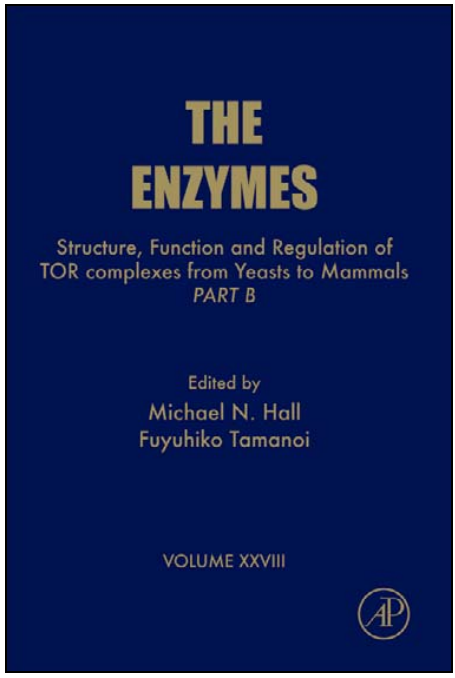

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TORC2 and Chemotaxis in Dictyostelium discoideum

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I. Abstract

Chemotaxis involves the cellular reactions of motility and directional sensing, which enable cells to sense and move along extracellular chemical gradients. *Dictyostelium discoideum* cells display robust chemotactic responses to cAMP. Extensive characterization of these responses has provided insights into the mechanisms of chemotaxis. In this system, chemotaxis is composed of multiple signaling pathways including the TORC2–PDK–PKB module where target of rapamycin complex 2 (TORC2) and phosphoinositide-dependent kinase (PDK) function as upstream activators of protein kinase B (PKBs) through hydrophobic motif (HM) and activation loop (AL) phosphorylations, respectively. This module forms a unique signaling pathway where chemoattractant signals are separated and then converge on substrate phosphorylations mediated by two PKB homologues, phosphatidylinositol (3,4,5)-tris phosphate (PtdIns $(3,4,5)P_3$)-dependent PKBA and -independent PKBR1. PKBA and PKBR1 contribute minor, redundant and major activities, respectively. Consistently, *pkbR1*$^-$ cells are more severely impaired in chemotaxis than cells lacking PKBA or PtdIns $(3,4,5)P_3$. The TORC2–PDK–PKB module is selectively activated at the

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front of morphologically polarized cells by an upstream regulator, RasC, during chemotaxis. Spatial dysregulation of this pathway, exemplified by *pten*$^{-}$ cells or cells expressing an active form of RasC where PtdIns (3,4,5)P_3-PKBA or TORC2-dependent PKBR1 is overactivated, respectively, leads to extension of pseudopods all around cells and to severe chemotaxis defects. These results strongly suggest that this pathway has critical roles in linking chemotactic stimuli to cytoskeletal rearrangements. These studies shed light on the function and regulation of the TORC2–PDK–PKB pathway from a new perspective.

II. Introduction

Chemotaxis, the directed motility of cells in chemical gradients, is a fundamental process displayed by cells throughout evolution. It is required for proper organ formation in embryogenesis, wound healing, and immune responses including inflammation and infection control. In microorganisms, it is used for forging and cell–cell interactions. Although human neutrophils and *D. discoideum* amoebae are widely separated evolutionally, they display very similar chemotactic behavior with high sensitivity to chemoattractants and rapid amoeboid movement and both systems have provided important insights to chemotaxis [1, 2]. *D. discoideum* is a powerful model for studies of chemotaxis. It has a sequenced 34Mbp haploid genome, and contains many signaling pathways important in higher eukaryotes, including PI3K–PTEN as well as TORC2–PDK–PKB systems [3]. Moreover, the chemotactic response is very robust and has been extensively studied.

3′,5′-Cyclic adenosine monophosphate (cAMP) acting as a chemoattractant triggers activation of a series of signaling pathways by binding to the cAMP receptor (cAR1). cAR1 is a G-protein coupled receptor (GPCR) that signals through the heterotrimeric G-proteins to mediate many events including activation of small GTPases, phosphatidylinositol (3,4,5)-tris phosphate (PtdIns (3,4,5)P_3) production, adenylyl cyclase activity (ACA) activation, and actin polymerization. Gradients of chemoattractants induce a polarized morphology and many of cAMP-stimulated events occur locally at the front or back. PtdIns (3,4,5)P_3 accumulates at the front of chemotaxing cells by the opposing actions of two enzymes, phosphatidylinositol-3-kinase (PI3K) and phosphatase and tensin homologue deleted on chromosome ten (PTEN) [4–7]. PI3K and PTEN, respectively, add or remove a phosphate at 3 position of the inositol ring of PtdIns (3,4,5)P_3 lipids. PtdIns (3,4,5)P_3 is synthesized at the pseudopod where actin polymerization occurs and drives the cell forward. *D. discoideum* cells lacking the PTEN form pseudopods all around the perimeter, resulting in a severe impairment of chemotaxis.

This argues for an important function of PtdIns (3,4,5)P_3 [7]. However, PtdIns (3,4,5)P_3 is not essential for chemotaxis because cells lacking PI3K activity can still carry out chemotaxis [8, 9]. This discrepancy appears to be explained by cAMP stimulation of PtdIns (3,4,5)P_3-dependent and -independent pathways which are mediated by two PKB homologues, PKBA and PKBR1 [10]. Activities of PKBA and PKBR1 are regulated in a PtdIns (3,4,5)P_3-dependent or -independent manner, respectively [5, 11]. Activation of both kinases requires phosphorylations by TORC2 and PDK [10, 12, 13]. While PKBA is also regulated by PtdIns (3,4,5)P_3 through its PH domain, PKBR1, a myristoylated molecule, is heavily dependent on TORC2 and PDK for activation. Recent studies have shown that TORC2 is regulated downstream of RasC activation. This review summarizes our recent understanding of chemotaxis, with a particular focus on the TORC2–PDK–PKB pathway.

III. The Life Cycle of *D. discoideum*

D. discoideum cells proliferate as a unicellular organism that feeds on bacteria and yeasts in the soil (Figure 6.1). Metabolites from microorganisms, for example folate from bacteria, act as chemoattractants. In nutrient poor environments, the cells begin a developmental program that leads to the formation of a multicellular structure. First, starvation triggers the expression of a series of genes required for early development. Cells begin to spontaneously secrete cAMP every 6 min, which functions as a chemoattractant. About a million cells aggregate to form a ''mound.'' A tip forms on the mound and elongates into a slug-like structure. The ''slug'' moves around sensing temperature and light. Finally, it transforms into a fruiting body that is composed of a basal disc, a stalk, and spores that endure in harsh environments. These cells use chemotaxis repeatedly at various stages of their life cycles.

In the laboratory, we can grow cells in defined liquid nutrient media or together with bacteria on an agar surface where they form plaques, starve, and differentiate. Using this system, a wide variety of developmentally defective mutants have been isolated and characterized. The most severe ones are unable to aggregate on bacteria lawns and form ''smooth'' plaques. Other mutants arrest at various different stages of development, such as mound, slug, etc. The early developmental phase is easily recapitulated by transferring growing cells into a nonnutrient buffer and incubating them until they reach ''aggregation competence.'' These cells are used to assess chemotactic ability and biochemical events during cAMP stimulation. Numerous assays for chemotaxis have been described. One key biochemical event is cAMP-mediated cAMP production. A method to measure

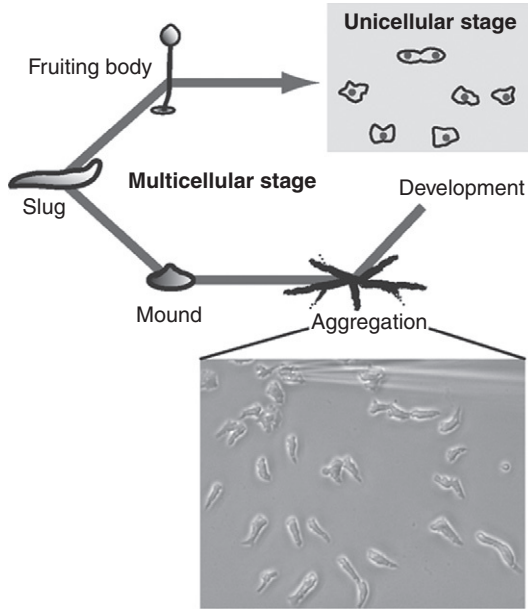

FIG. 6.1. The typical lifecycle of *D. discoideum* cells is depicted. In nutrient-rich environments, individual cells proliferate mitotically. However, once nutrients are deprived from their environment, cells begin a development program to form a multicellular aggregate by cAMP-mediated chemotaxis. An image of cells during chemotaxis toward a cAMP-filled micropipette is shown in the aggregation stage. The multicellular structure transforms to fruiting body through typical morphological steps, including mound and slug. When conditions are appropriate, spores germinate to grow vegetatively. More details are found in the text.

activation of ACA *in vitro* was established where nonhydrolyzable GTP, GTPγS, stimulates the enzymes acting through heterotrimeric G-proteins and small GTPases as described below. The cell lysate assay is also applied to a variety of other biochemical assays, such as TORC2–PDK-dependent PKB phosphorylations [10].

IV. The Components of TORC2–PDK–PKB Pathway in *D. discoideum*

A. PKB (PKBA AND PKBR1)

AKT or PKB has important roles in diverse biological circumstances, including cell growth, development, and cell motility. The kinase domains of PKBs are highly conserved among different species. PKBA and PKBR1,

two PKB homologues in *D. discoideum*, are structurally very similar except for the N-terminal region [5, 11] (Figure 6.2A). While PKBA is a canonical PKB and has a pleckstrin homology (PH) domain at the N-terminus, PKBR1 does not have PH domain but is myristoylated. Like mammalian PKB, PKBA localizes to PtdIns (3,4,5)P_3-enriched regions on the plasma membrane through its PH domain. In contrast, PKBR1 localizes to the plasma membrane constitutively and uniformly through its myristoylation.

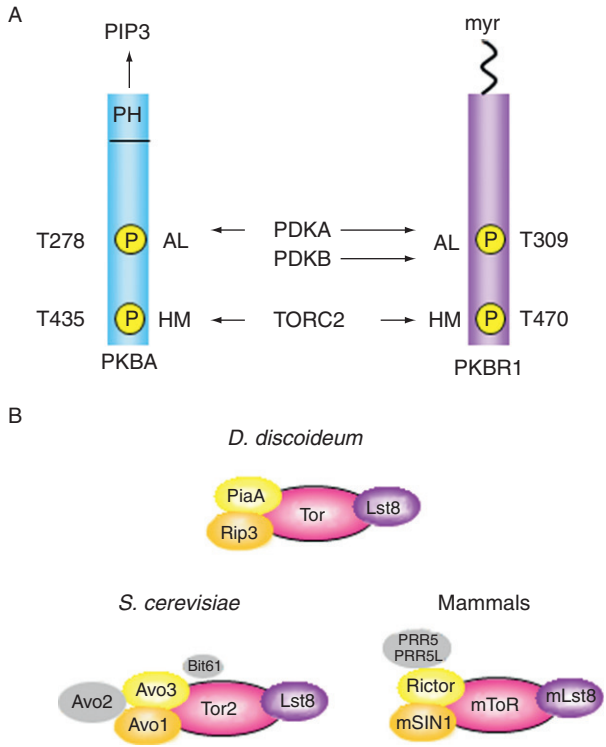

FIG. 6.2. The TORC2–PDK–PKB module in *D. discoideum* cells is shown. (A) The two PKB homologues, PKBA and PKBR1, are regulated by phosphorylations at the activation loop (AL) and the hydrophobic motif (HM). PKBA has a PtdIns (3,4,5)P_3 (PIP3)-dependent pleckstrin homology (PH) domain and PKBR1 is a myristoylated (myr) protein. Upon cAMP stimulation, PKBR1 and PKBA provide major and redundant, lesser activities, respectively. TORC2, composed of Tor, PiaA, Rip3, and Lst8, phosphorylates the HM of PKBA and PKBR1. The two PDK homologues, PDKA and PDKB, function as AL kinases. PDKA mainly phosphorylates PKBA and PKBR1 at the AL. PDKB phosphorylates only PKBR1. (B) The comparison of TORC2 structure in *D. discoideum*, *S. cerevisiae*, and mammals is shown.

Activation of PKB requires phosphorylations at the AL and at the HM. Extensive studies have shown that PDK and TORC2 are kinases responsible for the AL and HM phosphorylations [14–16]. PKBA and PKBR1 also have these consensus phosphorylation sites. The AL and the HM, respectively, at Thr278 and Thr435 in PKBA and at Thr309 and Thr470 in PKBR1 are essential for activity. Using PDK or TORC2 mutant cell lines, we found indeed PKBA and PKBR1 are phosphorylated at the AL and HM by PDK and TORC2, respectively [10, 13, 17]. However, the mechanisms of activation of PKBA and PKBR1 are somewhat complex. PKBA and PKBR1 with alanine mutations in the AL have impaired functions and, furthermore, reduced phosphorylations in the HM. The PKBs with alanine mutations in the HM are also not functional and display either no or greatly reduced AL phosphorylation for PKBR1 or PKBA, respectively [10, 17]. These results suggest that PKBA and PKBR1 activations require the initial phosphorylation at the HM by TORC2 and subsequent phosphorylation at the AL by PDKs. Moreover, the AL phosphorylation affects the stability or the efficiency of the HM phosphorylation. The reciprocal regulation between AL and HM is also supported by the experiments using *pdkA*$^-$*pdkB*$^-$ and TORC2 mutant cells [10, 13].

Mammalian AKT/PKB becomes constitutively active with phosphomimetic substitutions of both the AL and HM sites, suggesting that these phosphorylations are sufficient for its activation. However, activation of the PKBs in *D. discoideum* is not exactly the same as AKT/PKB in mammalian cells and requires further steps for activation [10]. First, PKBR1 with aspartate or glutamate in the AL is not active. Second, TORC2 appears to have additional functions beyond its role in HM phosphorylation because these substitutions at Thr470 of PKBR1 are sufficient for phosphorylation at the AL but not for function in cells lacking TORC2 subunit PiaA. We do not know whether PKBR1 with phosphomimetic substitutions at the HM in *piaA*$^-$ cells is impaired in kinase activity or another function, for example, substrate recognition. Although the exact mechanism remains unknown, another possible target of TORC2 is the turn motif (TM) of PKBs [18–21]. Several studies have shown the importance of TM phosphorylation by TORC2 in AGC kinases. Since both PKBA and PKBR1 conserve these sites, it is interesting to consider this possibility.

Cells lacking PKBA, PKBR1, or both PKBA and PKBR1 display different phenotypes, suggesting redundant and distinct functions *in vivo*. The *pkbA*$^-$ cells show weak chemotaxis defects and cells display slightly less polarity in early differentiation; however, they finally make normal fruiting bodies [5]. The *pkbR1*$^-$ cells are defective in chemotaxis and arrest at the ''mound stage'' [11]. While *pkbA*$^-$ or *pkbR1*$^-$ cells can grow in liquid medium, *pkbA*$^-$ *pkbR1*$^-$ cells cannot, suggesting overlapping functions for the two kinases in macropinocytosis [10, 11].

B. TORC2

D. discoideum express the counterparts of each of the subunits of TORC2, Tor, PiaA, Rip3, and Lst8 (Figure 6.2B). Tor encodes the catalytic subunit. The *tor* gene appears to be essential because targeted disruption of the gene by homologous recombination was not successful [12]. PianissimoA (PiaA) is an orthologue of Avo3 in budding yeast and RICTOR in mammals. PiaA was originally found in a genetic screen for cells defective in early development [22]. Independently, a mutant cell line that was temperature sensitive for early development was found to have an amino acid mutation of Asp at Gly917 in PiaA [23]. Ras interacting protein 3 (Rip3) is an orthologue of Avo1 in budding yeast and mSIN1 in mammals and was isolated as a factor that interacted with an activated form of mammalian Ha-Ras (Ha-Ras Gly12Val) in a yeast two-hybrid assay [24]. As the other homologues, Rip3 contains a Ras binding domain (RBD) and mutational analysis of the domain has demonstrated its functional importance [12]. The *lst8* gene was identified in the *D. discoideum* genome as an Lst8 orthologue of budding yeast or mammals [12].

Gene disruptions of all of the TORC2 subunits except for the *tor* catalytic gene were created and found to display similar phenotypes [10, 12, 22, 24]. First, they are unable to aggregate on bacteria lawns showing defects in early development upon nutrient depletion. Second, activation of PKBs, PKBA and PKBR1 upon cAMP stimulation of aggregation competent cells, are abolished. Furthermore, ACA activation and chemotaxis are abrogated in these mutants.

In addition to the similar phenotypes of the null mutant cells, the following observations also suggest that these factors function as a complex [12]. First, in coimmunoprecipitation experiments PiaA associated with Rip3 or Lst8, and both Tor and Lst8 were coimmunoprecipitated with PiaA in the presence of protein cross-linker. Second, in ACA activation experiments where extracts from the different mutant cell lines were mixed, no combinations yielded ACA activity, suggesting that a preformed complex is required. Recently, we found that PiaA proteins coimmunoprecipitate with the Tor catalytic subunit from wild-type cells but not from *rip3*$^{-}$ cells [25]. This suggests that TORC2 is unstable in *rip3*$^{-}$ cells. So far, purification of TORC2 has not been successful in *D. discoideum* and the exact composition of TORC2 awaits further examinations.

C. PDK (PDKA AND PDKB)

PDK1 belongs to the AGC family of kinases and was originally isolated as the kinase that catalyzes the AL phosphorylation of PKB in mammalian cells. *D. discoideum* cells have two PDK orthologues, PDKA and PDKB,

that contain a kinase domain and a C-terminal PH domain. PDKA and PDKB share 67% and 58% similarities in the kinase and PH domains, respectively [13]. Although both PDKs are structurally similar, they are not completely redundant. PDKA is the major AL kinase of PKBA and PKBR1 and PDKB contributes to phosphorylation of only PKBR1. This preference could arise from the respective kinase domains, as the deletion of PH domain of PDKA does not change its specificity. Since PDKA and PDKB are the only structurally related homologues of PDK1 in *D. discoideum*, it was expected that *pdkA*$^-$ *pdkB*$^-$ cells would completely lack AL phosphorylations of PKBs. However, in *pdkA*$^-$ *pdkB*$^-$ cells, some residual AL phosphorylations of the PKBs remain, suggesting that another kinase is involved in the AL phosphorylation. Nonetheless, the *pdkA*$^-$ *pdkB*$^-$ cells display greatly reduced phosphorylations of the PKBs and show several phenotypes including the ''mound arrest'' and chemotaxis defects. These phenotypes are similar to those seen in *pkbR1*$^-$ or *pkbA*$^-$*pkbR1*$^-$ cells. These results confirm the fundamental role of the TORC2–PDK–PKB pathway in chemotaxis.

Although mammalian PDK1 requires its PH domain for binding to PtdIns (3,4,5)P_3 on the membrane and for its AL phosphorylation of AKT/PKB, the PH domains of the *D. discoideum* PDKs are not essential for their activities [13]. The localizations of PDKA and PDKB proteins are different. PDKB-GFP is found in the cytosol, while PDKA-GFP localizes in the cytosol and on the plasma membrane and translocates to the membrane during chemotactic stimulation. It is also found on macropinosomes which are membraneous vesicular structures enriched in PtdIns (3,4,5)P_3 and involved in endocytosis of liquid. To determine the dependency of the membrane localization of PDKA on PtdIns (3,4,5)P_3, cells lacking PI3K activity were examined. These cells indeed lack the PDKA-GFP signals on macropinosomes and the translocation does not occur. However, PDKA-GFP still localizes to the plasma membrane in unstimulated cells. In spite of these observations, PtdIns (3,4,5)P_3 binding of the PH domain of PDKA cannot be detected in biochemical assays using filters spotted with phospholipids [13, 17]. Together, these results suggest that the PH domain of PDKA can recognize PtdIns (3,4,5)P_3 and also other unidentified molecules on the membrane. Next, function of the PH domain of PDKA was examined by deleting it. Surprisingly, PDKAΔPH can rescue AL phosphorylation of the PKBs as well as the ''mound arrest'' defects of the *pdkA*$^-$ *pdkB*$^-$ cells. This result shows that the PH domain of PDKA is not essential for its function. However, when PDKAΔPH was combined with a point mutation in the kinase domain with reduced ATP binding activity, the double mutant showed reduced function compared with either single mutant, suggesting that the PH domain may enhance the function of the enzyme under certain conditions.

D. Other AGC Kinases in *D. discoideum*

In *D. discoideum*, 21 AGC kinases are assigned and some of them other than PKBA and PKBR1 have HM-like sequences. The genes carrying most closely related HMs to those found in the PKBs are uncharacterized DDB0220670 and DDB0220702. DDB0220670 also has a conserved TM and has a longer C-terminal tail following the HM than does PKBA and PKBR1. This structure is reminiscent of the budding yeast Sch9 and mammalian S6K1 [26]. Moreover, since DDB0220670 also has a motif in its C-terminus similar to the autoinhibitory pseudosubstrate domain of S6K1, the same molecular regulation may also be conserved in DDB0220670. Sch9 and S6K1 have been shown to be phosphorylated by TORC1 and involved in cellular growth. The *D. discoideum* genome encodes a homologue of the budding yeast Kog1/mammalian RAPTOR suggesting that TORC1 is present. Together this evidence suggests that DDB0220670 might be an orthologue of Sch9 and S6K1 and regulated by TORC1. It is also possible that DDB0220670 is a substrate of TORC2.

As described above, since upon nutrient starvation TORC2 mutant cell lines do not initiate the development process resulting in a ''smooth plaque'' while *pkbA*$^{-}$ *pkbR1*$^{-}$ cells arrest at the ''mound stage,'' TORC2 may have an additional target in early development other than the PKBs. DDB0220702 could be one of the candidates.

V. The Signal Transduction Pathway for Chemotaxis

Chemoattractant-triggered signaling networks integrate processes of motility, directional sensing, and polarity to achieve efficient chemotaxis [1]. Studies of cAMP-mediated chemotaxis of *D. discoideum* cells in early development have provided the general insights into the process. These amoeboid cells move around randomly even in the absence of a chemoattractant gradient. Motility is driven largely by remodeling of the actin cytoskeleton to generate pseudopodial extensions and contraction force. Directional sensing is the process that decodes the extracellular gradient of chemoattractants. Cells detect gradient steepness even with differences of only 2% across the cell, over a wide range of midpoint concentrations. Cells amplify the gradient to produce localized changes in intracellular signaling molecules. Chemotaxing cells display morphologically distinct fronts and backs which are referred to as polarity. Pseudopod extensions occur at the front of cells and myosin II-mediated contraction occurs at the back. At the molecular level, many events also are spatially localized in a coordinated fashion that facilitates efficient chemotaxis. A working

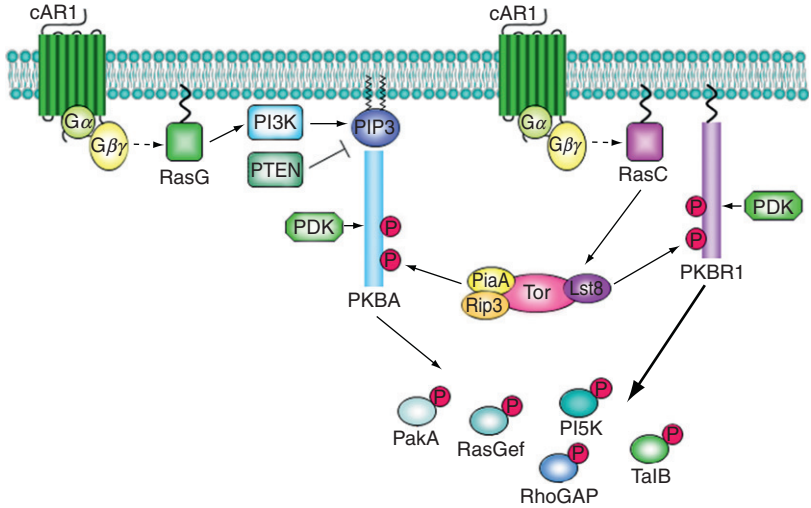

FIG. 6.3. The signaling network of cAMP-stimulated chemotaxis is summarized. cAMP binding to cAR1 stimulates dissociation between gα2 and gβγ subunit of the heterotrimeric G protein. RasG and RasC, small GTPases, are locally activated downstream of the heterotrimeric G protein at the front of cells. PI3 kinase (PI3K) is also recruited to the front and produces PtdIns (3,4,5)P_3 (PIP3) with RasG. PIP3 is detected by PIP3-specific PH domain containing proteins including PKBA. RasC functions as an activator of the PIP3-independent TORC2–PDK–PKBR1 pathway. PKBR1 and PKBA provide major and redundant, lesser activities, respectively. Both PKBs contribute to the phosphorylation of common substrates including TalB, RasGEF, PakA, PI5K, and RhoGAP linking chemotactic stimuli to rearrangement of cytoskeleton.

model of the signaling networks involved in chemotaxis is summarized in Figure 6.3 and is discussed below in more detail.

A. GPCR AND HETEROTRIMERIC G-PROTEIN

cAMP released from cells is recognized by a series of cAMP receptors (cAR1–4) that comprise a class of GPCR [27]. Guanine nucleotide exchange within the α2 subunit, one of 12 encoded α subunits, leads to dissociation of the βγ subunit, which are unique in the genome. In *cAR1*$^-$, *gα2*$^-$, *gβ*$^-$, and *gγ*$^-$ cells, all early development events including chemotaxis are impaired [27]. Using fluorescently labeled cAMP and single molecule imaging, it was shown that cAR1 binds with affinities of 2–200 nM and an average half-life of about 1.5 s [28]. Fluorescent resonance energy transfer (FRET) experiments designed to monitor the dissociation between the gα2- and the gβ-subunit showed that the extent of subunit dissociation parallels

the external gradient, suggesting that the G-protein activation is not amplified [29]. This implies that amplification occurs downstream of this module. Although the direct target of the heterotrimeric G-proteins is unknown, they mediate events including small GTPases activation, PtdIns (3,4,5)P_3 production, ACA activation, and actin polymerization. Most of these downstream events are polarized to the front or back of cells.

B. PtdIns (3,4,5)P_3-Dependent Pathway

PtdIns (3,4,5)P_3 accumulation at the front of chemotaxing cells is controlled by reciprocal regulation of PI3K and PTEN. The *D. discoideum* genome encodes five typical type-I PI3Ks, which have a RBD and a catalytic domain at the C-terminus [9]. PI3K1 and PI3K2, the major expressed PI3Ks, localize at the front of cells during chemotaxis through the N-terminal regions, which lack known motifs [6]. The RBDs of these PI3Ks have been shown to bind to an activated form of RasG, one of 15 Ras homologues in *D. discoideum*, in a yeast two-hybrid assay. RasG as well as other Ras proteins are activated at the front of cells. RasG activation depends on GefR as RasGEF and DdNF1 as RasGAP. Cells lacking GefR show only partially reduced RasG activation suggesting there are additional RasGEFs for RasG [30]. DdNF1, a homologue of human RasGAP neurofibromin 1 (NF1), found by the screening for impaired chemotaxis among RasGAP deletions was shown to be involved in RasG deactivation [31]. Importantly, cells lacking DdNF1 display abnormal activation of not only RasG but also PI3Ks resulting in chemotaxis defects with spatially unregulated pseudopod formation. Together, both recruitment to the membrane and activation by RasG contribute to the spatially localized activation of the PI3Ks. It is important to determine the binding sites for PI3Ks at the plasma membrane and determine the exact role of RasG in activation. As described above, PTEN localizes at the back of wild-type cells and helps confine accumulation of PtdIns (3,4,5)P_3 to the front. Cells lacking PTEN lose polarity and normal chemotactic activity since they accumulate PtdIns (3,4,5)P_3 along the entire cell.

The localized signal provided by PtdIns (3,4,5)P_3 is conveyed by PtdIns (3,4,5)P_3-specific PH domain containing proteins including PKBA, cytosolic regulator of adenylate cyclase (CRAC), and PH domain protein A (PHDA) in *D. discoideum* [4, 5, 32, 33]. These proteins translocate to the front of cells and when disrupted cause slight defects in chemotaxis. CRAC has an additional essential function for ACA activation [34]. Since CRAC and PHDA have no obvious domains, other than a PH domain, identification of interacting proteins will be important for determining the functions of these proteins. Among these PH domain containing proteins, PKBA

appears to have an important role in chemotaxis, since the chemotaxis defects of *pten*$^-$ cells are largely suppressed by concomitantly deleting the *pkbA* gene (M. Tang, M. Iijima, Y. Kamimura, and P. Devreotes, manuscript in preparation). Although they still display highly elevated PtdIns (3,4,5)P_3 levels on the plasma membrane, *pten*$^-$ *pkbA*$^-$ cells display much stronger polarity than the *pten*$^-$ cells, with spatially restricted pseudopod extensions occurring primarily at the front of cells.

C. TORC2–PDK–PKB Pathway

Studies of the phosphorylation of PKB substrates have shown that most of these phosphorylation events are unaffected in the cells lacking PtdIns (3,4,5)P_3 production or PKBA but significantly reduced in *pkbR1*$^-$ cells [10]. This shows that PKBR1 and PKBA contribute major and redundant, lesser activities, respectively. Furthermore, in spite of the uniform localization of PKBR1 on the plasma membrane, its activation, as that of PKBA, occurs selectively at the front of cells. Consistently, chemotaxis is abrogated much more severely in *pkbR1*$^-$ cells than in *pkbA*$^-$ cells. Furthermore, cell lines lacking the other key activators of PKBs, TORC2 and PDKs, also display reduced PKB activities as well as chemotaxis defects. These results show that the TORC2- and PDK-mediated activation of PKBR1 is an important PtdIns (3,4,5)P_3-independent pathway in chemotaxis. Most of the phenotypes of mutants related to PKBA and PKBR1 can be explained by the following model (see Figure 6.3). In wild-type cells, both PKBR1 and PKBA are activated. Cells lacking PtdIns (3,4,5)P_3 or PKBA still retain enough PKBR1 activity for almost normal chemotaxis. This explanation implies that TORC2 as well as PDKs can be activated independently of PtdIns (3,4,5)P_3. However, since *pten*$^-$ cells overaccumulate PtdIns (3,4,5)P_3, PKBA misregulation leads to severe chemotaxis defects.

Implication of Ras proteins in TORC2 activation was suggested by several early findings. First, Rip3, a subunit of TORC2, has a RBD which binds to an activated form of RasG [24]. Second, GTPγS addition to cell extracts from aggregation competent cells can trigger TORC2-dependent PKBR1 activation [10]. Third, in *rasC*$^-$ *rasG*$^-$ cells, phosphorylation of the HM of PKBR1 is decreased by 70% [10]. Recently, we have shown that in *rasC*$^-$ cells PKB phosphorylation is equally reduced [25]. Moreover, *rasC*$^-$ cells share some phenotypes with TORC2 mutant cells, including a failure to aggregate and a reduction of ACA activation, although chemotaxis defects of *rasC*$^-$ cells are milder. Moreover, expression of an activated form of RasC(Gln62Leu) prolongs PKB activation and causes a severe

loss of polarity and chemotaxis defects. These results assign RasC, but not RasG, as an activator of TORC2. Early screens for chemotaxis mutants led to identification of Aimless (AleA) which carried a RasGEF domain and was later demonstrated to be an exchange factor specific for RasC [30, 35]. The GAP for RasC has not been identified. Consistently, *aleA*$^-$ and *rasC*$^-$ cells show similar decreased levels of PKB phosphorylation [10, 25]. These results indicate the importance of AleA- and RasC-mediated activation of TORC2 in PKB regulation. Although the exact mechanism of this activation is unknown, we can speculate as to whether activated RasC directly regulates TORC2 or renders PKBs as more suitable substrates for TORC2.

D. Other PtdIns (3,4,5)P_3-Independent Pathway

In addition to the TORC–PDK–PKBR1 pathway, additional PtdIns (3,4,5)P_3-independent pathways appear to be needed for proper chemotaxis. Phospholipase A_2 (PLA_2) was found by screening for mutants that were supersensitive to PI3K inhibitors in early development [8]. In addition, depletion of PLA2 activity enhances the chemotaxis defects of PI3K mutant cells and of cells treated with PI3K inhibitors. These observations strongly suggest that PLA_2 also has a role in chemotaxis in the PtdIns (3,4,5)P_3-independent manner.

Yet, another PtdIns (3,4,5)P_3-independent pathway is cGMP-mediated Myosin II filament formation [36]. cGMP production upon chemoattractant addition is regulated by membrane-bound and soluble guanylyl cyclases, GCA and sGC, and a cGMP-specific phosphodiesterase, PdeD. Myosin II molecules, composed of Myosin heavy chain (MHC) and light chain (MLC), mostly form filaments at the back of cells and aid in producing contraction force during chemotaxis. Accordingly, *mhcA*$^-$ cells show reduced motility speed compared to wild-type cells and cells lacking guanylyl cyclase activity or the cGMP binding protein (GbpC) have a similar phenotype to that of *mhcA*$^-$ cells. Thus, these factors are thought to regulate Myosin II filament formation.

E. Downstream Events

One of most important downstream events is pseudopod formation through actin polymerization. The TORC2–PDK–PKB pathway has functions in actin polymerization, since dysregulated actin polymerizations occur in *pten*$^-$ cells or Ras(Gln62Leu) expressing cells [7, 25]. Some PKB substrates have already been identified, for example, Talin B (TalB), RasGEFs (GefS and GefN), p21 activated kinase (PakA), RhoGAP

(GacG and GacQ), and PI5K (Table 6.1) [10, 37] (M. Tang, M. Iijima, Y. Kamimura, and P. Devreotes, manuscript in preparation). Since many of these homologues are reported to have roles in actin polymerization dynamics in other organisms, further characterization is likely to link these factors to regulation of pseudopod formation.

cAMP-dependent ACA activation is critical for the signal relay responses between cells needed to efficiently form multicellular aggregates. Curiously, this pathway shares components with chemotactic responses, including the TORC2–PDK–PKB pathway. ACA is regulated by TORC2 and CRAC, independently, since ACA activation can be restored by mixing cell lysates from *piaA*$^-$ and *crac*$^-$ cells while only wild-type lysates will restore activation of *piaA*$^-$ *crac*$^-$ cells [22]. In *pten*$^-$ cells or in cells expressing RasC(Gln62Leu) ACA activation is prolonged [7, 25]. The effects of active RasC(Gln62Leu) are suppressed in *piaA*$^-$ cells, again confirming that TORC2 functions downstream of RasC. Furthermore, we have recently shown that *pkbA*$^-$ *pkbR1*$^-$ cells also impair ACA activation. Together, these results suggest that a PKB substrate, regulated by phosphorylation of TORC2-mediated PKB, could be an essential component for ACA activation.

TABLE 6.1

IDENTIFIED CAMP-STIMULATED PKB SUBSTRATES IN *D. DISCOIDEUM*

Gene DDB number	MW (kDa)	Putative PKB phosphorylation sites (RxRxxS/T)	Localization during chemotaxis	Motifs et al.	Ref.
talB DDB0191526	277.7	RIRGQT396	ND	Talin homologue	[10]
gefN DDB0167277	185.4	RLRSFT749 RSRAQT800	ND	RasGEF	[10]
pakA DDB0191313	134.6	RSRSHT585	Back	p21 activated kinase homologue	[37]
gacG DDB0233879	147.0	RRRTSS365 RERSSS480	ND	RhoGAP	[a]
gefS DDB0191324	116.9	RVRHST387 RIRSPS623	Cytosol	RasGEF	[10]
N.D. DDB0234212	80.5	RVRLNT262	Plasma membrane	Phosphatidylinositol-4-phosphate 5 kinase	[10]
gacQ DDB0233774	59.0	RQRSNT528	Cytosol	RhoGAP	[10]

[a]M. Tang, M. Iijima, Y. Kamimura, and P. Devreotes, manuscript in preparation.
ND is not determined.

TABLE 6.2

IDENTIFIED FOLATE-STIMULATED PKB SUBSTRATES IN *D. DISCOIDEUM*

Gene DDB number	MW (kDa)	Putative PKB phosphorylation sites (K/RxK/RxxS/T)	Localization during chemotaxis	Motifs et al.	Ref.
PHAPS DDB0307127	85.9	KKRTTT102	ND	SAM, PH	[17]
SHAPS DDB0306661	52.0	RPRASTT281	ND	BAR, SH3	[17]

ND is not determined. SAM is sterile alpha motif. PH is pleckstrin homology. BAR is Bin/Amphiphysin/Rvs.

F. FOLATE CHEMOTAXIS

D. discoideum cells use folate as a chemoattractant for seeking bacteria. Although folate chemotaxis is less efficient than cAMP chemotaxis, the two phenomena appear to share similar but not identical signaling pathways. While folate receptors have not been identified, they are certainly not cAR1. They are likely GPCRs since Gα4 (instead of Gα2) and Gβγ are required for folate chemotaxis [27]. Whereas PKBR1 is the major PKB activity stimulated by cAMP, folate primarily activates PKBA [17]. Moreover, the phosphorylation profiles of PKB substrates differ during folate versus cAMP stimulation. In folate-stimulated cells, PKBA preferentially phosphorylates PH/AKT-preferential substrate (PHAPS) and SH3/AKT-preferential substrate (SHAPS) (Table 6.2). Interestingly, although the transient stimulation of PKBA is prevented, basal phosphorylations of PKBA at both the AL and HM are retained in TORC2 mutant cell lines, *piaA*$^{-}$, *rip3*$^{-}$, and *lst8*$^{-}$ cells. In contrast to PKBA, PKBR1 phosphorylations are completely lost in TORC2 mutant cells. This result suggests that under certain circumstances PKBA can be phosphorylated by an unidentified kinase. It is important to determine how folate chemotaxis is affected in cells lacking components of the TORC2–PDK–PKB pathway.

VI. Conclusion

The TORC2–PDK–PKB module undergoes spatially confined activation and has critical roles in chemotaxis. The spatial activation of this module is dependent on RasC, a subtype of Ras small GTPases. Since little is known about TORC2 regulation in other organisms, further characterization is needed to understand the molecular basis of activation mechanism. Especially, it is important to discriminate whether the target of RasC is TORC2,

PKBs, or both. As described here, the activation of PKBs by TORC2 is complex. Our evidence suggests that PKBs require regulation in addition to phosphorylation in the HM. One possible mechanism might be phosphorylation of the TM. Alternatively, TORC2 also might have uncharacterized mechanism for the PKB activation. Related to this, AL phosphorylation of PKBs in *D. discoideum* cells is interesting. The PDK homologues can phosphorylate PKBs independently of both PtdIns (3,4,5)P_3 and TORC2, as long as the HM site is substituted with a phosphomimetic residue. Complete understanding of PKB activation by TORC2 and PDK could provide general insights for the spatial and temporal activation of this module.

The TORC2 complex appears to be fragile in cell lysates. Although interactions between TORC2 subunits can be seen, it has not been possible to purify the TORC2 complex to homogeneity. This may suggest that complex formation itself is regulatable. Like mammalian cells, *D. discoideum* cells share a common catalytic subunit between TORC1 and TORC2. The transition between these two different complexes could be important for certain cellular phenomenon. Furthermore, the TORC2 complex is more unstable in *rip3*$^-$ cells than in wild-type cells, suggesting, perhaps, that some subunits may regulate the amount of functional TORC2. To address these questions, it is essential to establish a biochemical method for purification of TORC2.

In addition to its function in chemotaxis, TORC2 is also required for the early developmental program itself. When starved, TORC2 mutant cell lines cannot initiate the developmental process, while cells lacking PKB activity progress further. This result suggests that TORC2 has substrates other than the PKBs. These may be other AGC family kinases or other substrates such as those found in yeast. Once additional substrates are identified, it will be interesting to explore RasC dependency as well as the cellular sites of activation.

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