

CHEMOATTRACTANT SIGNALING IN *DICTYOSTELIUM DISCOIDEUM*

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■ **Abstract** *Dictyostelium* is an accessible organism for studies of signaling via chemoattractant receptors. Chemoattractant-mediated signaling events and components are reviewed and presented as a series of connected modules, including excitation, inhibition, G protein-independent responses, early gene expression, inositol lipids, PH domain-containing proteins, cyclic AMP signaling, polarization acquisition, actin polymerization, and cortical myosin. The network incorporates information from biochemical, genetic, and cell biological experiments carried out on living cells. The modules and connections represent current understanding, and future information is expected to modify and build upon this structure.

CONTENTS

INTRODUCTION TO CHEMOATTRACTANT SIGNALING	224
Unique Advantages of <i>Dictyostelium</i>	224
Modular View of the Signaling Pathway	225
EXCITATION: TRANSDUCING EXTRACELLULAR SIGNALS	
INTO THE CELL	225
Chemoattractant Receptors	225
Heterotrimeric G Proteins	233
YakA, a Prototypical DyrK	234
INHIBITION: MULTIPLE POINTS OF SIGNAL ATTENUATION	234
Properties of Adaptation	234
Candidate Inhibitor Molecules	235
EARLY GENE EXPRESSION: SIGNALING PATHWAY REGULATES ITS OWN EXPRESSION	236
INOSITOL LIPIDS: CENTRAL NODE IN CHEMOATTRACTANT SIGNALING	237
Local Production of PI(3,4,5)P ₃	237
Local PI(3,4,5)P ₃ Degradation	238

PH DOMAIN-CONTAINING PROTEINS: ACTIVATION BY RECRUITMENT TO LOCAL PI(3,4,5)P ₃	239
cAMP SIGNALING: REGULATION OF cAMP ACCUMULATION	240
Adenylyl Cyclase and Cell-Cell Signaling	240
Components Involved in ACA Activation	241
cAMP Phosphodiesterases	242
G PROTEIN-INDEPENDENT RESPONSES: SIGNALING WITHOUT Gαβγ	242
POLARIZATION ACQUISITION: COORDINATING CELL-CELL SIGNALING WITH CHEMOTACTIC COMPETENCE	243
ACTIN POLYMERIZATION: SIGNALING TO PSEUDOPODIA	244
CORTICAL MYOSIN: DETERMINING THE BACK OF A CELL	245
SUMMARY	247

INTRODUCTION TO CHEMOATTRACTANT SIGNALING

Unique Advantages of *Dictyostelium*

Most chemoattractants act through G protein-linked signaling pathways to mediate directional migration and also modulate cell adhesion, gene expression, and cell-cell signaling. Many features of these pathways in mammalian leukocytes are similar to those in the model organism *Dictyostelium discoideum*. Analysis of signaling processes in these social amoebae has led to discoveries that have subsequently been confirmed in mammalian systems. There have been a number of recent reviews (Kimmel & Parent 2003, Meili & Firtel 2003, Williams & Harwood 2003, Iijima et al. 2002).

Advantages of *Dictyostelium* as a model organism include its genetic, biochemical, and cell biological accessibility. Genetic tools such as gene targeting by homologous recombination; structure-function analysis by random mutagenesis; insertional mutagenesis, which allows easy cloning of the gene disrupted; and multicopy suppression libraries are available (Landree & Devreotes 2003). In addition, double and triple knockouts can be obtained in single or multiple transformation steps. Many of the genes involved in signal transduction, development, and cell motility have been targeted by forward and reverse genetics. The *Dictyostelium* genome is approximately 40 Mbp, with approximately 10,000–13,000 genes on six chromosomes. It has been sequenced and annotated and is available at www.Dictybase.org. *Dictyostelium* grow and divide quickly, and mutants can be easily isolated, propagated, and stored. It is also straightforward to label amoebae with isotopes and express proteins exogenously, facilitating the analyses of specific proteins. Up to 10¹² identical cells can be prepared in a few days for protein purification. The flat, 20-μm cells are accessible for imaging, and the use of green-fluorescent protein (GFP) fused to proteins of interest allows researchers to visualize their location during chemotaxis. In addition, appropriate markers for all organelles and compartments facilitate subcellular fractionation studies.

Growing amoebae chemotax toward folic acid and other nutrients, whereas starved cells undergo a developmental process that transforms them into cAMP-sensing machines. Signaling proteins are upregulated within a few hours and the cells gradually develop a polarized cell morphology. Oscillatory pulses of cAMP with a period of 6 min propagate as waves through cell monolayers. Centers secrete cAMP and recruit chemotaxing cells into aggregates containing 10^5 amoebae. Because the early stages of development require chemoattractant signaling, laboratories studying these processes isolate mutants that fail to aggregate and then assess chemotaxis, cell-cell signaling, and gene expression.

Modular View of the Signaling Pathway

In this review, we focus on our current understanding of chemoattractant-signaling pathways primarily on the basis of studies of cell lines lacking the genes depicted in Figure 1. Because our knowledge of the signaling networks is incomplete, we present a modular view of the pathways. The modules group components and signaling events perceived to perform steps in the pathways leading to chemotaxis, cell-cell signaling, and gene expression (Table 1). One of the advantages of the modular view is that one can begin to analyze signaling network interactions without knowledge of every component and interaction. The modular structure can also be modified and built upon as more knowledge is obtained.

A cell first uses an excitation module to sense extracellular cues and transduces information into cellular second messengers. The inhibition module gathers together as yet poorly understood components that negatively regulate the pathways. Other modules describe G protein-independent responses and early gene expression. The inositol lipid and pleckstrin homology (PH) domain modules are prominent nodes in which upstream signals are amplified and transmitted downstream. The cAMP-signaling module describes events, including an autocrine loop, that generate oscillatory cAMP signals and enable cells to communicate with each other. The signaling apparatus also includes polarization acquisition, actin polymerization, and cortical myosin modules, which describe the ability of a cell to create well-defined anterior and posterior regions.

EXCITATION: TRANSDUCING EXTRACELLULAR SIGNALS INTO THE CELL

Chemoattractant Receptors

In *Dictyostelium*, folic acid, factors from bacteria, as well as cAMP serve as chemoattractants. Four cAMP receptors (cAR1-4) are most homologous to a 7-transmembrane receptor in *Arabidopsis thaliana* and, secondarily, to calcitonin receptors in mammals (Hereld & Devreotes 1992). Although there is 60% identity between the cARs, the receptors are expressed sequentially during development and differ in their affinity for cAMP (Kim et al. 1998). The key receptor involved

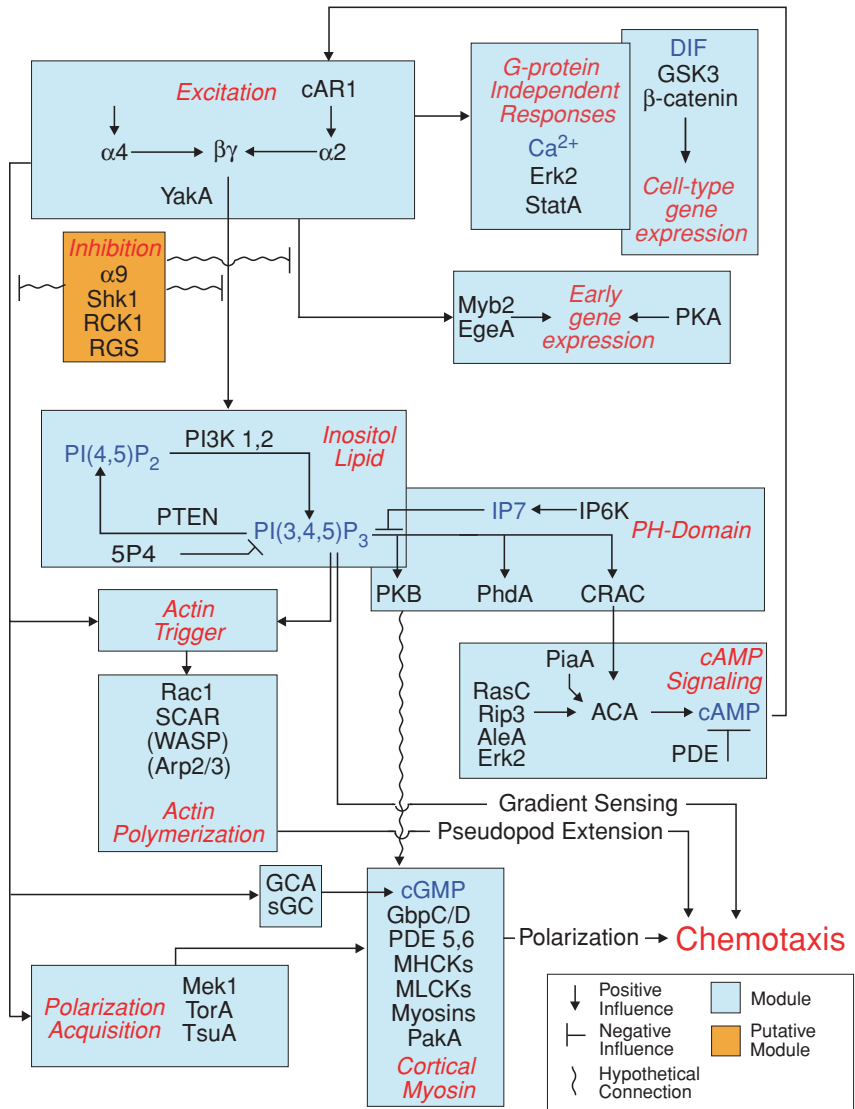


Figure 1 Modular view of the chemoattractant-induced signaling pathway in *Dictyostelium*. Except for those in parenthesis, the proteins depicted in this pathway have been shown to be involved in chemotactic signaling through analysis of cells in which the genes have been deleted. Shown in blue are key small molecules that participate in the pathways.

TABLE 1 *Dictyostelium* proteins involved in chemoattractant signaling

Dd protein	Phenotype of disruption	Homologs	Localization	Role in signaling
cAR1, cAR3	No aggregation No early gene expression	G protein-coupled receptors	Plasma membrane	Receptor for cAMP Essential for chemotaxis
G α 2	No aggregation No early gene expression	G protein α subunits	Plasma membrane and cytosol	Links to cAR1 Essential for cAMP chemotaxis
G α 4	Aggregation small plaque	G protein α subunits	Plasma membrane	Required for folic acid chemotaxis
G β γ	No aggregation No early gene expression Small plaque	G protein $\beta\gamma$ subunits	Plasma membrane and cytosol	Essential for cAMP and folic acid chemotaxis
YakA	No aggregation No early gene expression Small plaque	DyrK-related kinases	Cytosol	Essential for cAMP and folic acid chemotaxis
Rck1	Rapid development Mound delay	Regulators of G protein signaling (RGS)	Cytosol Translocates to membrane Leading edge of migrating cells	Negative regulator of development
Shk1	No aggregation Express early genes	None identified	Cortex	Unclear
G α 9	Many aggregation territories	G protein α subunits	Plasma membrane	Negative regulator of development
PI3K1, PI3K2	No aggregation on bacteria Aggregation on minimal media Express early genes	Class Ia PI3Ks	Cytosol Translocates to membrane Leading edge of migrating cells	Produces PI(3,4,5)P ₃ Amplification of signal

TABLE 1 (Continued)

Dd protein	Phenotype of disruption	Homologs	Localization	Role in signaling
PTEN	No aggregation Express early genes Small plaque	PTEN in mammals	Plasma membrane and cytosol Translocates to cytosol Rear of migrating cells	Degrades $PI(3,4,5)P_3$ Amplification of signal
PI5P1-4	No phenotype (PI5P1-3) Small territories (PI5P4) Slow growth	Inositol 5-phosphatases	ND*	None identified Degrades $PI(3,4,5)P_3$
CRAC	No aggregation Expresses early genes	None identified	Cytosol Translocates to membrane Leading edge of migrating cells	Essential for activation of adenylyl cyclase, reconstitutes in vitro
PKB	Mound arrest Express early genes	PKB in mammals	Cytosol Translocates to membrane Leading edge of migrating cells	Polarization, involved in myosin disassembly via activation of PakA
PhdA	No aggregation Small territories No streams	None identified	Cytosol Translocates to membrane Leading edge of migrating cells	Unknown Possible adaptor protein
AleA	No aggregation Expresses early genes	Ras guanine nucleotide exchange factor (GEF) <i>S. cerevisiae</i> Cdc25	Cytosol	Essential for activation of adenylyl cyclase
Erk2	No aggregation Expresses early genes	Mitogen-activated-protein kinase	Cytosol	Essential for activation of adenylyl cyclase

PiaA	No aggregation Expresses early genes	Homologs in yeast to human	Cytosol	Essential for activation of adenylyl cyclase, reconstitutes in vitro
ACA	No aggregation Expresses early genes	Mammalian adenylyl cyclase	Plasma membrane Rear of migrating cells	Essential for cAMP production
cAMP PDEs	No aggregation (Pde2) Normal aggregation (Pde6)	Both classes of mammalian PDEs	Plasma membrane (Pde1 and Pde4) Cytosol (Pde2 and Pde6)	Degradation of cAMP required for periodicity of cAMP signal
TorA	Small aggregation territories Expresses early genes	None identified	Sphere in one end of mitochondria	Coordinates acquisition of polarization with cell-cell signaling
Mek1	Small aggregation territories Expresses early genes	Mammalian MEKs	Cytosol Translocates to membrane	Coordinates acquisition of polarization with cell-cell signaling
TsuA	Small aggregation territories Expresses early genes	None identified	ND*	Coordinates acquisition of polarization with cell-cell signaling
Racs (15)	Rac1(a,b,c)—weak motility defects Others—no phenotype	Mammalian Racs	Cortex and cytosol	Involved in lamellipod formation in other systems
SCAR	Multi-tipped structures	SCAR/WAVE	Cytosol Translocates to cortex Leading edge of migrating cell	Activates Arp2/3 in other systems Pseudopod extension
WASP	ND*	WASP	Cortex and cytosol	Activate Arp2/3 complex in other systems

(Continued)

TABLE 1 (Continued)

Dd protein	Phenotype of disruption	Homologs	Localization	Role in signaling
Arp2/3	ND*	Arp2/3	Cortex and cytosol	Branching and nucleation of actin filaments in lamellipodia in other systems
GCA, sGC	Delayed development <i>gca-/sgc-</i>	Mammalian guanylyl cyclases	Membrane (GCA) Cytosol (sGC)	cGMP production after exposure to chemoattractant
Myosin Is	Delayed development Half as many fruiting bodies	Myosin I	Cytosol Translocates to cortex Leading edge of migrating cell	Role in polarity and motility Suppresses lateral pseudopods
Myosin II	Mound arrest	Myosin II	Rear of migrating cells	Role in polarity and motility Suppresses lateral pseudopods
MHCK	No aggregation	MHCK	Cortex	Phosphorylates myosin II, resulting in disassembly of myosin
PakA	No aggregation or aggregation (see text)	p21-activated serine/threonine protein kinase	Rear of migrating cells	Phosphorylates MHCK, resulting in disassembly of myosin at front of cell
cGMP phosphodiesterases	Large streams or no phenotype (Pde5) No phenotype (Pde6)	Not mammalian phosphodiesterases	Cytosol	Required for polarization
PKA	No aggregation No early gene expression	PKA	Cytosol	Required for upregulation of genes for early development

ND* = not determined.

in chemotaxis in early development is cAR1, although cAR3, expressed slightly later, partially overlaps in function. Cells lacking cAR1 and cAR3 fail to chemotax and do not aggregate (Kim et al. 1998). Although cells lacking cAR3 appear normal (Johnson et al. 1993), an effect on the prestalk/prespore ratio has been reported (Plyte et al. 1999). Deletion of cAR2 results in cells arrested in the tight mound stage, and deletion of cAR4 affects culmination (Louis et al. 1994, Saxe et al. 1993). In addition to the cARs, there are 3 cAMP receptor-like proteins, designated CrIA-CrIC, that may have roles in growth and development (Raisley et al. 2004). There are also over 30 G protein-coupled receptors identified in the *Dictyostelium* genome, representing the cAMP receptor, frizzled, glutamate/GABA, PAF, and secretin families (D. Hereld, unpublished observations; L. Eichinger, unpublished observations).

When cAR1, cAR2, and cAR3 are constitutively expressed, they couple to the same downstream effectors and activate responses normally mediated by cAR1 but with appropriately shifted EC₅₀s (Kim et al. 1998). The C-terminal regions of the cARs are highly variable. Unlike some classes of mammalian receptors, however, this region does not appear to confer major differences in regulation of receptor-mediated responses (Kim et al. 1998). Differences in affinity between the cARs can be attributed to specific amino acids in the second extracellular loop (Kim et al. 1998). It is likely that the different affinities are critical for chemotaxis and proper morphogenesis at the sequential stages when the cARs are expressed (Dormann et al. 2001, Kim et al. 1998).

Chemoattractants induce a dose-dependent phosphorylation of 3–4 serines in the C terminus of cAR1 (Klein et al. 1987). Phosphorylation is independent of G proteins. It occurs within seconds of stimulus, reaches a steady state within a few minutes, and declines when the stimulus is removed. Phosphorylation of Ser 303 or Ser 304 causes a shift in electrophoretic mobility and is correlated with an agonist-induced decrease in the affinity (Caterina et al. 1995). Purified, phosphorylated receptors also have lowered affinity, suggesting that the effects are intramolecular (Xiao et al. 1999). Surprisingly, phosphorylation of cAR1 is not essential for adaptation of adenylyl cyclase activation, actin polymerization, chemotaxis, or gene expression (Kim et al. 1997b). Phosphorylation does play a role in receptor internalization and turnover and cells overexpressing receptors lacking phosphorylation sites arrest in development at later stages (D. Hereld, unpublished observations).

Random mutagenesis studies on the region from the third transmembrane region through the cytoplasmic tail, or just the third intracellular loop, reveal that the receptor is remarkably tolerant of mutations; only 2% were loss-of-function mutants (Kim et al. 1997a, Milne et al. 1997). The receptor mutants that fail to rescue *car1-car3* cells are divided into four broad classes. Class III mutants, which form the largest group, display lower affinity for cAMP and fall into subclasses that have different narrow ranges of sensitivity. Class II mutants also have reduced cAMP affinity, but can be switched to high affinity with ammonium sulfate. Class IV mutants bind cAMP, are not phosphorylated, and do not carry out

signal transduction. Class I mutants are phosphorylated but are blocked for signaling. Some of these mutants also fail to carry out G protein-independent responses such as ligand-induced Ca^{2+} influx, possibly because the receptor must attain a certain conformation to induce these responses (Kim et al. 1997a, Milne et al. 1997).

Many studies indicate that gradient sensing is not achieved by relocalization of receptors or binding sites to the leading edge of the cell (Figure 2). When expressed

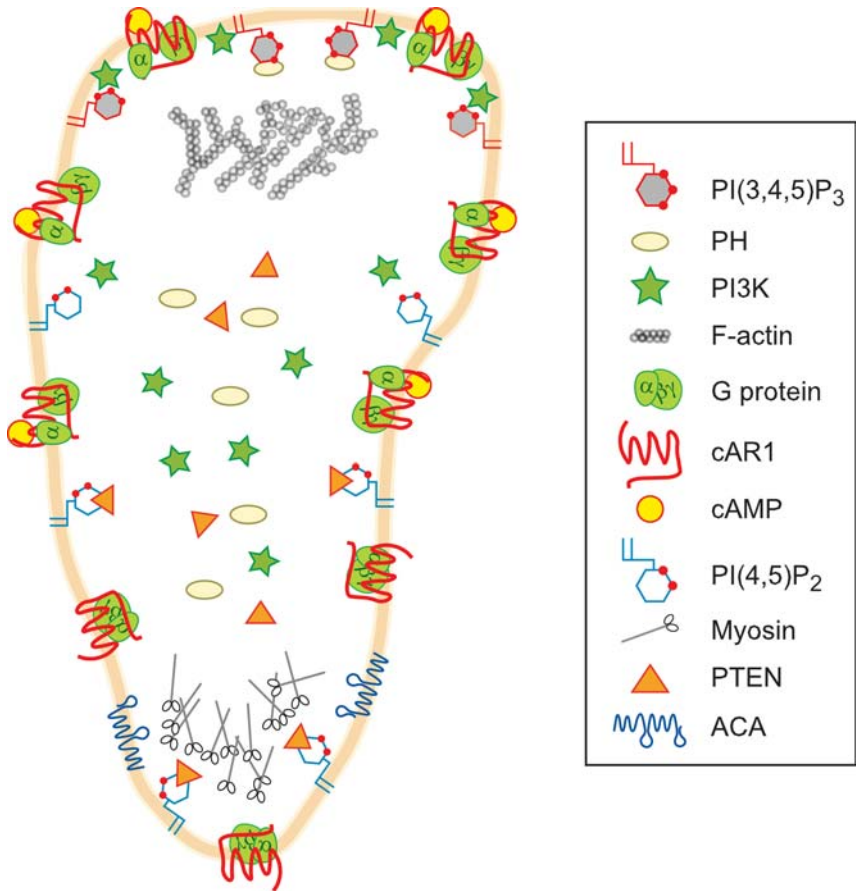


Figure 2 Spatial localization of key components of the signaling pathway in *Dictyostelium*. The cAMP receptor cAR1 is uniformly distributed and G protein dissociation is believed to mirror receptor occupancy in the presence of an external cAMP gradient. In contrast, several components are found preferentially on the front [phosphatidylinositol 3-kinase (PI3K), PI(3,4,5)P₃, PH domain-containing proteins, F-actin] or back [PI 3-phosphatase (PTEN), adenylyl cyclase (ACA), myosin II] of migrating cells. The cell is migrating toward the top of the page.

in a *car1*- cell line, cAR1-GFP remains uniformly distributed along the membrane in cells in chemotactic gradients (Xiao et al. 1997). Electron microscopy also shows a uniform distribution of cAR1 along the membrane. If cAR1, which fractionates quantitatively with cholesterol-rich, detergent-resistant membranes, is localized in rafts, these must be extremely small and mobile (Xiao & Devreotes 1997). Single-molecule imaging of receptor-bound fluorescent cAMP (Cy3-cAMP) also reveals that binding sites are uniformly distributed and highly diffusible along the membrane (Ueda et al. 2001). The Cy3-cAMP binds to the receptors for only a few seconds; the kinetics of dissociation at the front of a polarized cell are faster than at the posterior. Studies performed earlier on cAMP binding show that dissociation was multiphasic, which may be consistent with this finding (van Haastert 1983). In membranes, GTP increases the dissociation rate of Cy3-cAMP and this rate depends on G protein, which suggests that these proteins stabilize ligand binding (Ueda et al. 2001), consistent with previous studies (Wu et al. 1995).

Heterotrimeric G Proteins

G protein heterotrimers (consisting of α , β , and γ subunits) bind to and receive the signal from chemotactic receptors and $\beta\gamma$ subunits transmit them to downstream effectors. There are 11 reported G protein α subunits (Brzostowski et al. 2002, Parent & Devreotes 1996), one β (Lilly et al. 1993) and one γ (Zhang et al. 2001) in *Dictyostelium*. All of the α subunits are 40–50% identical to each other and are most closely related to the mammalian $G_{\alpha i}$ family (Brzostowski et al. 2002). $G\alpha 2$ is coupled to the cARs, whereas $G\alpha 4$ appears to be linked to folic acid receptors. The β subunit is 70% identical to mammalian β subunits, except within the first 50 residues, which interact with the γ subunit.

Although genetic studies suggest that $G\alpha 2$ and $\beta\gamma$ mediate cAMP signaling downstream of cAR1, proof of the linkage was provided only recently (Janetopoulos et al. 2001). By tagging $G\alpha 2$ with cyan fluorescent protein (CFP) and $G\beta$ with yellow fluorescent protein (YFP), G protein signaling was monitored in real time by fluorescence resonant energy transfer (FRET). The heterotrimer composed of $G\alpha 2$ and $\beta\gamma$ appeared to rapidly dissociate upon addition of cAMP, as evidenced by loss of FRET and reassociated upon removal of ligand (Janetopoulos et al. 2001).

Mutations that impair GTPase activity of $G\alpha 4$ or $G\alpha 2$ inhibit the accumulation of cGMP and cAMP, aggregation, and chemotaxis. The mutated $G\alpha 4$ subunit cannot rescue *ga4* cells and acts as a dominant-negative in wild-type cells (Srinivasan et al. 1999). Similar mutations in the $G\alpha 2$ subunit inhibit cAMP as well as folic acid chemotaxis (Okaichi et al. 1992). These observations suggest that $G\alpha$ subunits directly inhibit downstream effectors or, alternatively, that persistent release of $\beta\gamma$ leads to adaptation or down-regulation of the signaling system. $G\alpha 2$ is also phosphorylated upon cAMP stimulation, but loss of the phosphorylated serine does not result in a chemotaxis phenotype (Chen et al. 1994, Gundersen & Devreotes 1990).

Genetic evidence suggests that the $\beta\gamma$ subunit is the active signaling component for chemotaxis. The $g\beta^-$ cells are motile but do not chemotax, grow slowly on bacterial lawns, and are defective in GPCR-mediated cGMP accumulation, adenylyl cyclase activation, and actin polymerization (Wu et al. 1995, Zigmond et al. 1997). In $g\beta^-$ cells, the GTP-regulated high-affinity chemoattractant-binding sites are lost, indicating that G protein heterotrimers cannot associate with cAR1 (Wu et al. 1995). Use of a temperature-sensitive mutant showed that G β function is required at all stages of development (Jin et al. 1998b). A series of random mutations in G β , which do not interfere with GTP regulation of chemoattractant binding, but inhibited physiological responses, map to the shared G α /effector face of the protein (Jin et al. 1998a). This study shows that there are subdomains for G α and effector interactions. The G β subunit purifies with a single G γ subunit that contains the typical CAAX motif, required to target G $\beta\gamma$ to the plasma membrane (Zhang et al. 2001). Removal of the CAAX-motif and overexpression in wild-type cells shifts endogenous G β to the cytosol and impairs G protein signaling. GFP-tagged $\beta\gamma$ subunits are localized along the membrane of polarized cells in a shallow gradient (Figure 2), suggesting that they do not focus the signal to the front (Jin et al. 2000, Zhang et al. 2001).

YakA, a Prototypical DyrK

YakA was originally identified in *Dictyostelium* as a protein kinase required for the transition from growth to development (Souza et al. 1998) and as a mutant that phenocopies $g\beta^-$ cells (van Es et al. 2001a). YakA belongs to a family of kinases involved in growth and development that includes Yak1 from yeast and the DyrK/MNB-related kinases found in most eukaryotes. Cells lacking YakA do not aggregate and fail to express early development genes. Overexpression of YakA results in growth arrest and the premature induction of early genes (Souza et al. 1998). Cells lacking YakA do not activate signal transduction events such as cGMP accumulation and actin polymerization in response to folic acid and cAMP, even though G proteins still couple to cAR1. Studies on temperature-sensitive alleles of YakA reveal that these activities of YakA are required at early and late stages of development (van Es et al. 2001a). It is likely that YakA's role in coupling nutrient sensing with development is because of its function in signal transduction (Souza et al. 1998, van Es et al. 2001a).

INHIBITION: MULTIPLE POINTS OF SIGNAL ATTENUATION

Properties of Adaptation

Adaptation is an essential feature of chemotactic signaling. Cells respond to changes in receptor occupancy and adapt when occupancy is held constant. The cells will transiently respond again if there is an increase in receptor occupancy or

if the stimulus is removed, thus allowing recovery, and then reapplied. Adaptation allows a cell to sense gradients independently of the mean cAMP concentration and thus allows a cell to chemotax when close or far from sources of chemoattractant. To effect adaptation, inhibitors of excitation are needed.

Inhibition may occur at multiple points downstream of cAR1 because cAMP-induced cell shape changes, PI(3,4,5)P₃ accumulation, actin polymerization, cGMP synthesis, and cAMP synthesis and phosphorylation of myosin heavy and light chain all adapt. FRET studies show that adaptation occurs downstream of the G protein subunit dissociation and, therefore, we place the module in this location. It is likely that multiple adaptation modules will ultimately be incorporated into the network.

FRET between α and β G protein subunits show that the heterotrimer remains dissociated in adapted cells (Janetopoulos et al. 2001). This suggests that occupied receptors, whether phosphorylated or not, continuously trigger the G protein cycle. Consistent with this, GTP inhibition of cAMP binding to cAR1 is unaffected in adapted cells (Snaar-Jagalska et al. 1991). These observations contrast with the visual and the β -adrenergic systems, where phosphorylated receptors interact with arrestin, thereby preventing continued G protein activation. In *Dictyostelium*, adaptation must prevent or counteract the interaction of activated G protein subunits with downstream effectors. These adaptation events are apparently mediated by receptor independently of G α 2 because pretreatment of *g α 2*- cells with cAMP causes a decrease in the capacity of GTP γ S to activate PI3K and adenylyl cyclase in vitro (Huang et al. 2003).

Candidate Inhibitor Molecules

The G protein α subunit, G α 9, is constitutively expressed and is implicated as a negative regulator of development (Brzostowski et al. 2002). Cells lacking G α 9 form more cAMP signaling centers and complete aggregation sooner and at lower cell densities than wild-type cells. Conversely, cells expressing constitutively active mutants of G α 9 inhibit early aggregation and form mounds two to three times as large as wild-type cells. Although evidence suggests that G α 9 plays a role in adaptation, this cannot be the sole mechanism because responses in the *g α 9*- cells do subside, and the cells are able to differentiate and chemotax (Brzostowski et al. 2002).

Regulators of G protein signaling (RGS) proteins bind to the transition state of G α subunits and accelerate GTP hydrolysis and thus could potentially contribute to inhibitory function. Six RGS-like proteins have been identified in *Dictyostelium* on the basis of sequence homology (D. Hereld & T. Jin, unpublished observations). Five of the six RGS proteins have been disrupted and four display weak developmental phenotypes. The lack of strong phenotypes may be due to functional redundancy. Supporting this, overexpression of one of the RGSs gives a morphological phenotype (T. Jin, unpublished observation).

Cells disrupted in RCK1 (RGS-domain-containing kinase), chemotax 50% faster than wild-type cells, suggesting that Rck1 has a role in inhibiting chemotaxis

(Sun & Firtel 2003). The *rck1*- cells develop rapidly at early stages of development but are delayed in development after the mound stage. In $\alpha 2$ - cells, Rck1 kinase activity is not activated (Sun & Firtel 2003), whereas activity increases to higher levels in cells expressing constitutively active $G\alpha 2$, suggesting that Rck1 acts downstream of the G protein. Upon stimulation of cells with cAMP, myc-tagged Rck1 translocates from the cytosol to the plasma membrane and back to the cytosol within 10 s (Sun & Firtel 2003), but kinase activity remains elevated until the stimulus is removed. These kinetics are consistent with a role in inhibition.

Shk1 (SH2-domain-containing kinase) is a dual-specificity kinase that contains an SH2 domain near its C terminus (Moniakis et al. 2001). *Shk1*- cells reportedly have aggregation defects, abnormal localization of F-actin, and random localization of coronin-GFP. Cells lacking Shk1 resemble *pkbA*- cells (Moniakis et al. 2001). In our laboratory, cells developed for 5 h are unable to activate PI3K, which is consistent with this finding, because PKB is activated by PI3K (Y. Huang, unpublished observation). On the other hand, Moniakis et al. report that PKB activation is ~fivefold higher in *shk1*- cells than in wild-type, suggesting that Shk1 is a negative regulator of the PI3K pathways. Further studies are needed to clarify this apparent inconsistency. Shk1 is uniformly distributed around the cortex of the cell, as visualized using FLAG-tagged Shk1 (Moniakis et al. 2001). This localization is dependent upon the SH2 domain, which is also required for function. Targeting of the SH2-deleted Shk1 to the plasma membrane by the addition of a myristoylation motif does not rescue Shk1 function (Moniakis et al. 2001).

EARLY GENE EXPRESSION: SIGNALING PATHWAY REGULATES ITS OWN EXPRESSION

It is well recognized that pulsatile cAMP signaling induces the expression of early genes, including many components of the signaling apparatus. For example, expression of the chemoattractant receptors, G protein subunits, and adenylyl cyclase all require periodic activation of the signaling system. The dependence of early gene expression on signaling events constitutes a developmental feedback loop. Thus most mutations that interfere with signaling also block early gene expression. Therefore, it is important to distinguish whether a mutation or condition blocks a chemoattractant-triggered event or the transition to chemotactic competence.

cAMP-dependent protein kinase (PKA) levels increase several-fold during differentiation. PKA is required for the regulation of early development genes, such as *ACA* and *cARI*, is essential for development, and plays a key role in cell-type differentiation. Cells lacking PKA-R are constitutively active for PKA, and early development genes are induced prematurely (Zhang et al. 2003). These cells form many lateral pseudopods, move more slowly, and are rounder than wild-type cells (Zhang et al. 2003), although they maintain their polarity. Activation of PKA can bypass the need for many upstream signaling molecules in development. For

example, overexpression of PKA-C in cells lacking ACA or the cytosolic regulator of adenylyl cyclase (CRAC) can bypass cAMP signaling and rescue development.

Two proteins have been identified that are essential for early gene expression, Myb2 and EgeA. Myb2 is homologous to myb transcription factors in plants and mammals. *Dictyostelium* cells lacking Myb2 do not aggregate and contain undetectable levels of ACA transcripts (Otsuka & van Haastert 1998). Expression of ACA from a constitutive promoter rescues differentiation. The results indicate a role for Myb2 in initiation of development via induction of ACA expression. Early gene expression (EgeA) is a novel protein that contains a C2 domain and is essential for expression of early genes in development (Zhang et al. 2002b). Cells overexpressing or lacking EgeA have similar phenotypes, suggesting that an optimal amount of this protein is required for function. Overexpression of cAR1 or PKA fails to rescue cells lacking EgeA, suggesting that loss of gene expression is not due to lack of cAMP signaling.

INOSITOL LIPIDS: CENTRAL NODE IN CHEMOATTRACTANT SIGNALING

Local levels of phosphatidylinositol trisphosphate, PI(3,4,5)P₃, at the cell surface regulate actin polymerization and possibly additional events during chemotaxis. There is a balance between the synthesis of PI(3,4,5)P₃ by PI3K and dephosphorylation of this lipid by the PI 3-phosphatase (PTEN) and PI 5-phosphatases. These proteins restrict the region of PI(3,4,5)P₃ accumulation to the front of the cell via their activities and limited localizations in the cell (reviewed in Iijima et al. 2002). In a chemotaxing cell, PI3K is focused at the leading edge and PTEN is restricted to the sides and rear of the cell (Figure 2).

Local Production of PI(3,4,5)P₃

In *Dictyostelium*, there are three PI3Ks most closely related to the mammalian class I family of PI3Ks. Reportedly, PI3K1 and 2 are most like p110 α (Class Ia) PI3Ks, which are activated by Ras and tyrosine kinases in mammals, whereas PI3K3 is most closely related to Class Ib PI3Ks, which have been shown to be activated by G protein $\beta\gamma$ subunits (Zhou et al. 1995). In *Dictyostelium*, bulk PI(3,4,5)P₃ levels increase in response to chemoattractant in part because all three PI3Ks are activated (Huang et al. 2003). PI3K activation depends on $\beta\gamma$, but not on PI(3,4,5)P₃ levels in the cell (Huang et al. 2003). The specific mechanism of activation of *Dictyostelium* PI3K is unclear; however, it requires localization to the plasma membrane, a signal through the heterotrimeric G protein, and Ras. PI3K1 and 2 translocate to the leading edge when cells are in a gradient (Funamoto et al. 2001). This translocation exhibits kinetics similar to that of the PH domain-containing proteins. The N-terminal domains of PI3K1 and 2, which lack catalytic activity, are sufficient for membrane association, but the membrane binding site(s) are unclear. Neither PI3K inhibitors nor disruption of PI3K1 and PI3K2 blocks localization (Funamoto et al.

2002), and PI3K-binding sites are created in the absence of PI3K in a reconstitution assay (Huang et al. 2003). Membrane localization is enough to activate the PI3K pathway because in cells expressing myristoylated or prenylated PI3K, there is a large increase in the basal level of PI3K activation (Funamoto et al. 2002, Huang et al. 2003; Y. Huang, unpublished observation). Point mutations in the Ras-binding domains of PI3K1 or PI3K2 do not block translocation to the plasma membrane but do destroy activity (Funamoto et al. 2002).

Polarity and chemotaxis are impaired in *Dictyostelium* treated with the PI3K inhibitors wortmannin and LY294002, or lacking PI3K1 and 2, suggesting that the lipid products of PI3K are needed for chemotaxis (Huang et al. 2003, Zhou et al. 1995). However, these experiments have been confusing. When PI3K1 or 2 are mutated individually, there are weak chemotaxis defects, and when both are deleted, chemotaxis has been reported alternatively to improve (Buczynski et al. 1997) or be impaired (Funamoto et al. 2001). In addition, these mutants have defects in growth, development, pinocytosis, chemoattractant-mediated F-actin assembly, and polarization (Buczynski et al. 1997, Funamoto et al. 2001, Zhou et al. 1995). In our hands, the *pi3k1-pi3k2*- cells do not aggregate on bacterial lawns, but do aggregate fairly normally on minimal media and display clear chemotaxis responses to a cAMP-filled micropipette. There is residual PI3K activity in *pi3k1-2*- cells in an in vitro assay (Huang et al. 2003) and PI(3,4,5)P₃ and PI(3,4)P₂ are present in HPLC assays of phospholipids from these cells labeled with [³²P] (Zhou et al. 1998). It remains to be seen if chemotaxis can occur completely independently of PI(3,4,5)P₃ or whether it requires the presence of the residual lipids. In neutrophil-like cells treated with PI3K inhibitors chemotaxis is impaired and the ability to form pseudopods is affected (Wang et al. 2002). Moreover, use of cells derived from knockout mice confirm that PI(3,4,5)P₃'s role in chemotaxis is evolutionarily conserved (Hirsch et al. 2000, Li et al. 2000, Sasaki et al. 2000).

Local PI(3,4,5)P₃ Degradation

Dictyostelium contains one major PI 3-phosphatase, PTEN, which is nearly essential for the degradation of PI(3,4,5)P₃, setting up polarity in chemotaxis. When PTEN is deleted, cells form small, aggregation-deficient plaques, consistent with slow growth and poor chemotaxis (Iijima & Devreotes 2002). The cells produce many pseudopods and follow an indirect route to the chemoattractant, compared with the direct migration of wild-type cells. PH domain-containing proteins localize in a broader region across the anterior membrane, indicating that in wild-type cells PTEN degrades PI(3,4,5)P₃ along the sides and rear of the cell (Funamoto et al. 2002, Iijima & Devreotes 2002). The sites of ectopic pseudopodia correspond to the elevated regions of PI(3,4,5)P₃. In addition, actin polymerization in response to cAMP stimulation is higher and prolonged (Iijima & Devreotes 2002, Iijima et al. 2004). This provides convincing evidence that PI(3,4,5)P₃ does regulate F-actin polymerization and pseudopod formation. PTEN hypomorphs with lowered PTEN expression were also generated by placing the gene under control of a tetracycline-inhibited promoter (Funamoto et al. 2002). In the presence of

tetracycline, the PTEN mRNA is reduced compared with that in wild-type, and the cells display decreases in cell movement. PTEN also plays a role in motility in mammalian cells. Overexpression of PTEN in fibroblasts reduces motility in a wound-healing assay (Tamura et al. 1998). Enhanced motility was shown in fibroblasts and myeloid cells from mice deficient in PTEN (Liliental et al. 2000). Increased random motility promoted by elevated PI(3,4,5)P₃ levels is consistent with the inhibition of chemotaxis in *Dictyostelium*.

When imaged in live amoebae, PTEN-GFP is present on the plasma membrane in unstimulated cells and is transiently released from the membrane upon stimulation with kinetics similar to PI3K relocalization (Funamoto et al. 2002, Iijima & Devreotes 2002). In chemotaxing cells, PTEN-GFP localizes to the lateral edges and back of the cell, in a pattern complementary to PI3K (Figure 2). The N-terminal PI(4,5)P₂ binding motif in PTEN is essential for localization and regulates phosphatase activity, although localization does not depend on phosphatase activity, actin polymerization, or intracellular levels of PI(3,4,5)P₃ (Iijima et al. 2004). These results, along with the PI3K observations, suggest a model whereby the reciprocal localization and activation of PI3K and PTEN mediate directional sensing (Devreotes & Janetopoulos 2003).

Four PI 5-phosphatases have been identified in *Dictyostelium* (PI5P1-4), but only inactivation of PI5P4 leads to defects in growth and development. Single and double mutations suggest that there is functional redundancy. Chemotaxis was improved in some single and double knockouts, but no deletions inhibited chemotaxis. Cells disrupted for PI5P4 grow slowly in axenic culture and on bacteria, and the mutant cells form multiple-tipped aggregates (Loovers et al. 2003).

PH DOMAIN-CONTAINING PROTEINS: ACTIVATION BY RECRUITMENT TO LOCAL PI(3,4,5)P₃

Pleckstrin homology-domain-containing proteins, such as CRAC, PKB, and PhdA, are recruited to PI(3,4,5)P₃ on the plasma membrane. When cells are placed in a gradient, these proteins bind to PI(3,4,5)P₃ formed at the leading edge (Parent et al. 1998). PH-binding is blocked by the PI3K inhibitors wortmannin and LY294002, and is dramatically decreased in *pi3k1/-2-* cells (Funamoto et al. 2001). However, actin polymerization is not required, because treatment of cells with latrunculin to disassemble the actin cytoskeleton does not prevent PH domain association to the membrane (Parent et al. 1998). Similar experiments and the use of antibodies specific for PI(3,4,5)P₃ confirmed the presence of the phosphoinositide at the leading edge of a neutrophil-like cell line (Weiner et al. 2002).

Cytosolic regulator of adenylyl cyclase (CRAC) was the first PH domain-containing protein shown to be recruited to the membrane (Lilly & Devreotes 1995). CRAC associates with the plasma membrane after exposure to cAMP and is required for ACA activation (Lilly & Devreotes 1995). Cells lacking CRAC or carrying mutated CRAC do not activate ACA. In membranes from *crac-* cells,

GTP γ S does not activate ACA, but this activity is reconstituted by adding back supernatants containing CRAC.

Because ACA is required for development, CRAC should also be involved. The requirement for CRAC in early development is overcome by pulsing the cells with exogenous cAMP or constitutive activation of PKA. After aggregation, CRAC is required for spore and prespore cell differentiation (Parent et al. 1998). The PH domain of CRAC is not required for later development because cells lacking CRAC can still undergo cell-type differentiation if the catalytic subunit of PKA is overexpressed (Wang et al. 1999).

PKB is activated upon recruitment to the membrane by PI(3,4,5)P₃, and activation is diminished tenfold when PI3K1 and PI3K2 are mutated or inhibited. Cells lacking PKB express cAR1 appropriately, but have poor polarization and do not chemotax well (Funamoto et al. 2001, Meili et al. 1999). The phenotypes of *pi3k1-/-* and *pkb-* are not identical, suggesting that PI(3,4,5)P₃ has multiple targets. PKB phosphorylates and activates PakA, which is needed for myosin II assembly at the back of the cell (Chung et al. 2001). Decreased myosin II assembly is reported in *pkb-* cells. This is puzzling because PKB is located at the leading edge, and PakA and myosin II are located at the rear of the cell.

PhdA was identified by its strong homology to the N-terminal domain of CRAC and localizes to the plasma membrane upon addition of chemoattractant. Also, this response is blocked when wild-type cells are treated with LY294002 in *pi3k1-/-* cells and when the PH domain is mutated (Funamoto et al. 2001). PhdA is not involved in PKB activation, guanylyl cyclase stimulation, or regulation of myosin II (Funamoto et al. 2001), but may act as a docking site for other cellular proteins at the leading edge. A mammalian homolog of PhdA has not been identified.

Chemoattractant stimulation increases the levels of diphosphoinositol pentakisphosphate (IP7) and bis-diphosphoinositol tetrakisphosphate (IP8) in the cell (Luo et al. 2003) and IP7 competes with PI(3,4,5)P₃ for PH binding in vitro and in vivo. Deletion of the inositol hexakisphosphate kinase (IP6K), which converts inositol hexakisphosphate to IP7, abolishes this production of IP7 and IP8. These mutant cells aggregate rapidly and exhibit increased sensitivity to cAMP. These observations suggest that IP7 and IP8 generated during cAMP stimulation may be involved in damping the signal by preventing the recruitment of specific PH domain-containing proteins to the membrane.

cAMP SIGNALING: REGULATION OF cAMP ACCUMULATION

Adenylyl Cyclase and Cell-Cell Signaling

The adenylyl cyclase for aggregation (ACA) is a 12-transmembrane domain enzyme resembling mammalian ACs, with two sets of 6 transmembrane domains and 2 homologous cytoplasmic domains. ACA is expressed during aggregation

and mediates cell-cell signaling via cAMP-induced cAMP production. Cells lacking ACA do not signal and do not express early aggregation genes such as *cAR1* (Pitt et al. 1992). However, cAMP stimuli given to *aca-* cells, mimicking the cAMP production in wild-type cells, allow the cells to develop. Even so, they are not as polarized and are less motile in the absence of chemoattractant (Kriebel et al. 2003). These differentiated cells display normal guanylyl cyclase activity, and $PI(3,4,5)P_3$ increases when $GTP\gamma S$ is added to cell lysates (Lilly & Devreotes 1995, Pitt et al. 1992). However, *aca-* cells are unable to stream as wild-type cells do, reflecting the requirement for ACA activity in cell-cell signaling (Kriebel et al. 2003).

ACA-YFP fusion proteins localize to the rear of polarized cells (Kriebel et al. 2003). Enzyme activity is not required for localization, but constitutive activity impairs it. It is possible that myosin is involved in establishing the cytoskeletal framework to localize ACA at the back of cells or could be important for trafficking ACA to the rear. ACA localizes to intracellular vesicles, and ACA may traffic to the rear of cells via this route.

Components Involved in ACA Activation

In addition to receptors, G proteins, and CRAC, ACA activation requires Aimless, Erk2, and Pianissimo and possibly RasC. Cells lacking RasC have strong aggregation defects, and evidence suggests that it is required for cAMP relay during development. PKB phosphorylation is dramatically decreased in *rasC-* cells (Lim et al. 2001), suggesting a link to the PI3K signaling pathway. However, the receptor and G protein-mediated activation of PI3K is normal in cells lacking RasC (Y. Huang, unpublished observations).

Two proteins that putatively interact with Ras are also involved in ACA activation. Aimless (AleA) is a homolog of *Saccharomyces cerevisiae* Cdc25, a Ras exchange factor. AleA is also required for receptor-mediated activation of ACA, as well as for proper chemotaxis (Insall et al. 1996). It is not yet clear which Ras is the target for AleA. Rip3 (Ras-interacting protein 3) was identified in a two-hybrid search with mammalian Ha-Ras (Lee et al. 1999). Rip3 appears to be required for the cAMP relay and has a phenotype similar to cells lacking AleA or RasC. Rip3 preferentially interacts with RasG, but not with RasC. Double mutants lacking both Rip3 and AleA are defective for chemotaxis, suggesting that both proteins may regulate Ras-dependent processes in chemoaxis (Lee et al. 1999).

Pianissimo (PiaA) is a cytosolic protein required for chemoattractant-mediated activation of ACA and resembles in phenotype Rip3, RasC, and AleA nulls (Chen et al. 1997). *PiaA* is a novel gene conserved from yeast to human. The *S. cerevisiae* homolog of *PiaA* is an essential gene. Activation of ACA in vivo (through chemoattractant receptor) and in vitro (directly activating the G protein with $GTP\gamma S$) is absent in *piaA*-cells (Chen et al. 1997). This defect can be corrected by adding back supernatant containing PiaA to the assay, suggesting that PiaA acts directly in the pathway. A temperature-sensitive mutation in PiaA was derived in a screen of chemically induced mutants for the aggregation minus phenotype (Pergolozzi

et al. 2002). The mutant displays a temperature-dependent dominant phenotype in wild-type cells, suggesting that PiaA interacts with other proteins. Interestingly, Rip3 and PiaA correspond to two proteins, Avo1 and Avo3, that form a complex with Tor2 in yeast believed to play a role in cytoskeletal rearrangements (Loewith et al. 2002).

The MAPK Erk2 is also required for ACA activation and is activated by chemoattractant, but the mechanism is unknown (Schenk et al. 2001, Segall et al. 1995). Furthermore, Erk2 also inhibits RegA, a cAMP phosphodiesterase (Pde2, discussed below). As internal cAMP levels rise, PKA is activated and inhibits Erk2. Because Erk2 is inactivated, RegA becomes activated and reduces intracellular cAMP, which decreases PKA activity. This cycle may be important for the increase and decrease in cAMP associated with waves during development.

cAMP Phosphodiesterases

Intracellular and extracellular cyclic nucleotide phosphodiesterases (PDE) can be divided into three functional groups. The first group (Pde1, Pde4) degrades extracellular cAMP. The second (Pde2/RegA and Pde6/GbpB/PdeE) and third groups (Pde3, Pde5/GbpA/PdeD and Pde6/GbpB) degrade intracellular cAMP and cGMP, respectively. This last group is discussed separately in the cortical myosin module.

Pde6 is expressed in aggregating cells and has been shown to be important for development (Meima et al. 2003). Pde6 has dual substrate specificity and is activated by both cGMP and cAMP (Bosgraaf et al. 2002b). The activation by cAMP contains a negative feedback loop that may regulate the production of cAMP. However, because the *pde6*- cells aggregate, Pde6 must not be the only mechanism for attenuating cAMP. It is possible that Pde6, Pde2, and cAR1-mediated adaptation of ACA act together to terminate cAMP signaling (Meima et al. 2003), resulting in a robust system that is difficult to characterize with single disruptions.

G PROTEIN-INDEPENDENT RESPONSES: SIGNALING WITHOUT $G\alpha\beta\gamma$

It was first shown in *Dictyostelium* that G protein-coupled receptors, in fact, can also signal independently of G proteins (Milne & Devreotes 1993). Because only single genes encode the G protein β and γ subunits, proof of G protein-independent processes was obtained in studies of *Dictyostelium* cells lacking $G\beta$. The G protein-independent responses module emanates from the excitation module as these signals are mediated by the cARs. G protein-independent signaling has recently been reviewed in depth (Brzostowski & Kimmel 2001).

A diverse series of G protein-independent responses have been uncovered. First, cAMP-stimulated Ca^{2+} entry is partially independent of G proteins. Cells expressing cAR1 or cAR3 but lacking $G\beta$ display cAMP-stimulated Ca^{2+} entry with wild-type kinetics, ion specificity, and agonist dependence, although the

magnitude is 50% lower. The Ca^{2+} response is directly correlated to the level of agonist binding, and mutations in the third intracellular loop of cAR1 impair this signaling pathway (Caterina et al. 1994). Second, Erk 2 is transiently activated in *gβ*- cells, but requires cAR1 or cAR3. Again, the response is reduced by 50% compared with that of wild-type cells (Maeda et al. 1996). Third, cAMP triggers tyrosine phosphorylation of StatA and its translocation to the nucleus. This activation requires cAR1 or cAR3, but not Gβ, suggesting that its activation is G protein independent (Briscoe et al. 2001). Fourth, the zinc-finger G-box binding transcription factor (GBF) is necessary for expression of genes needed for the switch from aggregation to post-aggregation. Constitutive expression of GBF in cells lacking Gβ or Gα2 rescues the cAMP-induced GBF-dependent reporter gene expression. However, this does not occur in *car1*- or *car3*- cells, suggesting that the cARs are required for post-aggregation gene expression. Finally, prespore and spore cell fates require cAMP-induced cAR1 activation and downstream activation of GskA (Harwood et al. 1995). cAMP also inhibits GskA activity via cAR4 by dephosphorylation at Tyr215 on GskA (Kim et al. 1999). cAMP repression of stalk cell formation is lost, and abnormal gene expression is observed in *gskA*- cells. All these events influencing cell-type gene expression appear to be independent of Gβγ (Jin et al. 1998b).

POLARIZATION ACQUISITION: COORDINATING CELL-CELL SIGNALING WITH CHEMOTACTIC COMPETENCE

An interesting series of mutations involving Mek1, TorA, and TsuA develop normally and begin to relay cAMP signals at the appropriate time but are unable to migrate directionally up spatial gradients of cAMP. Cells lacking Mek1 develop and produce cAMP normally, but respond to chemoattractant with attenuated movements (Ma et al. 1997). Temperature shift experiments on *mek1*- cells expressing a temperature-sensitive Mek1 show that Mek1 activity is required throughout aggregation (Ma et al. 1997). Incidentally, activation of Erk2 is not affected in *mek1*- cells, suggesting that there are independent MAP kinase cascades involved in development.

A novel gene, *Tortoise* (*TorA*), was identified in a screen for aggregation-defective mutants. Cells lacking TorA generate cAMP waves with the same speed and frequency as wild-type cells, but the chemotactic steps are smaller than wild-type and the cells form many lateral pseudopods (van Es et al. 2001b). Much like cells lacking Mek1, *torA*- cells continuously make waves of cAMP, but do not form mounds with a normal time course. Eventually, the waves stop and *torA*- cells reorganize into tiny mounds and form small fruiting bodies (van Es et al. 2001b). TorA-GFP localizes to a small region in the mitochondria that stains intensely with Mitotracker, suggesting a role for mitochondria in chemotaxis. Interestingly, TorA and Mek1 can suppress defects in each other, consistent with the suggestion that

they are in the same genetic pathway. An insertional mutant, *Tsunami* (*TsuA*), has a phenotype similar to *mek1*- and *torA*- cells, although it is not clear if the defects are due to loss of function (L. Tang, unpublished observations). *TsuA*- cells differentiate and begin cell-cell signaling, indicating that they can sense and respond to cAMP. However, similar to *mek1*- and *torA*- cells, *Tsunami* cells are not polarized and do not chemotax toward the propagating cAMP waves.

ACTIN POLYMERIZATION: SIGNALING TO PSEUDOPODIA

There are two phases of actin polymerization triggered by a uniform stimulus that closely parallel the biphasic accumulation of PI(3,4,5)P₃. The first peak begins 5 s after stimulation and falls rapidly after 15–30 s, whereas the second peak occurs at 90 s (Chen et al. 2003, Postma et al. 2003). As cells develop and become more polarized, the second peak is diminished (Chen et al. 2003). In cells lacking PTEN, the slow phases of PI(3,4,5)P₃ and actin polymerization are larger (Iijima & Devreotes 2002). Exposure of wild-type and *pten*- cells to PI3K inhibitors abolishes most PI(3,4,5)P₃ production and the slow phase of actin polymerization (Chen et al. 2003). These results indicate a causal link between lipid production and actin polymerization, as represented by the actin trigger module in Figure 1. This module indicates that PI(3,4,5)P₃ and another process mediate actin polymerization.

Rho GTPases are thought to play a key role in actin rearrangements and 15 members have been identified in *Dictyostelium* (Rivero & Somesh 2002). In vitro experiments suggest that certain small GTP-binding proteins must be involved in actin polymerization (Zigmond et al. 1997). Studies have been performed with overexpression of wild-type or dominant-negative Racs, and some of the Rac genes have been disrupted. Rac1(a,b,c) has been implicated in chemotaxis. Expression of Rac1b in *Dictyostelium* results in the formation of large lamellipodia, increased in intracellular F-actin and increased chemotaxis to folic acid (Palmieri et al. 2000), whereas expression of activated Rac1b causes inefficient chemotaxis owing to decreased speed and an increase in lateral pseudopods (Chung et al. 2000). Overexpression of dominant-negative Rac1b produces immobile cells that are unable to extend pseudopods (Chung et al. 2000).

Targets for activation by Rho family GTPases are the WASP (Wiskott-Aldrich syndrome protein) family members (WASP, Scar/WAVE), which stimulate Arp2/3 via direct interactions. Scar was first identified in *Dictyostelium* as a suppressor of cAR2. Four human homologs of Scar have been identified and these interact with the p21 subunit of the Arp2/3 complex via their C termini in vitro (Machesky & Insall 1998). *Dictyostelium* cells lacking Scar perform chemotaxis but have abnormal cell morphology and actin distribution (Bear et al. 1998). Growing *scar*-cells have reduced levels of F-actin staining and have a multiple-tip formation defect in later stages of development (Bear et al. 1998). Scar associates with Pir121, which is thought to hold it in an inactive complex because disruption of Pir121

generates hyperactive cells with elevated levels of actin polymerization (Blagg et al. 2003). Surprisingly, the cAMP-triggered actin polymerization changes are normal in *scar-*, *pir121-*, and *scar-/pir121-* cells, suggesting that chemoattractant-mediated actin polymerization does not go through Scar. Cells also contain a WASP homolog that may mediate this process; however, this gene has not been disrupted.

Seven subunits of the Arp2/3 complex are found in *Dictyostelium*, and six of the subunit sequences are more similar to human than to *S. cerevisiae* (Insall et al. 2001). Attempts to disrupt the subunits have been unsuccessful, suggesting that these proteins are essential for growth. In lamellipodia, GFP-tagged Arp3 and p41Arc localize along the actin filaments instead of concentrating at the ends where actin polymerization is predicted to occur (Insall et al. 2001). Upon stimulation, Arp3 increases by twofold and accumulates at new chemotactic-induced leading edges, consistent with a role in chemotaxis. However, without disruptions or additional experiments, a conclusive role for the Arp2/3 complex in chemotaxis versus general motility cannot be identified.

CORTICAL MYOSIN: DETERMINING THE BACK OF A CELL

Early in development, cells are able to create new pseudopods at the sides and back upon a shift in the gradient, whereas more developed cells become relatively insensitive at the back. Some of the proteins critical for developing this polarity include those regulating PI(3,4,5)P₃ (see above) and those regulating cGMP and myosin filament formation. The differences between polarization and directional sensing have been reviewed recently (Devreotes & Janetopoulos 2003, Iijima et al. 2002).

Chemoattractants trigger a transient increase in cGMP that peaks at 10 s and returns to basal levels by 30 s. Guanylyl cyclases (GC) are activated briefly and the cGMP produced is rapidly degraded by a cGMP-induced, cGMP-specific phosphodiesterase (PDE) (Bosgraaf & van Haastert 2002). Two guanylyl cyclases, GCA and sGC, homologous to 12-transmembrane and soluble adenylyl cyclases, respectively, have been identified (Roelofs et al. 2001). *Gca-/sgc-* double mutants generate no cGMP, aggregate slowly, and exhibit reduced chemotactic activity. Specific phosphodiesterases, Pde3, Pde5, and Pde6, degrade cGMP generated by exposure to chemoattractant. Pde5 and Pde6 are novel cGMP-stimulated PDEs that are not homologous to known cGMP-binding proteins in higher eukaryotes (Bosgraaf et al. 2002b). Pde6 is a phosphodiesterase with dual substrate affinity and is activated by both cGMP and cAMP. Pde5 is deficient in *stmF* mutants, cells previously shown to be highly polarized (Bosgraaf et al. 2002b, Meima et al. 2003). Cells deleted for Pde5 and Pde6 display higher chemotactic indices, presumably owing to the suppression of lateral pseudopods (Bosgraaf et al. 2002a).

GbpC and GbpD are novel cGMP-binding proteins, and cells lacking these proteins move at slower speeds and with decreased chemotactic indices (Bosgraaf et al.

2002a, Goldberg et al. 2002). The phenotypes are similar to those in *gca-/sgc-* cells, suggesting that these proteins mediate the cGMP effects. Although their function is unclear at this time, the proteins have interesting signaling domains, such as RasGEF and protein kinase domains (Bosgraaf et al. 2002a).

Three myosin heavy chain kinases (MHCKs) in *Dictyostelium* are functionally redundant. MHCK A and MHCK B are expressed constitutively, whereas MHC protein kinase C (PKC) is expressed only in developed cells. All three probably phosphorylate the same sites in the tail of myosin II (Steimle et al. 2001). Upon cAMP treatment of cells, MHC-PKC translocates to the membrane and is autophosphorylated (Rubin & Ravid 2002). The amount of membrane-associated MHC-PKC correlates with the intracellular cGMP concentration. cGMP does not directly activate MHC-PKC, so a cGMP-dependent kinase is proposed to be the first step in activation of this protein. Myosin light chain kinase (MLCKa) phosphorylates myosin II. This modification is not essential but does augment the activity of filamentous myosin four- to sixfold (Zhang et al. 2002a). Deletion of MLCKa does not result in severe defects, but there must be other unidentified MLCKs because the basal level of regulatory light chain (RLC) phosphorylation can be detected in cells deleted for MLCKa (de la Roche & Cole 2001).

Taken together, these studies suggest that cGMP is required to induce the myosin filament formation at the rear of the cell, suppressing pseudopod formation at the lateral edges and back. cGMP is implicated in the transient phosphorylation of RLC and MHC of myosin II, which results in myosin II disassembly at the front of the cell (van Haastert & Kuwayama 1997). The cells that lack cGMP do have residual increases in RLC phosphorylation that may be mediated by PakA (Chung & Firtel 1999). cGMP may have additional roles in chemotaxis because the *gca-/sgc-* cells are more affected than cells lacking myosin II (Bosgraaf et al. 2002a). Although myosin formation in neutrophils appears to be mediated by a Rho-stimulated kinase, the result is the same. Myosin filament formation at the rear of neutrophils inhibits pseudopod formation at the sides and back of the cell and allows contraction of the rear of the cell (de la Roche & Cole 2001).

Cells mutated for one or more myosin I (Myosin A-F and MyoK) exhibit more lateral pseudopods during chemotaxis (Falk et al. 2003, Schwarz et al. 2000, Titus et al. 1993). Overexpression of myosin I impairs cell migration (Novak & Titus 1997), and MyoB is recruited to the cell membrane upon stimulation with cAMP (Titus et al. 1993). The SH3 domains in these proteins make direct contacts with 116-kDa protein, and MyoB and C are concentrated with Arp2/3 and p116 along the leading cortex of polarized cells. Myosin I may bring Arp2/3 to the leading edge via its interaction with p116.

Myosin phosphatases have not received much attention but have the potential to be important regulators of myosin activity. They act on myosin II RLC or MHC. One of these phosphatases has been recently identified and named PP2A-heterotrimeric protein phosphatase 2A (de la Roche & Cole 2001).

PakA colocalizes to the posterior cortex with myosin II (Chung & Firtel 1999, Muller-Taubenberger et al. 2003). PakA (p21-activated serine/threonine kinase) is

structurally similar to the mammalian Paks and contains an N-terminal regulatory/localization domain, a central Rac1^{GTP} binding or CRIB domain, and a C-terminal kinase domain (Chung & Firtel 1999). Chung & Firtel found that disruption of PakA results in defects to chemotaxis, polarity, and cytokinesis (Chung & Firtel 1999). Muller-Taubenberger et al. found that disruption caused no discernible phenotype, whereas overexpression of the C-terminal region has dominant-negative effects on chemotaxis and phagocytosis (Muller-Taubenberger et al. 2003).

SUMMARY

Owing to the numerous links among proteins and the large gaps in our knowledge of the underlying network, chemoattractant-induced signaling is not easily represented as a simple linear pathway. A modular representation is preferable as it allows us to depict functional units of the cell even when some of the components and biochemical reactions are unknown. Moreover, it can be easily modified as new connections are established and relationships between proteins are identified. Because of its powerful biochemical, genetic, and cell biological advantages over other systems that chemotax, *Dictyostelium* provides the ideal vehicle for studying chemotactic pathways. Parallel analysis of wild-type cells with those lacking specific genes is essential to elucidate the role of the protein. In addition, members of gene families and modules can be disrupted combinatorially to determine the role of individual components and of the module. Because many of the proteins and mechanisms are conserved in higher organisms, it is not surprising that *Dictyostelium* is leading the way in the discovery of the key events in chemoattractant signaling.

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