PldB, a Putative Phospholipase D Homologue, Mediates Quorum Sensing in Dictyostelium discoideum Development

Yi Chen

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PldB, a Putative Phospholipase D Homologue, Mediates Quorum Sensing in *Dictyostelium discoideum* Development

by

YI CHEN

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

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ABSTRACT

PldB, a Putative Phospholipase D Homologue, Mediates Quorum Sensing in

*Dictyostelium discoideum* Development

by

Yi Chen

Adviser: Derrick Brazill, Ph.D.

Quorum sensing, the ability to measure the local cell density, is required for animal cells to achieve proper cell growth, differentiation and development. However, because of the genetic intractability of mammalian cells, we study quorum sensing in the genetically pliable unicellular eukaryote *Dictyostelium discoideum*. *D. discoideum* cells live as individual cells under vegetative conditions. Starvation sets off development to a multicellular organism. But cells won’t develop unless enough cells are under starvation conditions. Approximately $10^5$ cells are needed to form a fruiting body, the final stage of the development. When starved, cells are able to calculate the concentration of surrounding starving cells by simultaneously secreting and sensing a glycoprotein named conditioned medium factor (CMF). A high density of starving cells results in a high level of CMF, which allows cell aggregation and development. Here, we describe the role of a putative phospholipase D (PLD) homologue, PldB, in quorum sensing pathways. PldB is present during early development when quorum sensing is occurring as well as in later development. *pldB−* cells can aggregate at low cell density. This phenotype is cell autonomous. Cells overexpressing *pldB* are unable to aggregate or form fruiting bodies even at high cell density. These observations imply that PldB is involved in quorum
sensing. CMF decreases cAMP-stimulated GTPase activity in wild-type cells, but not in $pldB^{-}$ cells, confirming that PldB mediates quorum sensing in the CMF signal pathway. $pldB^{-}$ cells develop faster than wild-type cells, indicating that $pldB$ is involved in the timing of development. Analysis of early developmental gene expression shows that cAMP receptor cAR1, required in aggregation, is expressed at higher levels earlier in $pldB^{-}$ cells than in wild-type cells, which may explain the rapid development phenotype of $pldB^{-}$ cells. However, $pldB^{-}$ cells have normal chemotactic rate while $pldB^{OE}$ cells do not chemotax. Cell fractionation and Western blot analysis reveal that PldB protein is localized to cytoplasmic membrane as well as vesicles. PldB takes effect in quorum sensing through its phosphatidylcholine hydrolysis product, phosphatidic acid, since exogenous phosphatidic acid inhibits cell aggregation at low cell density, mimicking the phenotype of overexpressing $pldB$. Rapamycin promotes cell aggregation at lower cell densities for wild-type, $pldB^{-}$ and $pldB^{OE}$ cells, implying that TOR may have a role in quorum sensing downstream of PldB. The knowledge of PLD in quorum sensing will help us further understand this phenomenon in mammals, which may lead to therapeutic strategies for tissue/organ repair and cancer treatment.
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CHAPTER 1

Introduction

1.1 Quorum sensing

Quorum sensing, the ability to measure the local cell density, is required for animal cells to achieve proper cell growth, differentiation and development. Typically, cells secrete signaling molecules that turn on one or more signaling cascades, which steer the developmental path of the cells. Quorum sensing mechanisms are present in mammals [1], Dictyostelium discoideum [2] and bacteria [3].

Bacteria use quorum sensing to co-ordinate their behavior and respond quickly to changes in environmental conditions in order to survive. These responses include adaptation to availability of nutrients, defense against other microorganisms, which may compete for the same nutrients, and the avoidance of toxic compounds potentially dangerous for the bacteria. Vibrio fischeri, the first bacterium studied for quorum sensing, can secrete and sense a homoserine lactone molecule and therefore monitor the cell density [4]. They don’t emit light in the free-living planktonic state. But when they colonize the light organs of certain luminescent fish or squid and accumulate to a large number, the high concentration of the homoserine lactone molecule will turn on the luminescence genes of the bacterium, causing light production.

As to mammalian cells, mice express and secrete an obese protein [1]. The level of expression of this obese gene signals the size of the adipose depot. A high level signal
may act on the central nervous system to inhibit food intake and/or regulate energy expenditure as part of a homeostatic mechanism to maintain constancy of the adipose tissue.

Cell-cell communication and group organization are important for bacteria to evade host defenses and antibiotics in persistent bacterial infections. With the knowledge of quorum sensing in bacteria, we will be able to develop new therapeutic agents to control the virulence and treat these infectious diseases through regulating bacterial group behavior, instead of just killing or stopping the growth of individual bacteria. In mammals, knowledge of quorum sensing will pave the way for therapeutic strategies for tissue or organ repair/regeneration to benefit patients with lost limbs, nerve injuries, or damaged organs. Although promising for quorum sensing research, mammalian cells are genetically intractable. Hence we study eukaryotic quorum sensing in *Dictyostelium discoideum*, which is easily accessible to techniques of molecular cell biology, genetics and biochemistry.

### 1.2 A model organism

1935 saw the entry of *Dictyostelium discoideum* for biological research [5]. For the past 70 years, it has been extensively studied worldwide for intracellular and intercellular signal transduction, cell motility, differentiation and morphogenesis. A unicellular eukaryote, *D. discoideum* provides a system to study cellular and developmental biology at a level of complexity greater than yeasts, but simpler than plants and animals. A proteome-based eukaryotic phylogeny shows that *D. discoideum* diverged from the
animal-fungal lineage after the plant-animal split [6]. Despite its early divergence, proteins of *D. discoideum* are more similar to human orthologues than are those of yeasts [7]. With its better approximation to the function and regulation of mammalian cells, its genetic pliability, and its multicellularity during development, *D. discoideum* makes it possible to study human disease orthologue proteins in an organism that shares both great similarity with higher eukaryotes and experimental tractability with yeasts. Moreover, *D. discoideum* cells are easy to grow with liquid nutrients. Cell number duplicates in 8-10 hours. Cell development is initiated by simply removing the nutrients and normally completes within 24 hours. Mutations can affect development without affecting growth. Mutant strains can be easily selected by inserting a specific drug-resistant cassette into the genome. With the ease of mutating any gene of interest, its corresponding function or contribution to a certain biological process can be readily assessed by observing the changes in phenotype. These characteristics make *D. discoideum* a good model organism to work with.

1.3 Life cycle

A social amoeba (cellular slime mold), *D. discoideum* lives in soil, feeds on bacteria and reproduces by binary fission. Lack of bacteria or other nutrient sources forces vegetative individual cells to stop division and develop. This involves cell aggregation, differentiation and morphogenesis. Through multiple stages of development, approximately $10^5$ cells will aggregate to form a fruiting body in 24 hours (Figure 1). A fruiting body consists of a mass of spore cells on the top of a column of stalk cells. The 20% stalk cells are dead while the 80% spore cells are dormant living cells [8, 9]. Upon
availability of nutrient sources, the spore cells will germinate and develop back into individual cells, thus reinitiating the life cycle. In other words, starvation initiates the transformation from individual cells to a multicellular organism and nutrients reverse the process. The relatively short and simple life cycle of *D. discoideum* makes it ideal for study in development and differentiation. Moreover, an international consortium has completed the sequencing of *D. discoideum* genome [6]. *D. discoideum* has a 34 Mb haploid genome, which consists of 6 chromosomes encoding approximately 12,500 genes. It is now easy to identify a gene with its homologue in mammalian species and study its physiological functions.

Figure 1. Post-aggregation stages of *D. discoideum* development.
Moving clockwise from lower right, the stages are the loose aggregate, the tight aggregate (mound), the tipped aggregate, the finger, a slug, and then culmination stages from the Mexican hat stage to the final fruiting body. This scanning electron micrograph is by R. Lawrence Blanton & Mark Grimson at Texas Tech. University.
1.4 cAMP-cAR1-Gα2 signaling

Starving *D. discoideum* cells have the ability to sense the density of other starving cells. Without this mechanism, small groups of cells that happen to starve at the same time would form small, ineffectual fruiting bodies. Normally when the number of starving cells reaches a certain threshold (~$10^5$ cells), they will initiate aggregation by chemotaxing to extracellular cyclic AMP (cAMP) pulses, which occur every 6 minutes [10]. Cells have cAMP receptor, cAR1, on the cell surface [11]. This seven trans-membrane domain glycoprotein is coupled with a heterotrimeric G protein (Gα2βγ) on the cytosol side of the plasma membrane [12]. Upon binding of cAMP to cAR1, GTP binds to Gα2, replacing GDP, and Gα2-GTP separates from βγ subunits [13]. Gα2-GTP will then activate the downstream guanylyl cyclase, which is needed for activation of genes required for motility [14] (Figure 2). Gβγ, together with cytosol regulator of adenylyl cyclase (CRAC) that is recruited to the cell membrane, will activate adenylyl cyclase (ACA) [15]. cAR1 is evenly distributed along the cell membrane [16]. Yet CRAC responds to the differential concentrations of cAMP across a cell and binds rapidly to the membrane side that encounters the highest concentrations of cAMP, translating the polarity of a cAMP wave into a polar response [15, 17-19]. ACA is a 12 trans-membrane domain protein with 2 large intracellular loops [20]. It increases intracellular cAMP that is believed to regulate the gene expression needed for development and releases cAMP into the extracellular space to relay this aggregation signal to other starving cells [21].
cAMP binds to cell surface receptor cAR1, which is coupled with Ga2βγ protein. GTP replaces GDP on Ga2 and causes separation of Ga2-GTP from Gβγ. Upon separation, Ga2-GTP and Gβγ activate guanylyl cyclase and adenylyl cyclase respectively.

Figure 2. cAMP signal pathway.

1.5 CMF-Gα1 signaling

It has been found that an 80-kDa secreted glycoprotein, conditioned medium factor (CMF), plays an indispensable role in the activation of cAMP signaling cascade [22-25]. CMF is heat stable and loses <10% of its activity after incubation at 80°C for 25 minutes [23]. Its glycosylation may help confer the stability. Neither pH (7.0 or below) of the environment nor light affects the accumulation or the activity threshold of CMF [26]. CMF has a diffusion trait, such that the level of CMF in the vicinity of an isolated starving cell will be too low to induce the expression of CMF-requiring genes, while the level in the vicinity of a high density of starving cells will induce the expression of CMF-requiring genes [26]. All these characteristics make CMF a good quorum sensing factor.
Cells synthesize CMF under vegetative conditions. But only under starvation conditions do cells secrete and sense CMF [27]. When only a few cells are starving, the CMF level is low and cells do not respond to cAMP [23]. Once a large number of starving cells releases a high level of CMF (above 0.3 ng/ml), cells start to chemotax and aggregate [23]. Therefore, CMF helps coordinate the development of fruiting bodies with appropriate size by allowing aggregation only when most cells in a given area are starving, as characterized by a high level of CMF.

CMF exerts control over development by regulating cAMP signaling through cAR1 [24]. With high cell density, and thus a high level of CMF, the cAMP-cAR1-Go2 signaling cascade is activated. In the absence of CMF, cAMP can still bind to cAR1, and the activation of cAR1 can still cause Go2 to release GDP and bind GTP. However, activation of downstream adenylyl and guanylyl cyclases is greatly inhibited [28]. This inhibition of adenylyl and guanylyl cyclases can be removed by a 10-second exposure of CMF, showing that lack of CMF is the direct cause of inhibition [28]. The presence or absence of CMF has no effect on the levels of cAMP-cAR1 induced binding of GTP to membranes [25], arguing that the lack of CMF has no effect on the interaction between cAR1 and its G protein in vitro. Thus, CMF controls aggregation by regulating cAMP signaling at a point after G protein activation but before the activations of adenylyl and guanylyl cyclases.

CMF accomplishes this by controlling the GTPase rate of Go2 [25]. It has been found that in cell lysates, approximately 250 molecules of GTP bind to a cell’s membrane in
response to a pulse of cAMP, regardless of whether CMF is present or absent. After

After cAMP stimulation, GTP is hydrolyzed to GDP at a rate of approximately 240 molecules in 3 minutes in the absence of CMF. However, in the presence of CMF, the rate of GTP hydrolysis is drastically reduced to approximately 51 molecules in 3 minutes. Since lysates from cells lacking Ga2 have no cAMP-stimulated GTP binding or hydrolysis, these results argue that quorum sensing through CMF is accomplished by controlling the cAMP-stimulated GTPase activity of Ga2.

CMF exerts its effect on cAMP-cAR1-Ga2 signaling through its own G protein signaling cascade. CMF has its receptor on the cell surface [29], which is coupled with Ga1βγ protein [30]. After CMF binds to its receptor, Gβγ will be able to activate phospholipase C (PLC), which in turn regulates the GTPase activity of Ga2 [30]. PLC is stimulated to produce IP3 and DAG in mammals [31]. This IP3 increases intracellular [Ca2+], which is known to activate protein kinase C (PKC) [32-34]. DAG also activates PKC directly [35]. CMF has been found to stimulate PKC activity in D. discoideum (unpublished data). Thus it is reasonable to imagine that PKC is likely downstream to PLC in the CMF-Ga1 pathway (Figure 3).
Figure 3. CMF signal pathway.
CMF binds to its cell surface receptor, which is coupled with $\text{Ga}\beta\gamma$ protein. $\text{G}\beta\gamma$ activates downstream PLC and PKC.

In mammalian cells, PKC regulates downstream phospholipase D (PLD) [36]. Additionally, phosphatidic acid, a product of phosphatidylcholine hydrolysis by PLD, has been shown to control the GTPase activity of $\text{Ga}$ proteins via RGS (regulator of $\text{G}$ protein signaling) proteins [37]. Together, these findings suggest the involvement of PLD in quorum sensing. In this project, we have a hypothesis that PldB, a homologue of PLD, mediates quorum sensing in the CMF signal pathway (Figure 4). CMF signal pathway decreases the GTPase activity of $\text{Ga}2$ and prolongs the lifetime of $\text{Ga}2$-GTP. This keeps $\text{Ga}2$ in its active form, allowing the activation of adenylyl and guanylyl cyclases, and makes following chemotaxis and aggregation possible.
Figure 4. CMF pathway regulates cAMP pathway by decreasing the GTPase activity of Ga2. We hypothesize that PldB has a negative role in aggregation by increasing the GTPase activity of Ga2 and lies downstream of phospholipase C in the CMF signal pathway. PldB activity is inhibited upon CMF binding.
1.6 Phospholipase D

1.6.1 Definition

A phospholipase is an enzyme that converts phospholipids to fatty acids and other lipophilic substances. There are four major classes: A, B, C and D. Phospholipase D (PLD) has two biochemical activities: phosphodiester bond hydrolysis and phospholipid transphosphatidylation. As a phospholipid hydrolysis enzyme, PLD cleaves phosphatidylcholine (PC) to yield phosphatidic acid (PA) and choline, using H$_2$O as an electron donor (Figure 5). If alcohol replaces H$_2$O, PLD can generate phosphotidylalcohols. In mammalian cells, there are two classes of PLD activities [38]. One class is phosphoinositide-independent, fatty-acids-activated PLD. No corresponding cDNA sequences have been reported for this class. The other is phosphoinositide-dependent PLD, which is inhibited by free fatty acids. cDNA cloning revealed two proteins of this class: PLD1 from human HeLa cells and PLD2 from mouse cells. They share 50% homology.

![Figure 5. Phosphatidylcholine hydrolysis.](image)

In the presence of water, PLD cleaves the phosphodiester bond in phosphatidylcholine to produce phosphatidic acid and choline.
1.6.2 Structure

The structure of PLD contains two conserved catalytic ‘HKD’ motifs (also known as conserved region (CR) II and IV, or the transphosphatidylation domains), disruption of which leads to loss of catalysis function [38] (Figure 6). An ‘IYIENQFF’ motif (CRIII) is found only in those that have bona fide activity, with a possible role of interacting with the choline headgroup of PC. CRI and a pleckstrin homology (PH) domain in the structure interact with phosphoinositol-4,5-biphosphate (PIP$_2$) in the membrane, keeping the PLD membrane bound and catalytically active [39]. A phox consensus sequence (PX) motif is reported to mediate protein-protein interactions [40]. The amino-termini of PLDs are poorly conserved and can tolerate significant modifications, such as fusion to green fluorescent protein (GFP) without changes in enzyme activity [41, 42]. On the contrary, the carboxy-termini are relatively well conserved and intolerant to modifications [41, 42]. PLD1 encodes a loop region of 100-150 amino acids at the center of the protein that is not present in PLD2 or most other PLDs from lower organisms [43, 44]. It is highly variable and does not seem to affect enzyme activity or localization when modified [45].

Figure 6. Schematic depiction of human PLD1.
Boxes, location of regions of highly conserved sequence or regions unique to PLD1 (loop sequence). P, site of tyrosine phosphorylation in mPLD2. This figure is from Reference #38.
1.6.3 Localization and regulation

PLD2 is localized to plasma membrane and only activated by lipids PIP$_2$ and phosphoinositol-3,4,5-triphosphate (PIP$_3$) [46]. PLD1 is usually found in perinuclear regions and vesicles [46]. Besides PIP$_2$ and PIP$_3$, PLD1 can also be activated by GTP binding proteins (ARF and Rho families of GTPases), protein kinase C (PKC), tyrosine kinases and Ras subfamily of small G proteins [38, 46-49]. PKC activates PLD either through phosphorylation of PLD itself, or a phosphorylation-independent mechanism where certain PKC isoenzymes activate PLD [50, 51]. Down-regulation of PKC or addition of PKC inhibitors impairs receptor mediated PLD activation [52].

1.6.4 Function in mammals

Besides the obvious role in phospholipid turnover and maintenance of the structural integrity of cellular or intracellular membranes, PLD and its phospholipid hydrolysis product PA are involved in a multitude of cellular events [53]. PLD1 activity is implicated in the budding of COPI-coated Golgi vesicles, the formation of nascent secretory vesicles in the trans-Golgi network, ARF-dependant clathrin coat assembly in the endosomal/lysosomal system, and the regulated exocytotic secretion [54]. PLD1 also plays a role in survival pathways that prevent apoptosis [55]. PLD2 has suggested functions in actin cytoskeletal rearrangement and receptor-triggered ARF-mediated endocytic process [54]. Moreover, PA is proposed to mediate the activation of NADPH oxidase, or the respiratory burst, leading to the release of active oxygen radicals [53]. Thus, determining the roles of PLD in various cellular responses will greatly benefit our
understanding of the corresponding molecular cell biology and biochemistry, which may guide us in therapeutic designs for different diseases.

1.6.5 PldB in *D. discoideum*

In *D. discoideum* cells, inhibition of PLD activity by butanol leads to aberrant relocalization of F-actin and impaired phagocytosis and pinocytosis [56]. These effects by butanol can be reversed by exogenous PA, confirming the effects are caused by inhibition of PLD activity. These findings suggest that localized PLD activity is essential for normal actin distribution and endocytosis. In this project, we describe the role of a putative phospholipase D homologue, PldB, in *D. discoideum* development.

To identify genes that encode PLD proteins in *D. discoideum*, a BLAST search is performed against the database of the Japanese *Dictyostelium* cDNA project by using the human phosphatidylcholine-specific PLD1 amino acid sequence as a query. Two different partial cDNAs are identified. Another BLAST search is performed against the database of the *Dictyostelium* Genome project by using the partial cDNAs as queries. Contigs containing these sequences are identified, yielding large putative open reading frames for both genes. These genes are named *pldA* and *pldB*. *pldA* is expressed consistently in both vegetative and developing cells, while *pldB* is differentially regulated (unpublished data). *pldB* is chosen for further study due to its expression pattern.

The *pldB* gene resides on chromosome 3, is 2,747 nucleotides long with one predicted intron, and codes for a hypothetical protein of 867 amino acids with a molecular weight
of ~100-kDa. The translated protein contains the conserved regions found in all PLD1’s including a PH domain, catalytic region I (CRI), CRII, a loop, CRIII, CRIV, and the C-terminal tail (Figure 7). Overall, the protein shares 32% similarity and 21% identity with PLD1 from humans. Excluding the N terminus and loop domains, which vary widely from species to species, PldB has 51% similarity and 34% identity to PLD1 from humans.

Figure 7. Comparison of the predicted amino acid sequences of the catalytic regions of PLDs. PldB (D. disc), human PLD1 (H. sap1), human PLD2 (H. sap2), and Drosophila melanogaster PLD (D. mela) are aligned at the three catalytic regions. Amino acid identities are in black boldface type, while amino acid similarities are in gray. Similarities between PldB and human PLD1: CR II, 89%; CRIII, 90%; CRIV, 95%.

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1.7 Significance

Quorum sensing is crucial to achieve normal cell growth, differentiation and cell restoration for mammalian cells. Without the ability to sense the number of cells present, an organ or tissue will not stop cell division when it reaches the appropriate size nor will it regenerate cells when damaged. While the genome of mammalian cells is not easy to manipulate, the single-celled eukaryote *D. discoideum* gives us easy genetic access to study quorum sensing. At the initial stage of its transformation to a multicellular organism, starving cells chemotax toward a common aggregation center using cAMP as chemoattractant. Previous studies show that cells’ sensing ability comes from a secreted glycoprotein CMF. CMF regulates cAMP signal pathway and relay by a signaling cascade involving $G\alpha 1\beta\gamma$ protein, PLC and PKC. Here, we demonstrate that a PLD homologue is involved in the CMF signal pathway, contributing to the further understanding of quorum sensing signal transduction. The knowledge of quorum sensing in *D. discoideum* will give us a framework to examine this phenomenon in mammalian cells, which can be applied in tissue and organ repair and cancer cell treatment.
CHAPTER 2

Materials and Methods

2.1 Cell culture

Wild-type cells (Ax2) are grown in axenic HL5 medium (0.5% (w/v) yeast extract, 0.5% protease peptone, 0.5% thiotone peptone, 1% dextrose, 4.7 mM Na$_2$HPO$_4$, 2.5 mM KH$_2$PO$_4$, pH 6.5) on a 180 rpm shaking platform at 22°C. pldB$^{-}$ cells and pldB$^{OE}$ cells are grown in the same shaking culture as above containing 10 μg blasticidin/ml and 20 μg G418/ml respectively. Wild-type and pldB$^{-}$ cells are also grown on SM/5 agar plates with Klebsiella aerogenes. pldB$^{OE}$ cells are also grown on GYP agar plates with the blasticidin and G418 resistant strain of Escherichia coli.

For development, cells at mid-log phase (2-5×10$^6$ cells/ml) are washed with PBM (20 mM KH$_2$PO$_4$, 10 μM CaCl$_2$, 1 mM MgCl$_2$, pH 6.1 with KOH), plated on filter pads at 1×10$^7$ cells/pad and incubated at 22°C. For aggregation competent cells, cells are starved in shaking PBM for 5 hours at 22°C.

2.2 Low cell density aggregation assay

Vegetative cells are collected and washed with PBM. They are then starved as submerged monolayer culture in a 24-well plate (BD, Franklin Lakes, NJ) at surface densities of 224×10$^3$, 112×10$^3$, 56×10$^3$, 28×10$^3$, 14×10$^3$, and 7×10$^3$ cells/cm$^2$ in 400 μl PBM, PBM supplemented with 5 μM, 25 μM, or 50 μM cell permeable phosphatidic acid, 1,2-Dioctanoyl-$sn$-Glycero-3-Phosphate (Avanti, Alabaster, AL), or PBM supplemented with
1 \mu M rapamycin (Calbiochem, San Diego, CA). The plate is then incubated at 22°C for 18 hours or longer, and examined with an inverted microscope.

2.3 GTPase assay

Cells are starved in shaking PBM at $10^7$ cells/ml for 5 hours. Cells are then washed twice with PBM and resuspended in lysis buffer (40 mM HEPES/NaOH, 0.5 mM EDTA, 250 mM sucrose, pH 7.7) to $10^8$ cells/ml. One set of cells is lysed immediately by passage through a 5 \mu m nylon filter (GE Water & Process, Trevose, PA) on ice. The other set is incubated with 1 ng/ml CMF for 30 seconds and then lysed. Both sets of cells are centrifuged at 14,000 \times g at 4°C for 3 minutes. The pellets are washed with wash buffer (10 mM triethanolamine-HCl, pH 7.4, with 0.5 mM EDTA) and resuspended in assay buffer (10 mM triethanolamine-HCl, pH 7.4) to $10^8$ cells/ml.

1.5 ml tubes on ice are preloaded with 70 \mu l reaction mixture ([$\gamma$-32P]GTP (0.1 \mu Ci/assay), 2 mM MgCl$_2$, 0.1 mM EGTA, 0.2 mM AMP-PNP, 0.1 mM ATP$_{\gamma}$S, 10 mM DTT, 5 mM creatine phosphate, 0.4 mg/ml creatine phosphokinase and 2 mg/ml BSA in 50 mM triethanolamine-HCl, pH 7.4). 10 \mu M cAMP and 0.01 \mu M or 50 \mu M cold GTP are also preloaded if required.

After 5 minutes preincubation of the reaction mixture at room temperature, the reaction is initiated by adding 30 \mu l cell membrane to the 70 \mu l reaction mixture. The reaction is carried out for 1 minute and terminated by 500 \mu l stop buffer (50 mM sodium phosphate buffer, pH 2.0, with 5% (w/v) activated charcoal). All tubes are then centrifuged at
14,000× g at 4°C for 5 minutes and 400 μl supernatant is removed and added to 3 ml scintillation fluid for scintillation counting. All samples are assayed in triplicates.

2.4 Northern blot analysis

Wild-type genomic DNA, pldB upstream primer 5’-CACCATTAGCTTCCCATGTC-3’ and downstream primer 5’-GGTGATCTTGCATTAGCATCCTC-3’ are used in PCR to produce pldB probe. The reaction is performed with NovaTaq PCR kit (EMD Biosciences, San Diego, CA) and contains 30 cycles of 1-minute denaturing at 94°C, 1-minute annealing at 55°C and 1-minute extension at 72°C. The PCR product is separated on a 2% agarose gel and visualized with ethidium bromide. The DNA is extracted and purified (Millipore, Billerica, MA). 25 ng DNA probe is labeled with 32P using α-32P dATP (Perkin Elmer, Wellesley, MA) and DNA labeling system (Invitrogen, Carlsbad, CA). The labeled probe is then purified by Micro Bio-Spin 6 chromatography column (BioRad, Hercules, CA).

RNA is isolated from cells starved on filter pads at the times indicated with the Rneasy mini kit (Qiagen, Valencia, CA) or Trizol reagent (Invitrogen, Carlsbad, CA). 5 μg of each RNA sample is run on an agarose gel, visualized by ethidium bromide staining, and transferred to Hybond-N+ nylon membrane (Amersham, Piscataway, NJ). Northern blot analysis is performed with the following partial gene fragments as DNA probes: pldB (250bp), cAR1 (710bp), CRAC (616bp), ACA (705bp), and IG7 (368bp). Blots with the same probe are incubated together in the same hybridization buffer and exposed to film
for the same length of time in the same film cassette. Densitometry is performed by using a Molecular Dynamics personal densitometer SI.

### 2.5 Western blot analysis

$1 \times 10^7$ cells at mid-log phase growth are starved on filter pads for 4, 8, 12, 16, 20 and 24 hours. They are then washed into 1.5 ml tubes with 1 ml PBM and centrifuged at $300 \times g$ for 5 minutes. Cell pellets are resuspended in 250 μl ddH$_2$O with 6× SDS-PAGE sample buffer and heated at 95°C for 3 minutes. 20 μl of each protein sample is loaded into two separate 10% Tris-HCl polyacrylamide gels (BioRad, Hercules, CA). Gels are run at 80V for 20 minutes and then 150V till the end. The gel with unstained protein marker is stained with Coomassie Blue Stain for protein quantity recognition. Proteins in the other gel with prestained protein marker are transferred to PVDF membrane (GE Healthcare, Piscataway, NJ). Afterwards, the membrane is blocked with 50 ml blocking buffer (1× PBS, 0.1% Tween-20, 5% w/v nonfat dry milk) for 1 hour at room temperature on a shaker. The membrane is then incubated with 1° antibody (custom made by immunizing a rabbit with the synthetic peptide GCFLIVYKKKKHDEDKPS from amino acids 30 to 47 of PldB and collecting the serum 8 weeks after the second injection and purified by using the above peptide attached to sepharose beads) at 0.4 μg/ml in 40 ml blocking buffer for 1 hour, washed with 50 ml PBST (1× PBS + 0.05% Tween-20) 3 times for 5 minutes, incubated with 2° antibody – Anti-rabbit IgG HRP-linked antibody (Cell Signaling, Danvers, MA) at 10.4 ng/ml in 40 ml blocking buffer for 1 hour and washed with 50 ml PBST another 3 times for 5 minutes. The membrane is incubated in
chemiluminescent detection reagent (Pierce, Rockford, IL) for 5 minutes and exposed to film for 1 minute.

2.6 Transphosphatidylation assay (PLD activity assay)

Cells are incubated at 10⁶ cells/ml in HL5 with [9,10-³H(N)]-Palmitic Acid (Perkin Elmer, Wellesley, MA) at 5 μCi/ml in a culture dish for 24 hours at room temperature. Cells are then washed with 5 ml PBM and starved in 10 ml PBM for 5 hours if required. Cells are resuspended in 5 ml PBM and mixed with 5 ml PBM containing 70 μl butan-1-ol or tertiary-butanol to achieve 0.7% final concentration, and incubated at room temperature for 22 minutes. PBM with butanol is removed and 500 μl of ice cold [Methanol:6N HCl (50:2)] is added. The cells are scraped off the plate and transferred to the first extraction tube (155 μl of 1N NaCl and 500 μl of ice cold chloroform) on ice. The tubes are then vortexed for 30 seconds and centrifuged at 14,000× g, 4°C for 2 minutes. The lower organic phase is transferred to the second extraction tube (350 μl ddH₂O, 115 μl methanol and 115 μl 1N NaCl), which is vortexed for 30 seconds and centrifuged at 14,000× g, 4°C for 2 minutes. 10 μl of the lower organic phase of the second extraction tube is used to determine the total CPM of the sample. All the samples are then normalized to the total CPM. The normalized samples are dried under a flow of nitrogen gas and resuspended in 30 μl of spotting solution (Chloroform:Methanol = 9:1). The tubes are then vortexed and let sit for 3 minutes. 10 μl of the sample is spotted at a time onto a silica-gel 60Å TLC plate (Whatman, Florham Park, NJ). The plate is dried at room temperature and placed into the TLC chamber containing 100 ml mobile phase (the upper organic phase of a mixture containing [Ethyl Acetate:Isooctane:Glacial Acetic Acid:ddH₂O] in the ratio of
110:50:20:100). The solvent front is allowed to reach the top of the plate. The plate is air dried and sprayed with $^3$H enhancer spray (Perkin Elmer, Wellesley, MA) and let dry for 20 minutes. It is then put into a transparent plastic bag and exposed to film for 10 days at -80°C. The films are scanned by a densitometer and phosphatidylbutanol bands are quantified by ImageQuant software (GE Healthcare, Piscataway, NJ). Background is calculated using “local average” in the software.

2.7 Cell fractionation

Wild-type and $pldB^-$ cells are allowed to develop on filter pads for 16 hours and 20 hours. Cells are then collected in PBM and sonicated in ice water bath (Misonix, Farmingdale, NY). Sonication products are centrifuged at 19,000× g, 4°C for 5 minutes to separate membrane and cytosol fractions. 20 μg protein is boiled for 3 minutes in SDS-PAGE sample buffer and loaded in a 10% Tris-HCl polyacrylamide gel (BioRad, Hercules, CA), and Western blot analysis is performed as described above.

2.8 Chemotaxis assay

Cells are starved in shaking PBM at $2 \times 10^7$ cells/ml for 5 hours. All the wells of a 24-well Transwell plate (Corning, Corning, NY) are preloaded with 600 μl PBM and inserts are put into half of the wells. $7.5 \times 10^5$ vegetative or starved cells in 100 μl PBM are loaded into each insert. After 10 minutes, each insert is transferred to a new well. The inserts sit for another 3 minutes, after which 6 μl 1 mM cAMP is added to half of the wells. The plate is incubated for 3 hours at 22°C. The inserts are then removed and 3 μl 2 mM Calcein-AM (Calbiochem, San Diego, CA) is added to all the wells and mixed gently.
The plate is then incubated at room temperature for 75 minutes, protected from light. PBM with Calcein-AM is removed afterwards and cells are resuspended in fresh PBM. Fluorescence of cells is measured by a Typhoon scanner and quantified by ImageQuant software (GE Healthcare, Piscataway, NJ).
CHAPTER 3

PldB mediates quorum sensing in the CMF pathway

Quorum sensing is first studied in bacteria [4] and found to be important in cell group behavior and survival. Later research utilizes cells from higher organisms including mammals where quorum sensing is involved in cell growth, organization, differentiation and morphogenesis. *Dictyostelium discoideum* also uses quorum sensing, and unlike mammalian cells, *D. discoideum* is amenable to genetic manipulation. This has made *D. discoideum* useful in the field of quorum sensing research.

Typically, a quorum sensing mechanism involves a cell-secreted molecule that cells can sense. This cell density sensing molecule needs to be stable under diverse environmental conditions and able to diffuse to a certain range so that cells in a given area can sense the accumulated amount of the molecule. The amount of this molecule will increase with cell density. When it reaches a threshold, certain genes will be induced in the cell, which may lead to physiological changes. In *D. discoideum*, two such mechanisms have been found during the early stages of development initiated by starvation [2].

One mechanism is mediated by a molecule called prestarvation factor (PSF). PSF is synthesized and secreted during growth stage and accumulates in the micro-environment according to the density of the cells [57]. A 65-kDa glycoprotein, PSF induces expression of several early developmental genes during late exponential growth, including those encoding for aggregation stage cAMP receptor (cAR1) and the aggregation-specific form
of cyclic nucleotide phosphodiesterase [58]. The gene inductive effect of PSF is inhibited by the presence of bacteria. Production of PSF declines rapidly when cells are shifted to starvation conditions [58]. These observations indicate that growing cells use PSF to monitor their own density in relation to their food supply. When appropriate conditions (cell number high, food approaching depletion) are detected, PSF will induce the production of gene products needed to initiate cell aggregation and development. When the food supply is depleted, PSF production declines, chromosomal DNA synthesis ceases while mitochondrial DNA synthesis continues [59], and a second density sensing pathway comes into play.

This second cell density sensing mechanism involves an 80-kDa glycoprotein named conditioned medium factor (CMF). Starving cells simultaneously secrete and sense CMF so as to coordinate cell aggregation and ensure an effectively-sized fruiting body. Cells will chemotax to cAMP and aggregate only when a large number of cells are starving in a given area, signified by a high CMF concentration (> 0.3 ng/ml) [2]. CMF realizes this control over aggregation by regulating the GTPase activity of Gα2 in the cAMP-cAR1-Gα2 signaling cascade [25]. Through its own signal pathway that includes a CMF receptor, Gα1βγ, PLC and a possible PKC, CMF eventually inhibits Gα2 GTPase activity, prolongs the lifetime of the cAMP activated Gα2-GTP configuration, and allows cAR1-mediated cAMP signal transduction to take place [30]. Thus, downstream adenylyl and guanylyl cyclases will be activated, genes required for further development will be induced, and chemotaxis and aggregation will occur (Figure 4).
This project is focused on the CMF-Gα1 signal pathway with an aim to find out downstream signaling molecules that lead to the regulation of Ga2 GTPase activity. Here, we present such a molecule – PldB.

3.1 PldB is a negative regulator of aggregation

CMF based quorum sensing involves PKC and a regulator of Gα GTPase activity. Since PKC activates PLD in mammalian cells [36] and RGS protein is regulated by PA – a product of PLD activity [37], it is reasonable to imagine that PLD activity is involved in CMF signal pathway of quorum sensing. A preliminary low cell density assay is performed to examine this possibility. The ability of cells to aggregate at different densities is measured after treating them with butanol, which inhibits PLD function by blocking its ability to produce phosphatidic acid [60]. The result shows that wild-type cells starved in the absence of conditioned medium are only able to form aggregates down to $28 \times 10^3$ cells/cm$^2$. Adding conditioned medium allows aggregation at $7 \times 10^3$ cells/cm$^2$. Butanol (0.1%) in the absence of conditioned medium also allows aggregation to occur as low as $7 \times 10^3$ cells/cm$^2$, thus mimicking conditioned medium. To ensure that this effect is due specifically to butanol and not the presence of alcohol, the same experiment is performed with tertiary-butanol (0.1%), an isomer of butanol, which has no effect on PLD activity; these cells behave exactly like the untreated cells, aggregating only down to $28 \times 10^3$ cells/cm$^2$. Therefore, when plated at low cell density, cells treated with butanol and presumably containing decreased PLD activity respond to starvation as if they are in the presence of CMF. This result strongly suggests that PLD activity is involved in quorum sensing.
Since butanol allows cells to aggregate at low cell density, it is reasonable to believe that disrupting one or both of the PLD genes would also allow cells to aggregate at low cell density. To examine this possibility, pldB− cells are created by inserting a blasticidin resistance cassette into the pldB locus by homologous recombination. Genomic integration and disruption of the pldB gene is confirmed by PCR [60]. Strains exhibiting proper integration of the disruption construct into the pldB gene are examined for their ability to aggregate at low cell density. While wild-type cells aggregate down to $28 \times 10^3$ cells/cm$^2$, pldB− cells are able to aggregate at $7 \times 10^3$ cells/cm$^2$ (Table 1), similar to the observation with the addition of butanol to wild-type cells. Thus, disruption of phosphatidic acid production by butanol or deletion of pldB both allow aggregation at low cell density. The addition of exogenous CMF had no apparent effect on aggregation in the pldB− cells [60], suggesting that pldB is required by cells to properly respond to CMF. Therefore, starving cells lacking pldB behave as though they are at high cell density and are nonresponsive to conditioned medium. This suggests that PldB suppresses aggregation and is negatively regulated by CMF. Loss of pldB mimics the presence of CMF.

Since pldB gene activity normally blocks aggregation, it is reasonable to believe that cells overexpressing pldB should be unable to aggregate at high cell density. To examine this possibility, strains overexpressing pldB are created by introducing a plasmid-borne copy of the pldB gene constitutively expressed from the actin 15 promoter into wild-type cells. RT-PCR [60] and Northern blot analysis (data not shown) are performed on RNA isolated from vegetative cells to confirm overexpression of pldB. For these pldB$^{OE}$ cells,
aggregation is inhibited even at $224 \times 10^3$ cells/cm$^2$, the highest cell density examined (Table 1). Addition of 0.1% butanol is able to partially reverse this inhibition and allow aggregation at $112 \times 10^3$ cells/cm$^2$ [60], suggesting that the inability to aggregate may be ameliorated by inhibition of PLD activity. In addition, exogenous CMF is unable to induce aggregation at a lower cell density [60], corroborating previous results suggesting that $pldB$ is involved in the cellular response to CMF.

If $pldB$ is a negative regulator of aggregation, then enhancing its activity would preclude aggregation at higher and higher cell densities; the data show that this is the case. Likewise, removing its activity would allow cells to aggregate at any density, including extremely low densities, which the data also show to occur. Taken together, the data suggest that $pldB$ is involved in quorum sensing as a negative regulator of aggregation and may lie in the CMF pathway.

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<td>wild-type</td>
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<td>$pldB^-$</td>
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<td>$pldB^{OE}$</td>
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Table 1. Ability of wild-type, $pldB^-$ and $pldB^{OE}$ cells to aggregate at low cell density.
Cells are starved at various cell densities in submerged monolayer culture. The field of cells is then examined with an inverted microscope at 18 hours. The presence of aggregates is represented by a plus sign, while the absence of aggregates is represented by a minus sign. These are the representative results of three separate assays.
3.2 The ability of \( pldB^- \) cells to aggregate at low cell density is cell autonomous

\( pldB^- \) cells can aggregate at low cell density while wild-type cells can not. It is possible that \( pldB^- \) cells improperly secrete a diffusible factor, such as CMF, that allows them to aggregate at low cell density. If this were true, a small amount of \( pldB^- \) cells, when mixed with wild-type cells, would secrete enough of the factor to cause the wild-type cells to aggregate at low cell density. To examine this possibility, wild-type cells and \( pldB^- \) cells are mixed together at ratios of 1:9, 1:1, and 9:1. Their ability to aggregate at low cell density is examined (Table 2). The result shows that the addition of 10% \( pldB^- \) cells to wild-type cells does not induce aggregation at low cell density. Conversely, the addition of 10% wild-type cells to \( pldB^- \) cells does not prevent aggregation at low cell density. In other words, cells making up the majority in the mixture determine the phenotype of the mixture. Therefore, the ability of \( pldB^- \) cells to aggregate at low cell density is not due to a change in cell secretion and is thus cell autonomous.
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<th>Cell type(s) (ratio)</th>
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<td>wild-type</td>
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<td>pldB$^-$</td>
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<td>wild-type: pldB$^-$ (9:1)</td>
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<td>wild-type: pldB$^-$ (1:9)</td>
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Table 2. Ability of mixed populations of wild-type and pldB$^-$ cells to aggregate at low cell density. Wild-type Ax2 and pldB$^-$ cells are mixed in different ratios and are starved at various cell densities in submerged monolayer culture. The field of cells is then examined with an inverted microscope at 18 hours. The presence of aggregates is represented by a plus sign, while the absence of aggregates is represented by a minus sign. These are the representative results of three separate assays.
3.3 PldB lies in the CMF signal pathway

While PldB is clearly involved in quorum sensing, it is possible that this is through a CMF-independent pathway. CMF is demonstrated to accomplish quorum sensing by decreasing the GTPase activity of Ga2, keeping Ga2 in the active GTP-bound form [25]. Low cell density aggregation assays have shown that PldB protein is involved in quorum sensing and possibly in the CMF pathway. Therefore, it is reasonable to presume that cells without PldB protein will not have a decrease in GTPase activity of Ga2 after exposure to CMF, since lack of PldB breaks the CMF pathway that regulates the GTPase activity of Ga2. For cells overexpressing pldb, CMF should not have as much a decreasing effect on the GTPase activity of Ga2 as it would for wild-type cells, if there is any. To test this postulation, we perform GTPase activity assays to measure the GTPase activity of Ga2 under the influence of CMF.

GTP hydrolysis in D. discoideum membranes is caused by a low-affinity GTPase and a high-affinity GTPase [61, 62]. cAMP binding enhances GTP hydrolysis by stimulating the high-affinity GTPase, but not the low-affinity GTPase [61]. This high-affinity GTPase activity is associated with Ga2 [25]. To assess the change in GTPase activity of Ga2, we need to measure the high-affinity GTPase with cAMP stimulation and presence of CMF. In the assay, wild-type, pldb and pldbOE cells are starved for 5 hours and lysed with or without 30-second incubation of CMF. Cell lysates are then added to the reaction mixture. Half of the samples are stimulated by 10 μM cAMP. [γ-32P] GTP in the mixture is hydrolyzed by the GTPase activity to GDP and 32P. 32P, is counted by liquid scintillation. Total GTPase activity is measured with the presence of 0.01 μM cold GTP.
Low-affinity GTPase activity is measured with the presence of 50 μM cold GTP. High-affinity GTPase activity is calculated by subtracting low-affinity GTPase activity from total GTPase activity. Percent stimulation of GTPase is calculated by comparing high-affinity GTPase with and without cAMP stimulation.

As the results show, wild-type, \textit{pldB}^\text{−} and \textit{pldB}^{\text{OE}} cells all have an increase in high-affinity GTPase activity with cAMP stimulation (Figure 8). There is no statistically significant (\(\alpha=0.05\)) difference in the cAMP-stimulated percentage of GTPase between wild-type and \textit{pldB}^\text{−} cells, or wild-type and \textit{pldB}^{\text{OE}} cells. This demonstrates that wild-type, \textit{pldB}^\text{−} and \textit{pldB}^{\text{OE}} cells have similar high-affinity GTPase activity after cAMP stimulation without the presence of CMF. However, with the presence of CMF, wild-type cells have a statistically significant (\(\alpha=0.05\)) decrease in the stimulated percentage of GTPase activity, while \textit{pldB}^\text{−} cells and \textit{pldB}^{\text{OE}} cells do not. Thus, wild-type cells respond to CMF by decreasing the cAMP-stimulated high-affinity GTPase activity of \textit{Ga2}. Cells lacking or overexpressing PldB protein appear to be nonresponsive to CMF. These findings match our postulation. They confirm that PldB protein is required by CMF signal pathway to regulate the cAMP signal pathway in quorum sensing. Therefore, PldB mediates quorum sensing in the CMF pathway.
Figure 8. Effect of CMF on cAMP-stimulated high-affinity GTPase.
Cell lysates are stimulated with extracellular cAMP with or without the presence of CMF. Percent stimulation of GTPase is the increase in high-affinity GTPase between samples stimulated with cAMP and samples without cAMP. Data are presented as mean ± S.E.M. Statistics for the effect of CMF (paired t test; one-tailed \( p \) value): wild-type cells, \( n=5, p=0.0095 \); \( pldB^- \) cells, \( n=3, p=0.2643 \); \( pldB^{OE} \) cells, \( n=3, p=0.0650 \). Statistics for the difference in percent stimulation of GTPase by cAMP (unpaired t test; two-tailed \( p \) value): wild-type cells vs. \( pldB^- \) cells, \( p=0.4260 \); wild-type cells vs. \( pldB^{OE} \) cells, \( p=0.0821 \).
3.4 Discussion

In *D. discoideum*, CMF is demonstrated to be an extracellular quorum sensing signaling protein that regulates aggregation by decreasing the GTPase activity of Ga2 [25]. This makes cAMP-mediated signaling possible through Ga2βγ-bound cell surface cAMP receptor cAR1, which leads to expression of genes needed for cell chemotaxis and aggregation [25]. This CMF regulation is via a signal pathway that involves Ga1βγ and phospholipase C [30]. In this project, we identify a gene with sequence similarity to phospholipase D, *pldB*, and present evidence to show that it has a role in quorum sensing pathways.

Cells lacking *pldB* can aggregate at low cell density, bypassing the requirement for CMF. This ability is cell autonomous. Cells overexpressing *pldB* are not able to aggregate at high cell density, and are thus blocked from any further development. Exogenous CMF will allow wild-type cells to aggregate at low cell density, but has no effect on *pldB*OE cells [60], showing that *pldB*OE cells are insensitive to CMF. These observations suggest PldB is a negative regulator of aggregation in the CMF signal pathway. PKC activity has been detected in *D. discoideum* [63], though its gene has not been cloned. This PKC activity is up-regulated by the presence of CMF (unpublished data). In mammalian cells, PLC activates PKC, which in turn activates PLD. PA, a product of PLD activity, regulates RGS, which regulates the GTPase activity of G proteins. These observations lead to our hypothesis: PldB lies downstream of PLC in the CMF signal pathway of quorum sensing. PldB increases the GTPase activity of Ga2. CMF triggers a decrease in PldB activity, and therefore GTPase activity of Ga2, allowing cell chemotaxis and
aggregation to occur. According to this hypothesis, cells lacking PldB have low GTPase activity of Ga2, as if CMF were present, and are able to aggregate at low cell density. Cells with high levels of constitutively expressed PldB have sustained high GTPase activity of Ga2 and insensitivity to CMF. cAMP signaling is therefore blocked. Obviously, the role of PKC in the CMF pathway needs to be clarified in future research, as well as the RGS protein that regulates Ga2.

If our hypothesis is true, then the GTPase activity of Ga2 should be insensitive to the presence of CMF in cells lacking PldB protein. We have shown that this is exactly the case with the GTPase assay. GTPase activity of Ga2 in cells overexpressing pldB seems to remain unchanged with CMF. This corroborates the finding that pldBOE cells are insensitive to CMF in the low cell density assay. All these results fit our hypothesis. This leads to further investigations to reveal the properties of PldB protein.
CHAPTER 4

Expression pattern, activity and localization of PldB

Proteins are expressed at different time points of development so as to provide various functions needed by the cell. Interference with the timing of protein expression may result in an abnormal development program. The localization of a protein is also important for proper protein functions. Mislocalization could lead to diverse physiological aberrations or even halt development altogether. Now that we have confirmed that PldB protein mediates quorum sensing, we would like to find out the temporal and spatial characteristics of PldB by examining the RNA and protein expression patterns, cellular PLD activity and PldB localization in the cell.

4.1 *pldB* mRNA is detected in developing cells

In order to confirm the developmental regulation of PldB expression, a Northern blot analysis is performed with total RNA from developing wild-type cells, using a DNA probe generated from a fragment of *pldB*. The 2.8 kb *pldB* mRNA exhibits little or no expression in vegetative cells (Figure 9). Expression is apparent by 8 hours and peaks by 16 hours. Thus *pldB* gene is transcribed during development.
Figure 9. Northern blot of total RNA from developing wild-type cells. Total RNA is visualized by ethidium bromide staining. Nylon membrane is probed with pldB DNA probe. RNA sizes are measured by RNA standard run in the same agar gel.
4.2 PldB protein production roughly parallels mRNA expression

To examine production of the PldB protein, antibodies are raised against a peptide of PldB. These antibodies are then affinity purified with the same peptide and used to probe Western blots. To ensure that the antibodies are specific for PldB, antibodies are used on Western blots of lysates from both wild-type Ax2 cells and \( pldB^- \) cells. Also, to examine developmental production of PldB, cell lysates from developing cells are used. A band at approximately 100-kDa appears in the wild-type sample but is absent from the \( pldB^- \) sample (Figure 10), suggesting that the antibodies specifically recognize PldB. PldB is found in vegetative cells and protein production largely increases during development, peaking at 20 hours. This pattern roughly parallels that of the \( pldB \) mRNA expression. Thus, PldB is present during early development when quorum sensing is occurring as well as in later development.

![Figure 10. Western blots of cell lysates from developing wild-type and \( pldB^- \) cells.](image)

PVDF membranes are probed with PldB antibody. Protein quantities are monitored by Coomassie Blue staining of another gel run simultaneously with the same samples. Protein size is measured by protein marker run in the same gel.
4.3 PLD activity of wild-type, \textit{pldB} knockout and \textit{pldB} overexpression cells

Western blots show that PldB protein is present throughout development. But whether PldB functions in cell signaling processes depends on its enzyme activity. Specifically, PldB is supposed to hydrolyze phosphatidylcholine to form phosphatidic acid and choline, which includes a phospholipid transphosphatidylation step. Thus, we examine the overall PLD activity in wild-type, \textit{pldB}^{-} and \textit{pldB}^{OE} cells with an \textit{in vivo} transphosphatidylation assay, also known as a PLD activity assay. In this assay, primary butanol replaces H\textsubscript{2}O in phosphatidylcholine hydrolysis, forming metabolically stable phosphatidylbutanol (Figure 11). Cells are preincubated with [9,10-\textsuperscript{3}H(N)] palmitic acid for 24 hours. Phospholipids are thus labeled with tritium atoms. The phosphatidylbutanol bands containing tritium atoms are separated on a thin layer chromatography plate and visualized on films. Tertiary-butanol can not compete as well with H\textsubscript{2}O in phosphatidylcholine hydrolysis. Therefore it is used as a negative control.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{transphosphatidylation_assay.png}
\caption{Transphosphatidylation assay.}
\end{figure}

In the presence of primary butanol, PLD cleaves phosphodiester bond to form phosphatidylbutanol and choline. Phosphatidylbutanol with tritium atoms is then visualized through thin layer chromatography.
Quorum sensing happens during early development. Thus we perform the assay on vegetative and 5-hour starved cells. As is shown previously, *D. discoideum* has a basal level of PLD activity [56]. In our assay, under both vegetative and starvation conditions, *pldB* cells show lower PLD activity than wild-type cells, while *pldB* cells show higher PLD activity than wild-type cells (Figure 12, Table 3). The data demonstrate that loss of PldB causes an overall decrease in cellular PLD activity while overexpression of PldB causes an overall increase in cellular PLD activity. PldB is thus a true PLD enzyme. PLD activity is present during quorum sensing and aggregation stages.

![Figure 12. Overall PLD activity of wild-type, *pldB* and *pldB* cells.](image)

*In vivo* transphosphatidylation assays are performed for vegetative and 5-hour starved cells. Tertiary-butanol is used as a negative control as opposed to butan-1-ol. This is representative of three experiments.

<table>
<thead>
<tr>
<th>Densitometry Readings (relative to wild-type)</th>
<th>wild-type</th>
<th><em>pldB</em></th>
<th><em>pldB</em></th>
</tr>
</thead>
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<td><strong>vegetative</strong></td>
<td>1</td>
<td>0.85</td>
<td>1.21</td>
</tr>
<tr>
<td><strong>5-hour starved</strong></td>
<td>1</td>
<td>0.35</td>
<td>1.39</td>
</tr>
</tbody>
</table>

**Table 3.** Overall PLD activity of *pldB* and *pldB* cells relative to wild-type cells.

Phosphatidylbutanol bands in transphosphatidylation assay are scanned by a densitometer and quantified. Densitometry readings of *pldB* and *pldB* cells are adjusted relative to that of wild-type cells under vegetative and starvation conditions.
4.4 PldB is localized to cellular vesicles and/or cytoplasmic membrane

Protein localization is important for normal enzyme activity and proper cellular functions. To determine the localization of PldB protein in the cell, total cell lysates are separated into membrane and cytosol fractions from cells at 16 and 20 hours of development on filter pads. These are the time points when PldB protein is at its expression peak. Western blot analysis is performed on both fractions. PldB protein is only observed in the membrane fractions of wild-type cells at 16-hour and 20-hour of development (Figure 13). This indicates that PldB protein is localized to cellular vesicles and/or cytoplasmic membrane.

**Figure 13. Western blot of membrane and cytosol fractions of wild-type and pldB^- cells.**
Cell lysates are prepared as described in Materials and Methods and probed with affinity-purified anti-PldB peptide antibodies.
4.5 Discussion

As shown by mRNA and protein expression, PldB is present during early development, when quorum sensing is occurring, as well as in later development. This implies that in addition to its role in quorum sensing, PldB may have roles in later cell development. In support of this, we have found that cells lacking PldB are preferentially sorted into the apical region of a mound when mixed with wild-type cells, indicating they have become prestalk cells (unpublished data). This is consistent with the later observation that almost all \( pldB^{-} \) cells in these mixtures end up in the stalk of the final fruiting body (unpublished data). This suggests that PldB is involved in cell differentiation and cell sorting during the mound stage when cells are divided into prestalk cells at the apical tip and prespore cells at the lower part. The mound stage occurs at 12 hours of development. Our Western blot shows that PldB is present at this time point. Also, Western blot analysis shows that PldB protein expression peaks at around 20 hours of development, the culmination stage, when the spore mass is pulled up the stalk to form a fruiting body. This suggests PldB may have a role in morphogenesis. Although our \( pldB \) knockout cells form similar fruiting bodies to those of wild-type cells, details in timing and morphology need to be examined. Total PLD activity is lower in \( pldB \) knockout cells and higher in \( pldB \) overexpressing cells. This indicates that PldB is a true PLD enzyme and its activity takes up a good part of the total PLD activity in \( D. discoideum \). The lack or overexpression of PldB, as demonstrated by the difference in total PLD activity, causes the divergence in phenotypes.
As demonstrated by cell fractionation and Western blot analysis, PldB is localized to membranes and vesicles. Additionally, GFP-tagged PldB is localized to cytoplasmic membrane as well as vesicles in cells starved for 5 hours (unpublished data). Cell organelle staining shows that PldB is co-localized with lysosomes and endosomes, but not mitochondria (unpublished data). This could be explained by PldB structure. Sequence analysis shows that PldB contains all of the PLD conserved regions including a PH domain, CRI to CRIV, a loop, and a C-terminal domain, suggesting a member of the PLD1 subfamily [38]. PH domains are best known as phospholipid binding domains, but they have also been shown to be involved in binding to the βγ-subunits of heterotrimeric G proteins and protein kinase C [64, 65]. In D. discoideum, the PH domains of CRAC and protein kinase B are believed to be responsible for translocating these proteins to the plasma membrane during chemotaxis [66-68]. It has been suggested that the selective localization of such PH domain-containing proteins to the leading edge of the cell is what allows a directional response to a chemoattractant [69, 70]. Specifically, it is proposed that the localized activities of phosphatidylinositol 3-kinase and PTEN cause the accumulation of phosphatidylinositol-(3,4,5)-triphosphate at the leading edge, forming binding sites for PH domain-containing proteins [66, 71]. Additionally, the PH domain in PLD1 is suggested to be vital in protein activity for its specific and high affinity binding to phosphatidylinositol-(4,5)-biphosphate in lipid surfaces [48]. It is therefore feasible that the PH domain of PldB also allows binding to phospholipids, thus resulting in localized PLD activity. The PH domain of CRAC binds specifically to PI(3,4,5)P₃ and PI(3,4)P₂ [66]. To find out the phosphoinositides that the PH domain of PldB specifically binds to, a dot blot assay can be performed by incubating PH_{PldB}\_eGFP-His fusion protein
with phosphoinositides spotted on a PVDF membrane, and probing the membrane with anti-GFP antibodies. Additionally, glutathione-S-transferase (GST) fusion protein comprising GST and PH\textsubscript{PldB} can be created to investigate the possibility of PH\textsubscript{PldB} binding to G\textsubscript{βγ}. Furthermore, visualizing single molecules of green fluorescent protein tagged to PldB or PH\textsubscript{PldB} may help reveal the dynamics of PldB localization before and during chemotaxis with cAMP stimulation. This technique has been demonstrated with CRAC [72].

In mammalian cells, PLD activity is found in multiple cellular membranes, including the nuclear envelope, endoplasmic reticulum, Golgi apparatus, transport/secretory vesicles and plasma membrane [54]. Specifically, PLD1 is enriched significantly in the Golgi apparatus in the perinuclear region and is also present in cell nuclei [73]. PLD1 associates closely with membrane fragments of Golgi apparatus and dissociates from the membranes with inhibition of PA synthesis. This is consistent with its role of vesicle trafficking from the Golgi apparatus. We show that PldB has similar subcellular localization in \textit{D. discoideum} and has a role in quorum sensing. Additionally, loss of PLD activity in vegetative \textit{D. discoideum} results in aberrant actin distribution and impaired endocytosis [56]. This suggests that PLD activity has a role in actin relocalization and thus phagocytosis and macropinocytosis, which are actin-based processes [74]. Vesicle trafficking is not only important for vegetative \textit{D. discoideum} cells, when it mediates the food intake, but also for developing cells, where it is required for signal secretion and spore coat formation. This observation raises the possibility that PldB may play a role in actin organization and vesicle trafficking. In fact, we have found that cells overexpressing
*pldB* do not form filopodia under starvation conditions, and therefore can not have proper chemotaxis or aggregation (unpublished data). This confirms that PldB may regulate actin polymerization.
Knowing that PldB mediates quorum sensing and that \( pldB \) is expressed during chemotaxis and aggregation stage as well as later developmental stage, we wonder whether \( pldB \) is required for proper development of the fruiting body. To examine this possibility, \( pldB^- \) cells are starved on agar and allowed to develop. The resulting fruiting bodies are relatively normal, with the possibility that \( pldB^- \) fruiting bodies may have slightly longer stalks than wild-type fruiting bodies (Figure 14).

![Images of wild-type and \( pldB^- \) fruiting bodies](image)

**Figure 14.** \( pldB^- \) cells form fruiting bodies with slightly elongated stalks. Wild-type Ax2 and \( pldB^- \) cells are plated with bacteria on agar. As the bacteria are consumed, development is triggered. Fruiting bodies form after 24 hours. A side view of these fruiting bodies is shown. Bar, 1 mm.
To find out whether pldB is involved in the timing of aggregation, the time it takes for pldB− cells to aggregate in submerged culture is examined. Wild-type cell aggregates appear at 16 hours, while pldB− cells form aggregates by 9 hours, demonstrating that they aggregate more rapidly than wild-type cells (Figure 15).

**Figure 15.** pldB− cells aggregate rapidly in submerged culture.
Cells are starved at 224×10^3 cells/cm² in PBM, and photos are taken 9 and 16 hours later. Bar, 1 mm.
We next examine whether this is a phenomenon associated with submerged cultures by determining whether \( pldB^- \) cells can develop as rapidly on filter pads. On filter pads, \( pldB^- \) cells show an accelerated rate of development compared to wild-type cells. \( pldB^- \) cells form aggregates by 4 hours, while wild-type cells show no signs of development. By 6 hours, \( pldB^- \) cells form tight aggregates, whereas wild-type cells only start streaming, the initial stage of aggregation. \( pldB^- \) cells develop consistently more rapidly, forming fruiting bodies by 16 hours, 6 hours ahead of the wild-type cells. Thus, loss of \( pldB \) expression allows an earlier initiation of the developmental cycle, leading to early aggregation and culminating in early fruiting body formation (Figure 16).

There are various reasons that might cause a rapid development phenotype. Here we examine two possible causes. One is increased rate of chemotaxis, which could make \( pldB^- \) cells move faster and aggregate sooner. The other is earlier expression of genes required for chemotaxis and aggregation, which could make \( pldB^- \) cells respond to external signaling molecules earlier than wild-type cells.
Figure 16. *pldB*<sup>−</sup> cells develop rapidly on filter pads.
Cells are deposited on filters and allowed to develop. Photos are taken at the indicated times. Bar, 3mm.
5.1 \textit{pldB}^− \textit{cells show normal chemotaxis to cAMP while pldB}^{OE} \textit{cells do not}

To determine whether the accelerated development seen in \textit{pldB}^− cells is due to altered chemotaxis, we perform a Transwell chemotaxis assay of wild-type and \textit{pldB}^− cells to extracellular chemoattractant cAMP. \textit{pldB}^{OE} cells are also included to examine their chemotactic ability. In this assay, cells are allowed to migrate through a membrane at the bottom of an insert to the cAMP-containing well, which accommodates the insert. The chemotactic index is the ratio of the number of cells migrated with cAMP to the number of cells migrated without cAMP. Therefore, a chemotactic index of 1 indicates that there is no chemotaxis to cAMP, while a chemotactic index larger than 1 indicates that there is chemotaxis to cAMP.

Under vegetative conditions, wild-type, \textit{pldB}^− and \textit{pldB}^{OE} cells all have a chemotactic index around 1 (Figure 17), indicating that there is no chemotaxis to cAMP. This is in accordance with the phenotype that vegetative cells live as individual cells and do not aggregate. When cells are starved for 5 hours, wild-type and \textit{pldB}^− cells show similar chemotactic index around 2, indicating that there is chemotaxis to cAMP. However, \textit{pldB}^{OE} cells still have a chemotactic index near 1, denoting no chemotaxis to cAMP. The similarity in chemotactic ability between wild-type and \textit{pldB}^− cells suggests that the rapid development of \textit{pldB}^− cells is not caused by an increased rate of chemotaxis.
Figure 17. Chemotactic ability of wild-type, pldB− and pldBOE cells. Vegetative and 5-hour starved cells are used as described in Materials and Methods. Chemotactic index = (cells migrated with cAMP)/(cells migrated without cAMP). These are the results of three separate assays with standard deviation.
5.2 cAR1 is expressed earlier in $pldB^-$ cells

To determine whether the accelerated development seen in the $pldB^-$ cells is due to earlier expression of genes required for development, a series of Northern blots are performed to examine the expression of three genes required early in development, cAR1 (cAMP receptor on cell surface), CRAC (cytosol regulator of adenylyl cyclase) and ACA (adenylyl cyclase A), in wild-type and $pldB^-$ cells. For CRAC and ACA mRNA expression patterns, we observe no apparent difference in the timing of expression between wild-type and $pldB^-$ cells (Figure 18). However, we find that cAR1 is more highly expressed in $pldB^-$ cells under vegetative conditions than in wild-type cells (Figure 19A).

To take a closer look at the expression pattern of cAR1 at the early stage of development, we isolate total RNA from vegetative cells and cells developed on filter pads at 1, 2, 3, 4, 5, and 6 hours. We confirm that cAR1 mRNA is higher in $pldB^-$ cells under vegetative conditions than in wild-type cells (Figure 19B). In addition, cAR1 expression in $pldB^-$ cells remains consistently higher than in wild-type cells throughout the first 6 hours of development. cAR1 expression in $pldB^-$ cells peaks at 3-4 hours of development, while that of wild-type cells doesn’t peak until 6 hours. Thus cAR1 is expressed earlier and at higher levels in $pldB^-$ cells. This suggests that altered cAR1 expression can be one of the reasons that cause the rapid development phenotype of $pldB^-$ cells and PldB is involved in the timing of development.
Figure 18. Wild-type and pldB− cells have similar CRAC and ACA mRNA expressions.
Northern blots of total cellular RNA isolated from wild-type Ax2 cells and pldB− cells at 4-hour intervals after starvation are probed with radiolabeled fragments of the crac gene and aca gene respectively. mRNA bands in agarose gels are stained with ethidium bromide and used to monitor RNA loading.
Figure 19. car1 is expressed strongly and early in pldB<sup>−</sup> cells. Northern blots of total cellular RNA isolated from wild-type Ax2 cells and pldB<sup>−</sup> cells at 4-hour intervals (A) or 1-hour intervals (B) after starvation are probed with a radiolabeled fragment of the car1 gene. The same blots are stripped and reprobed with a radiolabeled fragment of the IG7 gene to control for RNA loading. The densitometry measurements are a ratio of cAR1 to IG7, normalized to the wild-type Ax2 vegetative sample.
5.3 Discussion

In addition to allowing cells to aggregate in suboptimal density, loss of \( pldB \) also accelerates the developmental program. In both submerged monolayer conditions and on filter pads, \( pldB^- \) cells aggregate well before wild-type cells. If loss of \( pldB \) alters the chemotactic ability of cells, then it might explain the rapid development phenotype observed in \( pldB^- \) cells. Here we demonstrate that there is no change in chemotaxis rate between wild-type and \( pldB^- \) cells at the aggregation stage. Of course, there still could be changes in other aspects of chemotaxis, such as when cells become chemotactically active, or how fast cells recover from a chemotactic pulse to cAMP, or how much lateral movement cells make. Such parameters of chemotaxis can be monitored through videomicroscopy coupled with Digital Image Analysis Software (DIAS), as depicted previously [75]. \( pldB^{OE} \) cells lack chemotaxis to cAMP under starvation conditions. This may explain their non-aggregation phenotype.

If the genes required for early development are expressed earlier in \( pldB^- \) cells, then \( pldB^- \) cells might be able to initiate development earlier and thus have the rapid development phenotype. Therefore, we examine the expression patterns of the early development genes cAR1, CRAC and ACA. cAR1 is the cAMP receptor on the cell surface. It mediates cAMP signaling through its coupled \( \gamma_2 \beta \gamma \) protein. Upon cAMP binding, \( \gamma_2\text{-GTP} \) separates from \( \beta \gamma \). \( \gamma \beta \gamma \) and CRAC together will activate downstream adenylyl cyclase – ACA. ACA increases intracellular cAMP, which regulates expression of genes needed for development and gets released extracellularly to relay the cAMP chemotaxis signal. There is no apparent difference in the timing of
expression of CRAC and ACA between wild-type and *pldB* cells. The seemingly less expression of ACA and CRAC at 4 hours and 8 hours in *pldB* cells would only lead to delayed chemotaxis and aggregation, instead of accelerated development. Thus ACA and CRAC are not subject to further analysis. Meanwhile, cAR1 is expressed earlier and at higher levels in *pldB* cells than in wild-type cells. This suggests that *pldB* cells may respond to extracellular cAMP earlier with premature cAR1 on cell surface, and thus initiate development earlier, causing a rapid development phenotype. The data indicate that altered cAR1 expression is likely one of the factors that cause the rapid development of *pldB* cells, implying that PldB plays a role in the timing of development. Interestingly, a previous study shows that overexpressing cAR1 at growth and aggregation stages delays the onset of aggregation by 1-3 hours, though subsequent stages proceed normally [76]. This is somewhat counterintuitive since cells overexpressing cAR1 are supposed to respond to cAMP earlier and faster. The authors claim that the constitutively expressed cAR1 has similar affinity for cAMP to the endogenous one. So the possibility of exogenous cAR1 having lower affinity is ruled out. Our *pldB* cells have 2.2 fold more cAR1 than wild-type at growth stage and 1.1-2.8 fold during early development (densitometry readings in Figure 19). While in their study, cAR1 is constitutively overexpressed at 7-40 times over growth stage and 3-5 fold over aggregation stage. This might cause constant adaptation of the cAR1-ACA pathway, as has been suggested recently [77]. This adaptation may explain the aggregation delay phenotype.

Besides *pldB* cells, Gα1 cells or drug treatments, such as addition of PKC activators, also cause aggregation at low cell density ([30]; unpublished data). But they do not have
the rapid development phenotype. This argues that \( pldB \) may also play a role in aggregation or development that is outside of CMF signal pathway. It has been reported that overexpressing DdPK2 or DdPK3, which encode catalytic subunits of protein kinase A (PKA), will cause rapid development in \( D. discoideum \) cells [78, 79]. PKA is crucial at all stages of \( D. discoideum \) development. Knocking out the catalytic subunit of PKA will preclude transcription of genes required for early development, such as \( acaA, pdiA, carA \), and make cells non-aggregative [80]. It is easy to understand that overexpressing the catalytic subunits of PKA will cause higher PKA activity, which may lead to higher and earlier expression of developmental genes, and thus the rapid development phenotype. Since PKA regulates PLD activity in mammalian cells [81-84], it is conceivable that PKA may regulate PldB or vice versa in \( D. discoideum \). If PKA inhibits PldB activity, then overexpressing PKA activity will mimic lack of PldB, thus leading to a rapid development phenotype. Or if PldB inhibits PKA activity, then overexpressing PldB will nullify PKA activity, thus prevent expression of early developmental genes, resulting in a non-aggregation phenotype. These hypotheses can be tested by measuring PLD activity with PKA inhibitors and activators or PKA knockout and overexpressing strains, and measuring PKA activity with butan-1-ol to inhibit PLD activity or \( pldB^- \) and \( pldB^{OE} \) cells.

During \( D. discoideum \) chemotaxis, a cAMP phosphodiesterase (PDE) is secreted to extracellular space to reduce cAMP, terminate cAMP receptor adaptation, and prepare the cells for a new encounter with cAMP [85]. Overexpression of PDE leads to rapid aggregation, presumably because the cells spend less time in an adapted state, though development is arrested at mound stage [86]. Additionally, disruption of a gene that
contains a PDE sequence causes precocious accumulation of intracellular cAMP at vegetative and early development stages, and thus a rapid development phenotype [87]. In mammalian cells, Ca^{2+}-stimulated intracellular redistribution of cAMP-specific phosphodiesterase is dependent on the PLD-mediated generation of phosphatidic acid [88]. These observations suggest a possibility that PldB protein might regulate PDE in *D. discoideum*. Assessing the PDE activity and its localization and secretion with *pldB*− and *pldBOE* cells may reveal the connection.

Finally, EDTA-resistant cohesiveness is stronger in *D. discoideum* cells with rapid aggregation [89]. In human patients with myeloid leukemia, depression of glycosyl-phosphatidylinositol-specific PLD activity can increase the adhesion rate of bone marrow mononuclear cells [90]. These facts elicit the possibility that PldB might have an additional role in cell-cell adhesion in *D. discoideum*. This can be tested with a cell cohesion assay, which measures the number of non-aggregated and aggregated cells from a shaking flask of starving cells at different time points [91].
CHAPTER 6

Downstream signaling of PldB

We have hitherto shown that PldB mediates quorum sensing by suppressing aggregation. Cells lacking pldB can aggregate at low cell densities and have accelerated development. Cells overexpressing pldB can not chemotax to extracellular cAMP or form aggregates at even high cell densities. PldB takes effect in the CMF signal pathway that regulates the GTPase activity of Ga2. Naturally, we wonder what could be the downstream signaling molecules to PldB. Here we present evidence for two of these downstream molecules: phosphatidic acid (PA) and target of rapamycin (TOR).

The principal PLD activity in all organisms is to hydrolyze phosphatidylcholine to create PA and choline. PA has been shown to be a signaling molecule in mammalian cells [92]. It is very likely that PldB uses PA to implement its functions in D. discoideum.

The target of rapamycin (TOR) is a serine/threonine kinase highly conserved from yeasts to mammals that controls cell growth in response to nutrients [93]. As the name implies, the immunosuppressant rapamycin inhibits TOR activity by binding to the FRB region in the C terminus of TOR proteins [94]. In mammalian cells, mammalian target of rapamycin (mTOR) integrates input from multiple upstream pathways, including growth factors, nutrients and energy [95]. One mode of mTOR regulation by growth factor involves PLD1 and PA [96, 97]. The growth factor activates PLD1 via the small GTPase, Cdc42 (a Rho family protein), causing an intracellular increase of PA [97]. PA directly
interacts with the domain in mTOR that is targeted by rapamycin, and activates mTOR [96]. Recent studies in eukaryotic cells have identified two distinct TOR complexes, TORC1 and TORC2, which phosphorylate different substrates and have distinct physiological functions [98]. In *D. discoideum* cells, a TOR-containing complex that is related to the TORC2 of *Saccharomyces cerevisiae* regulates both chemotaxis and signal relay [75]. Three knockout strains of three distinct components of the complex all have non-aggregation phenotypes. The reasons are found to be loss of ACA activation and reduced chemotaxis rate with loss of cell polarity. This demonstrates that TOR is present during chemotaxis and aggregation stage and it plays a significant role in the process. Together, these observations imply that TOR might have a role in quorum sensing.
6.1 Phosphatidic acid inhibits cell aggregation

To determine whether PA may serve as a downstream messenger of PldB in quorum sensing pathways, we perform low cell density assays with exogenous cell permeable phosphatidic acid. Both wild-type and \( \textit{pldB}^{-} \) cells show aggregation inhibition with various concentrations of PA (Table 4), mimicking the phenotype of \( \textit{pldB}^{\text{OE}} \) cells. Obviously, overexpressing \( \textit{pldB} \) will raise the level of intracellular PA, thus having an effect on cell aggregation similar to that of adding exogenous PA. As could be predicted, PA has more of an effect on wild-type cells than on \( \textit{pldB}^{-} \) cells at threshold levels. This implies that PA may be the downstream signal of PldB in the CMF signal pathway.

<table>
<thead>
<tr>
<th>Cell type and treatment</th>
<th>Presence of aggregates at cell density (( \times 10^{3} ) cells/cm(^{2} )) of:</th>
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<td></td>
<td>224</td>
</tr>
<tr>
<td>Ax2</td>
<td>+</td>
</tr>
<tr>
<td>Ax2 + 5 μM PA</td>
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<td>+</td>
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<td>−</td>
</tr>
<tr>
<td>( \textit{pldB}^{-} )</td>
<td>+</td>
</tr>
<tr>
<td>( \textit{pldB}^{-} ) + 5 μM PA</td>
<td>+</td>
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<td>( \textit{pldB}^{-} ) + 25 μM PA</td>
<td>+</td>
</tr>
<tr>
<td>( \textit{pldB}^{-} ) + 50 μM PA</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 4. Effect of phosphatidic acid (PA) on low cell density aggregation.
Wild-type Ax2 cells and \( \textit{pldB}^{-} \) cells are starved at various cell densities in submerged monolayer culture in the absence or presence of PA. The field of cells is then examined with an inverted microscope at 22 hours. The presence of aggregates is represented by a plus sign, while the absence of aggregates is represented by a minus sign. These are the representative results of three separate assays.
6.2 Rapamycin promotes cell aggregation

To examine the possibility of TOR being involved in quorum sensing, a low cell density assay is performed with rapamycin. The results demonstrate that rapamycin promotes cell aggregation at lower cell densities for all cell types tested (Table 5). Rapamycin in wild-type cells mimics loss of pldB, making cells aggregate at 14×10^3 cells/cm^2. This suggests that TOR is involved in quorum sensing and it could be upstream or downstream of PldB. However, rapamycin also has an aggregation-promoting effect on pldB\(^{-}\) and pldB\(^{OE}\) cells. This implies that TOR is actually downstream of PldB.

<table>
<thead>
<tr>
<th>Cell type and treatment</th>
<th>Presence of aggregates at cell density (×10^3 cells/cm^2) of:</th>
</tr>
</thead>
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<tr>
<td></td>
<td>112 56 28 14 7 3.5</td>
</tr>
<tr>
<td>Ax2</td>
<td>+    +   +    -   -   -</td>
</tr>
<tr>
<td>Ax2 + 1 μM rapamycin</td>
<td>+    +   +    +   -   -</td>
</tr>
<tr>
<td>pldB(^{-})</td>
<td>+    +   +    +   +   -</td>
</tr>
<tr>
<td>pldB(^{-}) + 1 μM rapamycin</td>
<td>+    +   +    +   +   +</td>
</tr>
<tr>
<td>pldB(^{OE})</td>
<td>-    -   -    -   -   -</td>
</tr>
<tr>
<td>pldB(^{OE}) + 1 μM rapamycin</td>
<td>+    -   -    -   -   -</td>
</tr>
</tbody>
</table>

Table 5. Effect of rapamycin on low-cell-density aggregation.
Wild-type Ax2 cells, pldB\(^{-}\) cells and pldB\(^{OE}\) cells are starved at various cell densities in submerged monolayer culture in the absence or presence of rapamycin. The field of cells is then examined with an inverted microscope at 47 hours. The presence of aggregates is represented by a plus sign, while the absence of aggregates is represented by a minus sign. These are the representative results of three separate assays.
6.3 Discussion

The enzyme activity of PLD is the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. PA has been identified as a second messenger in cell signal transduction [92]. It is then natural to imagine that PA has a role in quorum sensing as a product of PldB activity. Low cell density assays are performed to investigate this possibility with a series of increasing PA concentrations. The results confirm the involvement of PA in quorum sensing. Exogenous PA makes cells unable to aggregate at high cell densities, just as overexpressing pldB will do. Obviously, overexpressing pldB will increase PA production, which is mimicked by adding exogenous PA. The data are consistent with PldB taking effect in the CMF signal pathway through PA production.

In view of PA regulating TOR in mammalian cells and the requirement of a TOR complex for aggregation in D. discoideum, we wonder whether TOR has a role in quorum sensing. Again, low cell density assays are performed with rapamycin to inhibit TOR activity. Addition of rapamycin causes wild-type, pldB− and pldBOE cells to aggregate at lower cell densities than they would without rapamycin. This phenotype is similar to that of knocking out pldB gene. This indicates TOR is involved in quorum sensing and probably downstream of PldB, and therefore PA.

With these data, it is reasonable to come up with this hypothesis: PA activates TOR, and TOR eventually increases GTPase activity of Gα2. Since PA and rapamycin both interact with the same domain in TOR, exogenous rapamycin will compete with endogenous PA in binding to TOR. TOR is then less activated, thus GTPase activity of Gα2 is lower. Gα2
is preferentially kept at active GTP-bound form, and chemotaxis and aggregation are allowed to occur. Conversely, overexpressing PldB results in elevated PA production, which makes TOR highly activated. Consequently, GTPase activity of Ga2 is high. Ga2 is preferentially kept at inactive GDP-bound form, and chemotaxis and aggregation are not allowed to occur.

It is interesting to notice that for the mammalian TOR-containing multiprotein complexes, TORC1 is sensitive to rapamycin while TORC2 is not [99]. Correspondingly, a putative TORC1 is present during growth of *D. discoideum* [75]. Rapamycin inhibits cell growth, and knocking out the TOR gene seems to be lethal to cells. Yet TORC2, mutation of whose components results in non-aggregation phenotype, is rapamycin-insensitive, because the signaling pathways that are genetically controlled by TORC2 are not inhibited by rapamycin [75]. Mutant strains of TORC2 do not have growth defects [75]. This implies that a TORC1 controls the growth of *D. discoideum* while a TORC2 plays a role during development. Our data demonstrate that the TOR involved in quorum sensing is sensitive to rapamycin. Suppressing its activity leads to aggregation at lower densities, instead of non-aggregation. Thus this TOR complex doesn’t seem to be TORC2, but TORC1. This implies that the TORC1 controlling cell growth may still be available during development, at least the initial quorum sensing stage. If so, then both TORC1 and TORC2 are present during the transition stage from growth to development. To determine whether TORC1 and TORC2 are directly involved in the CMF quorum sensing pathway, performing low cell density assay and GTPase assay of Ga2 on TORC2 mutant strains and TORC1 overexpressing strains will be helpful.
On the other hand, PA is a regulator of RGS (regulator of G protein) activity. Specifically, mammalian RGS4 is inhibited by PA [37]. RGS proteins act as GTPase activating proteins for heterotrimeric G proteins. The controlling effect of CMF on GTPase activity of Ga2 is indirect and involves a G protein-coupled signaling cascade. An RGS protein is most likely directly responsible for controlling the GTPase. This RGS protein could be regulated by PA produced by PldB. Therefore, PldB, through localization provided by its PH domain, could be involved in localized regulation of Ga2, the G protein mediating cAMP chemotaxis. Given that G proteins in chemotaxing cells are evenly distributed along the membrane [100], localized control of G protein activity could help to influence the polarization of a chemotaxing cell. Specifically, a gene encoding RGS domain-containing protein kinase (RCK1) has been identified in D. discoideum [101]. RCK1 is preferentially expressed at the aggregation stage of development. rck1 null cells chemotax approximately 50% faster than wild-type cells, while overexpression of wild-type RCK1 reduces chemotaxis speed by approximately 40%. This suggests that RCK1 plays a negative regulatory role in chemotaxis and is consistent with a putative function of activating GTPase activity of Ga2. Upon cAMP stimulation, RCK1 transiently translocates to the membrane region with similar kinetics of membrane localization of the PH domain-containing proteins. Further genetic evidence suggests that Ga2 may directly interact with RCK1. This report is intriguing in that this RGS domain-containing protein kinase can possibly be involved in quorum sensing and directly regulate the GTPase activity of Ga2. It would be interesting to examine the role of this RGS protein in quorum sensing with low cell density assay and GTPase assay of Ga2, identify its substrate with GST fusion protein technique, and deduce its connection with PA and TOR.
CHAPTER 7

Summary

We present evidence to show that a putative PLD homologue, PldB, mediates quorum sensing in *Dictyostelium discoideum* development by negatively regulating cell aggregation. Cells lacking *pldB* can aggregate at low cell density. This phenotype is cell autonomous. Cells overexpressing *pldB* are unable to aggregate even at high cell density. PldB functions in the CMF signal pathway that regulates the cAMP signal pathway by decreasing the cAMP-stimulated GTPase activity, allowing cell chemotaxis and aggregation to occur. cAMP-stimulated GTPase activities in *pldB*− cells and *pldB*OE cells are not responsive to CMF. Phosphatidic acid and TOR are two possible signaling molecules downstream of PldB in the CMF signal pathway, since addition of exogenous phosphatidic acid or TOR-inhibiting rapamycin inhibits and promotes cell aggregation at low cell density respectively.

Localized to cellular vesicles as well as cytoplasmic membrane, PldB is not only present during early development when quorum sensing is occurring, but also in later development. Besides the role in quorum sensing, *pldB* is involved in the timing of development. cAMP receptor cAR1 is expressed earlier and at higher levels in *pldB*− cells than in wild-type cells, contributing to the rapid development phenotype of *pldB*− cells. However, *pldB*− cells have normal chemotaxis rate to cAMP. *pldB*OE cells lose chemotactic ability, which may explain their non-aggregation phenotype.
As a model organism, the discovery of a PLD enzyme in *D. discoideum* quorum sensing pathways sheds light on the path to understanding quorum sensing mechanisms in mammalian systems. Monitoring cell number while manipulating PLD activities would be a feasible start. Moreover, our results indicate the involvement of a TOR-containing multiprotein complex in quorum sensing pathways, as rapamycin disrupts quorum sensing. This TOR complex is likely the one in charge of vegetative cell growth, i.e. TORC1, thus disclosing a crosstalk between cell growth and quorum sensing pathways.

A similar mechanism is seen in mammalian cells. The type 1 insulin-like growth factor receptor can send two seemingly contradictory signals, one for growth and one for differentiation, depending on a balance between two of its substrates [102]. In our case, TORC1 controls cell growth and also seems to mediate the quorum sensing pathway that leads to cell development. This possible mechanism is feasible. When cells are in vegetative growth and actively replicating, they do not aggregate or develop. This can be explained by a high PldB activity, leading to a high TORC1 activity. This eventually increases the GTPase activity of Gα2, inhibiting cAMP signaling. Thus, cells can not aggregate or develop. When starving cells enter the development program, cell growth stops. This can be explained by a low TORC1 activity caused by the presence of CMF and consequently inhibited PldB activity. This decreases the GTPase activity of Gα2, allowing cAMP signaling to occur. Cells can then aggregate and develop.

Although how TOR controls cell growth in *D. discoideum* has not been clarified, mammalian TOR (mTOR) coordinates cell growth and proliferation by enabling mRNA translation through three different downstream signal pathways [103]. One is that mTOR
inhibits 4E binding protein 1 (4E-BP1), which then dissociates from eukaryotic initiation factor 4E (eIF4E), making eIF4F complex formation possible. mRNA translation will then be initiated. The second is that mTOR activates ribosomal protein S6 kinase 1 (S6K1), which then activates ribosomal protein S6 (rpS6), a component of 40S ribosome. mRNA translation will then occur. The third is that mTOR inhibits eukaryotic elongation factor 2 kinase (eEF2K). This keeps eEF2 active, which can mediate translation step of peptide-chain elongation. A similar process may occur in *D. discoideum*.

Increased mRNA translation as a result of up-regulated mTOR activity has been strongly suggested in the genesis of human cancers [95]. mTOR is activated by an upstream Ras homolog enriched in brain (Rheb) protein, which is a mediator of nutrient signaling. Rheb is inhibited by tuberous sclerosis complex (TSC1/2), which can be activated by AMP-activated protein kinase (AMPK), a mediator of energy homeostasis. TSC1/2 can also be inhibited by the growth factor insulin through PI3K/Akt pathway. Thus, the big picture is that nutrients, energy and growth factors have a combined regulation of mTOR and therefore mRNA translation. Mutations in tumor suppressors, such as PTEN (reverses the PI3K signaling), LKB1 (activates AMPK to sense ATP depletion) and TSC1/2, will result in constitutively activated mTOR. Since mTOR is required in cell cycle progression and has anti-apoptotic activity [104, 105], constitutive mTOR activity enables cancer cells to survive and proliferate.

Recently, phosphatidic acid, a product of PLD activity, has been reported to activate mTOR as a way for cancer cells to overcome normal cellular responses to stress to
continue proliferation [106]. Elevated PLD activity has been implicated in various human cancers [107]. Similarly, our findings suggest that PldB may activate TOR in the quorum sensing pathway of *D. discoideum*. This implies that the PLD regulation of mTOR is possibly part of a quorum sensing mechanism. When cell size and number are below the optimal level, PLD activity is high. mTOR is activated and mRNA translation occurs. Cell cycle progression occurs. When cells reach the appropriate size and number, PLD activity is low. mTOR is less activated and mRNA translation slows down. Cells may be arrested at G₁ phase and enter dormant G₀ phase, or undergo apoptosis as needed. For tumor or cancer cells, their compromised quorum sensing mechanisms either can not sense the number of cells present or mistakenly sense a suboptimal level at all times. These cells have constitutively high PLD activity, therefore constitutively activated mTOR. Thus, cells keep increasing in size and number and will not go into dormancy or apoptosis. Rapamycin competitively binds to the region of mTOR that interacts with phosphatidic acid, preventing PLD activation of mTOR [108]. Therefore rapamycin displays a tumor suppressing effect.

Overall, investigation of TOR, PLD and their upstream signals in mammalian quorum sensing mechanisms is a promising direction for future research. This includes finding out the subcellular localizations of these proteins and possible crosstalks with Rheb, TSC1/2 and PI3K/Akt pathways. Our work in *D. discoideum* reveals multiple physiological roles for PLD and beyond, providing a practical guide for research in mammalian systems, thus leading to new therapeutic designs for human cancers and other diseases.
REFERENCES


