP now localized to the edge of the cell furthest from the chemoattractant source (Xu et al., 2007). This polarity inversion suggests that a stronger inhibitory signal is at the original leading edge in comparison to the rear. It will be interesting to relate these mechanistic observations to those involving the response to 8CPT-cAMP. Furthermore, these correlations may further our understanding of how *Dictyostelium* are able to remain directionally

polarized as they move relative to a cAMP wave, first experiencing an increasing temporal/spatial gradient of cAMP at the front of the wave, but then eventually a decreasing gradient at the back of the wave, as a cell and wave move relative to one another (Soll et al., 2002).

3.2. Feedback amplification within the PI3K pathway

PH-GFP imaging and other data show clearly that the initial response to cAMP within each region of the cell is proportional to the strength of the stimulus. This response rapidly resolves to the highly polarized state observed in cells chemotaxing within a stable gradient. Recent data suggest that a positive feedback loop among Ras, PI3K, and F-actin may amplify the cAMP signal, ensuring highly polarized, local levels of PIP₃ at the leading edge (Sasaki et al., 2004).

While the mechanistic activation of PI3K by cAMP is not fully defined, it appears to require CAR1/G protein-dependent activation of Ras GTPases (Figures 2). In its active form Ras is complexed with GTP, with GEFs (GTP-Exchange Factors) mediating a positive path, and GAPs (GTPase Activating Proteins) being inhibitory. Upon global cAMP stimulation, Ras proteins undergo transient activation, with activation/de-activation occurring prior to that of PI3K (Sasaki et al., 2004). Spatially, Ras-GTP levels are rapidly elevated at the leading edge of chemotaxing cells, even in the presence of the PI3K inhibitor LY2940002. In contrast, deletion of *RasC* or the Ras-GEF gene *AleA* or expression of a GDP-locked, RasG dominant-negative variant in either *aleA*- or *rasG*-nulls reduces PH-GFP translocation, as well as Akt/PKB activation and chemotaxis (Lim et al., 2001; Sasaki et al., 2004). Thus, Ras would appear to function upstream of PIP₃ signaling (Figure 2).

However, sensitive evaluation of Ras activation suggests a more complex interaction between Ras and PI3K. Ras activation is quantitatively reduced in cells treated with LY2940002 or the F-actin inhibitor Latrunculin A, suggesting that PI3K and F-actin participate in a regulatory feedback loop involving Ras. Low levels of PI3K are membrane associated in quiescent, basal cells, with cAMP-induced recruitment of PI3K to the membrane dependent upon F-actin polymerization (Sasaki et al., 2004). In response to a chemotactic gradient, the local increase in Ras-GTP may mediate the activation of the basal PI3K that is localized at the membrane independently of cytoskeletal organization. The resulting PIP₃ accumulation recruits PH-domain containing proteins that promote F-actin polymerization and a feedback cascade. F-actin further stimulates Ras activation and the translocation of PI3K, thus amplifying the signal at the leading edge to polarize the cell (Sasaki et al., 2004). Still, the strength of this feedback amplification is not entirely resolved (Xu et al., 2005).

3.3. PLA₂-Signaling

Although significant data infer a major role for PIP₃-signaling in control of chemotaxis, other data, as indicated above, are less supportive. *pi3k1/2*-nulls exhibit directionality and speed defects, but still chemotax towards high concentrations of cAMP (Funamoto et al., 2001). In addition, development of *pi3k1/2*-null cells in shaking culture for periods longer than that of WT is sufficient to rescue the majority of their chemotactic defects (Takeda et al., 2007). Treatment of *pi3k1/2*-null cells with LY2940002 further reduces chemotactic

speed and persistence, but does not eliminate chemotactic movement. Also, while the triple *pi3k1/2/3*-null strain cannot be rescued by increasing developmental times, it is still able to chemotax in steep cAMP gradients (Takeda et al., 2007).

Dictyostelium disrupted for all five *PI3K* genes are unable to establish PIP₃ gradients along the cell membrane whether or not PTEN is present, but can still reciprocally localize PI3K and PTEN, as visualized by GFP-tagged reporters. These cells also show a rapid rise in F-actin polymerization upon exposure to cAMP and are able to chemotax effectively to a chemoattractant source, albeit with reduced speed (Hoeller and Kay, 2007). Although PI3K activity may contribute to chemotaxis, it does not appear to be required (Figure 4). These results indicate that a parallel pathway for F-actin polymerization and chemotaxis must function independently of PIP₃ formation (Figure 4).

Two independent research groups have now identified PLA₂ as an alternative mediator of chemotaxis (Chen et al., 2007; van Haastert et al., 2007; Figures 4,5). PLA₂ catalyzes the cleavage of phospholipids into fatty acids (e.g. arachidonic acid, AA) and lysophospholipids. One group identified a putative PLA₂ in an insertional mutagenic screen to identify strains that are unable to aggregate in the presence of LY294002 concentrations (e.g. 50 μM) that do not inhibit WT cells (Chen et al., 2007). Biochemical data indicate that the isolated gene in *Dictyostelium* encodes a functional PLA₂ that can produce AA. Ultimately they showed that cells lacking PLA₂ develop and activate PIP₃-signaling normally, but display aggregation and chemotactic defects in the presence of the PI3K-inhibitor LY294002, arguing that PLA₂ and PI3K regulate “redundant” pathways. They further showed that *pi3k1/2* nulls could not aggregate or chemotax in the presence of the PLA₂ inhibitor bromoenol lactone (BEL) and that *pla2/pi3k1/2*-null cells were far more defective in sensing and polarizing within a cAMP gradient, than are either of the “parental” *pla2*-null or *pi3k1/2*-null cells. Cells deficient in both pathways exhibit a “synthetic” defect in chemotaxis, while cells deficient for only one component can chemotax in high cAMP gradients (Chen et al., 2007). These data confirm that PLA₂ and PI3K function in parallel pathways (Figures 4,5).

A separate group postulated that the intracellular signaling molecules that function downstream of CAR1 activation must accumulate rapidly and transiently (<10 sec.) and have rapid diffusion rates to effect the observed changes in F-actin during chemotactic response (van Haastert et al., 2007). Thus, they focused on phospholipids, fatty acids, and cytosolic Ca²⁺ and their corresponding regulatory components including PI3K, PLC, and PLA₂. Using combinations of pharmacological inhibitors and appropriate mutant strains, they analyzed chemotaxis to varying concentrations of cAMP in small cell population assays. The defining inhibitors were LY2940002 for PI3K, U73122 for PLC, quinacrine and p-bromophenacyl bromide (BPB) for PLA₂, and EGTA to chelate Ca²⁺ (van Haastert et al., 2007).

Cells deficient in PI3K or PLA₂ activity migrate normally to high concentrations (~1μM) of cAMP, but not as well to lower concentrations (~50 nM; Figure 4). Additionally, when all pathways except only PI3K or only PLA₂ are inhibited, the cells are still capable of chemotaxis (van Haastert et al., 2007). Cells simultaneously inhibited for both PI3K and

PLA₂ chemotax very poorly even to high concentrations of cAMP (van Haastert et al., 2007).

The inhibitory data also confirm previously predicted pathway linkages. *plc*-null cells, which are suggested to limit PIP₃ accumulation, are insensitive to the PI3K-inhibitor LY2940004, but their chemotaxis is blocked by treatment with PLA₂ inhibitors (Figure 5). Thus, it is argued that *plc*-null cells primarily utilize PLA₂ signaling to mediate chemotaxis. As noted, *plc*-null cells are unable to mount a PIP₃ response, as measured by a PH-GFP readout (van Haastert et al., 2007). In contrast, *iplA*-null cells, which lack a putative IP₃ receptor, essential for Ca²⁺ release and influx (Traynor et al., 2000), chemotax in the absence or presence of PLA₂ inhibitors, but are blocked with LY2940004 (Figure 5). Presumably, IplA-mediated Ca²⁺ signaling is integrated within the PLA₂ pathway, but functions independently of PI3K (van Haastert et al., 2007).

The exact relationship of Ca²⁺ signaling in the context of PLA₂ is still not clear. Ca²⁺ is not required to activate PLA₂ (Chen et al., 2007), but PLA₂ inhibitors suppress cAMP-dependent influx of Ca²⁺ and addition of the putative PLA₂ product AA rescues Ca²⁺ influx in the presence of PLA₂ inhibitors (Schaloske and Malchow, 1997). Nonetheless, cells genetically deficient for one of the several putative PLA₂ genes in *Dictyostelium* display normal Ca²⁺ influx and addition of AA to *pla2*-null cells treated with LY2940004 partially rescues the ability of these cells to aggregate (Chen et al., 2007).

3.4. cGMP-Signaling

Cells that have become highly polarized still chemotax in the presence of inhibitors that completely block PI3K, PLC, PLA₂, and Ca²⁺-signaling (van Haastert et al., 2007; Veltman and van Haastert, 2008), suggesting that other cAMP-activated pathways may also impact chemotaxis. One pathway involves cGMP (see Figures 4,5); cells, which are inhibited for both PI3K- and PLA₂-signaling and also unable to synthesize cGMP, chemotax very poorly (Veltman and van Haastert, 2008).

There are two guanylyl cyclases in *Dictyostelium*, GCA and sGC. During chemotaxis, pseudopod formation is generally restricted to the leading edge of the cell, as myosin filaments that localize to the rear inhibit pseudopod extension (Figure 3). Cells that are defective in cGMP production or that lack the cGMP binding protein GbpC are defective in myosin assembly and are unable to suppress lateral pseudopod extension (Bosgraaf et al., 2002; Veltman and van Haastert, 2006; Veltman and van Haastert, 2008). Although these cells have many protrusions that arise spontaneously around the cell periphery, they are still capable of effective directional chemotaxis, as these lateral pseudopods are less persistent than those at the leading edge.

Although *gca/sgc*-nulls also inhibited for both PI3K- and PLA₂-signaling are very poorly chemotactic (Figure 4), response to cAMP can be fully rescued by re-expression of full-length sGC (Veltman and van Haastert, 2006; Veltman and van Haastert, 2008). However, expression studies using mutant versions of sGC indicate a surprising complexity and bifurcation within the pathway (Figure 5).

In quiescent cells, sGC is localized in the cytosol; upon cAMP-stimulation sGC is rapidly recruited to the cell periphery, where it becomes activated. Thus during chemotaxis, sGC localization is restricted to the leading edge (Figure 3). As the N-terminal membrane localizing domain is separate from the central GC catalytic domain, it has been possible to dissect the independent functions of these individual domains. sGC variants were constructed that lacked GC catalytic activity but retained the N- and C-terminal domains or that lacked the N-terminal membrane localization domain but retained catalytic activity and the C-terminal domain (Veltman and van Haastert, 2006; Veltman and van Haastert, 2008). Remarkably, *gca/sgc*-nulls inhibited for both PI3K- and PLA₂-signaling, but engineered to accumulate normal levels of cGMP in response to cAMP stimulation, remained poorly chemotactic. However, expression of the catalytically inactive variant of sGC that retained the membrane localization and C-terminal domains was able to partially rescue chemotactic defects in *gca/sgc*-nulls also inhibited for both PI3K- and PLA₂-signaling (Veltman and van Haastert, 2008).

Thus, the GCs have 2 distinct functions (Figure 5). cGMP provides a signal that organizes myosin filament formation in the rear of chemotaxing cells. In addition, sGC localization at the leading edge functions independently of cGMP formation to promote chemotactic movement. It has been suggested that an activity located within the C-terminus of sGC may interact with actin and regulate pseudopod formation (Veltman and van Haastert, 2006).

4. Modeling perspectives

Oscillating cAMP wave propagation is essential for chemotactic-mediated aggregation in *Dictyostelium*. Apart from components that activate signal production and propagation, the cAMP oscillations also require a negative feedback (inhibitory) loop, encompassing a timing delay relative to that of the excitatory pathway (Figure 2). One can theoretically model the cAMP oscillation circuit in *Dictyostelium* through the negative feedback to CAR1 or ACA, (e.g. Martiel and Goldbeter, 1985; Maeda et al., 2004) and while such partial models can successfully predict an accurate ~6 min. cAMP oscillation frequency, they do not fully incorporate relevant experimental data or explain the more rapid activation/inactivation cycle of PI3K and other components in comparison to that of ACA (Brzostowski and Kimmel, 2006).

Putative excitatory/inhibitory actions can be modeled spatially. Excitation is suggested to be locally constrained, while inhibition acts globally. Local excitation/global inhibition (LEGI) would polarize cellular response to regions closest to a signaling source (Ma et al., 2004; Levine et al., 2006; Meier-Schellersheim et al., 2006) and asymmetry can be further enhanced by positive feedback amplification (Sasaki et al., 2004). Still, the inhibitory pathways are only poorly understood mechanistically, and although one can speculate that all the intracellular oscillations (e.g. cAMP, cGMP, Ca²⁺ flux, PI3K activity, ERK2 phosphorylation) are regulated by a primary inhibitory loop acting on a common upstream element, multiple or branching feedback mechanisms within the circuit cannot be excluded (see Figure 2).

Adaptation/inhibition appears to function independently of Gα₂, but two other Gα proteins have definitive inhibitory functions. Gα₉ antagonizes multiple chemoattractant responses that regulate directional cell movement, including ACA, GC, PI3K, and F-actin polymerization, but loss of Gα₉ only delays, and does not eliminate adaptive responses (Brzostowski et al., 2004). Gα₁ negatively regulates PLC (Bominaar and Van Haastert, 1994), and thus may impact PIP₃-signaling. Potentially, multiple Gα proteins coordinate inhibitory signaling. Indeed, PI3K may be required for activation and inhibition of ACA and both pathways are suggested to involve G protein signaling (Comer et al., 2006).

5. Connections: starvation, pulsatility, aging

Oscillatory signaling pathways are required in diverse organisms to maintain homeostasis and regulate processes essential for growth, development, and reproduction. Studies of the early stages of *Dictyostelium* development provide a detailed molecular description for pulsatory signaling among dispersed cells and the consequent intracellular responses that ultimately organize aggregation territories and multicellular development. Yet, signaling within multicellular organisms is more intricate, integrating both short- and long-range modes of intercellular communication among various specialized tissues to ensure homeostatic balance. Upon metabolic stress, various feedback and amplification mechanisms modulate intercellular pulsatile signaling to maintain stability within these pathways. Nonetheless, during prolonged stress or aging, oscillatory signaling may be subject to severe dysfunction, impacting fertility and survival (this volume).

Hormonal signals from peripheral tissues balance bioenergy status with nutrient intake and integrate within the hypothalamic-pituitary-gonadal (HPG) axis to regulate reproductive function (Martin et al., 2008; Veldhuis, 2008). Thus, dietary intake is a significant determinant for reproductive success. Yet, as caloric restriction can suppress pulsatile signaling in the gonadal axis of mammals and reduce fecundity, it can also extend the lifespan of species as diverse as yeast, worms, flies, fish, and mammals. A potential common pathway may involve the nutrient sensor TOR, which is suggested to function not only intracellularly, but also to integrate intercellular signaling among organ systems to control homeostasis (Arsham and Neufeld, 2006). However, it remains to be determined if these species share a conserved “anti-aging” mechanistic response and if specific nutrients are more critical than is energy intake, per se (Piper and Partridge, 2007; Lee et al, 2008).

In the context of dietary restriction, pulsatility, and aging, it is interesting that withdrawal of both energy (glucose) and amino acid sources is required to stimulate *Dictyostelium* to establish the extracellular cAMP oscillations that regulate multicellular formation and development. Although *Dictyostelium* do not have a specified lifespan in the presence of abundant nutrients and sufficient space, response to starvation allows cells to enter a protective non-reproductive, developmental cycle and to survive in the absence of exogenous nutrients. Furthermore, this growth-to-development transition involves responses that are mediated by TOR as both a nutrient sensor and signaling regulator (Lee et al., 2005; Rosel, Khurana, Majithia, Bhandari, Huang, and Kimmel, submitted; Liao, Majithia, and Kimmel, in preparation). Although the physiological consequences of nutrient depletion to

Dictyostelium and metazoa are clearly distinct, continued studies of *Dictyostelium* may provide new insights that relate starvation, signaling pulsatility, and aging.

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A.

B.

Figure 1. cAMP Oscillations in *Dictyostelium discoideum*

A. Dark-field, optical imaging of *Dictyostelium discoideum* wave patterns during early development.

B. Periodicity of cAMP signaling.

Dictyostelium discoideum were differentiated in suspension culture and the level of endogenous cAMP produced in the absence of an exogenous stimulus was measured at the indicated time points during an ~18-min period. The first cAMP peak was set arbitrarily to 100.

Figure 2. The Oscillating cAMP loop and signal-relay

AC activation: Extracellular cAMP stimulation of the CAR1/G protein complex mediates Ras activation, which leads to activation of PI3K and accumulation of PIP₃. PIP₃ recruits the PH domain proteins CRAC, Akt (et al.) to the membrane where they become functionally active. Activation of the Akt and membrane-bound PKBR1 kinases require phosphorylation by PDK1 and the TOR complex 2 (TORC2; TOR, Pia, RIP3, Lst8). CRAC and Akt/PKBR1 are both required to activate ACA at a distal site in the cell. TORC2 regulation by PI3K or of ACA is not known. Activated ACA synthesizes cAMP, which has both intracellular and extracellular functions. Secreted cAMP relays the original signal and directs chemotaxis. Intracellular cAMP activates PKA.

cAMP Degradation: The extracellular and intracellular pools of cAMP are degraded by separate phosphodiesterase (PDE) pathways. Developing *Dictyostelium* secrete a PDE that clears the extracellular cAMP signal. Intracellular degradation is more complex. Active RegA is a primary intracellular cAMP PDE, subject to phosphorylation and inhibition by ERK2. In quiescent cells, ERK2 is inactive, RegA is active and intracellular cAMP levels are low; upon receptor stimulation, an ERK2 kinase is activated and an ERK2 PPase is inactivated. ERK2 becomes active, RegA is inhibited, and cAMP levels rise. It is inferred that activated PKA has a negative feedback role for the intracellular accumulation of cAMP, although the mechanism is not defined. PKA does alter the phosphorylation of ERK2. RegA has a putative PKA site, potentially making it a regulatory motif.

Adaptation: The actions of the various PDEs are not sufficient to produce oscillations in cAMP; an inhibitory, adaptive response is also required. Many CAR1/G protein mediated responses to cAMP are only transiently active and adapt (de-activate) to a continuous cAMP stimulus. The Ras/PI3K pathway adapts in <30 sec., with ACA activity declining in <2 min. PTEN is able to degrade PIP₃. While the activating pathway for ERK2 is also attenuated, the PPase response is non-adaptive and remains inactive if extracellular levels of cAMP persist. The adaptive/inhibitory pathways are not understood mechanistically. They are impacted by G protein signaling, and perhaps by receptor phosphorylation, and could potentially involve the cyclical activations/de-activations of Ras-GEFs and Ras-GAPs. Direct feedback inhibition of ACA is also postulated.

Component descriptions are described in the text.

cAMP Source

Figure 3. Cell polarization in response to a directional cAMP source

In response to cAMP, cells move directionally toward the signal. CAR1 is distributed uniformly at the cell surface. G proteins are activated proportionally to cAMP signal-strength, e.g. in an ~10% gradient, there is only a relative 10% difference in G protein activation in comparing opposite poles of the cell. Polymerized F-actin is primarily associated with pseudopod formation at the cell anterior, but cortical F-actin is detected around the cell periphery. Ras-GTP, PI3K, PH proteins, and sGC are highly polarized to the leading edge of cells. PTEN is found at the posterior and at the lateral sides of the cell. ACA and myosin are polarized to the posterior. All are represented with varying intensities of green. Protein descriptions are described in the text.

CAR1, PI3K, PTEN, ACA, PH-domains, sGC, and Myosin localizations are detected by fluorescent, live cell imaging as fusions with GFP. F-actin is detected with various F-actin binding proteins in fusion with a fluorescent tag. Ras-GTP is detected with the RBD (Ras-GTP binding domain) of Raf1 in fusion with GFP. Gα2-GTP is inferred by loss of association with Gβγ by FRET analyses.

Figure 4. PI3K-, PLA₂-, and GC-signaling cooperate to regulate chemotaxis to cAMP
Chemotaxis of non-polarized *Dictyostelium* to different concentrations of cAMP. *Dictyostelium* were differentiated for times sufficient to develop responsiveness to cAMP but not to polarize; cells remained amoeboid. For each concentration, the WT response is arbitrarily set to 100. Data are adapted (van Haastert et al., 2007) and summarize the work of multiple groups (Chen et al., 2007; van Haastert et al., 2007; Hoeller and Kay, 2007; Takeda et al., 2007).
Chemotaxis of polarized *Dictyostelium* to 1 μM cAMP. *Dictyostelium* were differentiated for times sufficient to both develop responsiveness to cAMP and polarize. Data are adapted and summarized from studies of the van Haastert laboratory (Veltman et al., 2005; Veltman and van Haastert, 2006; Veltman et al., 2008; Veltman and van Haastert, 2008).

Figure 5. PI3K, PLA₂, and GC separately regulate chemotaxis

PI3K/PLC-signaling: Upon cAMP stimulation, both PI3K and PLC are activated and both target PIP₂. Phosphorylation of PIP₂ by PI3K produces PIP₃, while hydrolysis by PLC produces IP₃ and DAG. PIP₃ recruits PH domain proteins to the membrane, where they become functionally active. Pathway components, including CRAC, Akt/PKBR1, and TORC2, mediate chemotactic response. Loss of either PI3K or PLC reduces PIP₃-signaling, but chemotaxis can proceed in the presence of a fully functional PLA₂ pathway (Figure 4).

PLA₂/Ca²⁺-signaling: Upon cAMP stimulation, there is an activation of PLA₂ and an influx of Ca²⁺. Ca²⁺ influx is linked to the IP₃/IplA-mediated release of Ca²⁺ from intracellular stores. PLA₂ catalyzes the hydrolysis of phospholipid (PL) to fatty acid, like arachidonate (AA), and lysophospholipid (LysoPL). AA-signaling is a primary mediator of chemotaxis, although Ca²⁺ can enhance the response. In the absence of PLA₂, chemotaxis can proceed in the presence of a fully functional PI3K pathway (Figure 4). Inhibition of PI3K, PLC, PLA₂ and Ca²⁺-signaling has a more severe impact than if only PI3K and PLA₂ are inhibited.

GC-signaling: Two guanylyl cyclases (GC), GCA and sGC, regulate cGMP production in response to extracellular cAMP signaling. GCA is constitutively membrane-bound; sGC is localized to the cytosol of unstimulated cells, but is recruited to the leading edge during chemotaxis (see Figure 3), where it becomes activated. The predominant GC activity during aggregation is sGC. The cGMP-binding protein GbpC acts downstream of cGMP to regulate myosin filament formation at the cell posterior (see Figure 3) and suppress lateral pseudopod formation.

Cells lacking both GCA and sGC do not have detectable GC activity and exhibit chemotactic defects to low concentrations of cAMP. In the absence of PI3K/PLA₂, chemotaxis can proceed in polarized cells that express fully functional GC pathways, but not in *gca/sgc*-nulls

cells (Figure 4). Expression of a catalytically inactive version of sGC that can still localize to the cell anterior can partially rescue chemotaxis in *gca/sgc*-nulls cells that are also inhibited for PI3K-and PLA₂- signaling. In contrast, cAMP-stimulated accumulation of cGMP in the absence of anteriorly localized sGC is insufficient for such rescue.

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Figure 6. Polarity inversion in response to 8-CPT-cAMP

In response to cAMP, cells move directionally toward the signal; PH proteins are highly polarized to the leading edge of cells, while PTEN is found at the posterior and at the lateral sides of the cell. In response to 8CPT-cAMP, wild-type cells reverse polarity and migrate away from the signal source; PH proteins are highly polarized to the edge of the cells away from 8CPT-cAMP, while PTEN is found at regions closest to the signal and at the lateral sides of the cell.

Analyses are as described in Figure 3.