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Published in:
Molecular Biology of the Cell

DOI:
10.1091/mbc.E07-05-0407

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Kortholt, A., King, J. S., Keizer-Gunnink, I., Harwood, A. J., & Van Haastert, P. J. M. (2007). Phospholipase C regulation of phosphatidylinositol 3,4,5-trisphosphate-mediated chemotaxis. Molecular Biology of the Cell, 18(12), 4772-4779. https://doi.org/10.1091/mbc.E07-05-0407

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Molecular Biology of the Cell
Vol. 18, 4772–4779, December 2007

Phospholipase C Regulation of Phosphatidylinositol 3,4,5-trisphosphate-mediated Chemotaxis[V]

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Submitted May 4, 2007; Revised August 6, 2007; Accepted September 17, 2007
Monitoring Editor: John York

Generation of a phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] gradient within the plasma membrane is important for cell polarization and chemotaxis in many eukaryotic cells. The gradient is produced by the combined activity of phosphatidylinositol 3-kinase (PI3K) to increase PI(3,4,5)P_3 on the membrane nearest the polarizing signal and PI(3,4,5)P_3 dephosphorylation by phosphatase and tensin homolog deleted on chromosome ten (PTEN) elsewhere. Common to both of these enzymes is the lipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], which is not only the substrate of PI3K and product of PTEN but also important for membrane binding of PTEN. Consequently, regulation of phospholipase C (PLC) activity, which hydrolyzes PI(4,5)P_2, could have important consequences for PI(3,4,5)P_3 localization. We investigate the role of PLC in PI(3,4,5)P_3-mediated chemotaxis in *Dictyostelium*. *plc*-null cells are resistant to the PI3K inhibitor LY294002 and produce little PI(3,4,5)P_3 after cAMP stimulation, as monitored by the PI(3,4,5)P_3-specific pleckstrin homology (PH)-domain of CRAC (PH_{CRAC}GFP). In contrast, PLC overexpression elevates PI(3,4,5)P_3 and impairs chemotaxis in a similar way to loss of *pten*. PI3K localization at the leading edge of *plc*-null cells is unaltered, but dissociation of PTEN from the membrane is strongly reduced in both gradient and uniform stimulation with cAMP. These results indicate that local activation of PLC can control PTEN localization and suggest a novel mechanism to regulate the internal PI(3,4,5)P_3 gradient.

INTRODUCTION

Chemotaxis, or migration toward a concentration gradient of chemoattractant, is an essential response of many cells. It plays an important role in a multitude of biological processes and organisms, such as finding nutrients in prokaryotes, forming multicellular structures in prokaryotes, and tracking bacterial infections in neutrophils (Baggiolini, 1998; Campbell and Butcher, 2000; Crone and Lee, 2002). Chemotaxis is a complex cellular process involving several signaling pathways and molecules. The initial event is binding of chemoattractant to cell surface receptors. Receptors convert these signals to the interior of the cell were they activate a complex network of signaling pathways, resulting in a gradient of cellular components. This gradient induces coordinated remodeling of the cytoskeleton and cell adhesion to the substratum, which leads to formation of new actin filaments in the front that induce the formation or stabilization of local pseudopodia, and acto-myosin filaments at the back that inhibit pseudopod formation and retract the uropod (Van Haastert and Devreotes, 2004; Andrew and Insall, 2007). The final outcome is cellular movement up the chemoattractant gradient.

Dictyostelium cells are single-celled amoeba that feed on bacteria. On starvation, cells undergo a tightly regulated developmental process in which they secrete and chemotax toward cAMP, resulting in multicellular fruiting bodies. Because the mechanism of chemotaxis is essentially identical in all eukaryotes, *Dictyostelium* offers a genetically tractable model system in which to study chemotaxis (Devreotes and Zigmond, 1988; Van Haastert and Devreotes, 2004). An important response in both the establishment of cell polarity and chemotaxis is the formation and accumulation of phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P_3] at the leading edge (Parent *et al*., 1998; Funamoto *et al*., 2002; Huang *et al*., 2003). PI(3,4,5)P_3 is produced by the phosphorylation of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] by phosphatidylinositol 3-kinases (PI3Ks), and it is reversed by the inositol 3-phosphatase action of PTEN (Parent *et al*., 1998; Funamoto *et al*., 2002; Huang *et al*., 2003). A PI(3,4,5)P_3 gradient is accomplished by the reciprocal temporal and spatial regulation of PI3K at the leading edge and phosphatase and tensin homolog deleted on chromosome ten (PTEN) at the back of the cell. The accumulation of PI(3,4,5)P_3 at the leading edge results in recruitment of PI(3,4,5)P_3 binding signaling molecules and subsequent pseudopod extension (Parent *et al*., 1998; Funamoto *et al*., 2002; Iijima and Devreotes, 2002).

It has long been known that an early event after cAMP stimulation is the hydrolysis of PI(4,5)P_2 by phospholipase C (PLC) to form two important second messengers, diacylglyc-

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–05–0407) on September 26, 2007.

[V] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

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erol (DAG) and inositol-1,4,5-triphosphate [I(1,4,5)P_3] (Katan, 1998). *Dictyostelium* contains a single *plc* gene, structurally similar to mammalian PLC-δ and consists of N-terminal PH and EF hand domains followed by a split catalytic domain (all eukaryotic PLC enzymes have an insertion in the catalytic domain) and a C-terminal conserved C2 domain (Drayer and van Haastert, 1992; van Haastert and Van Dijken, 1997). The enzyme is Ca^{2+} dependent and upon cAMP stimulation PLC activity is increased approximately twofold. cAMP both stimulates and inhibits PLC activity via $G\alpha_2$ and $G\alpha_1$ G protein subunits, respectively (Bominaar and van Haastert, 1994; Bominaar *et al*., 1994; Drayer *et al*., 1995). Previously, a *plc*-null mutant was generated, and although the *plc*-null mutant had no measurable PLC activity, growth, development and chemotaxis seemed normal (Drayer *et al*., 1994). Surprisingly, *plc*-null cells also contain normal levels of I(1,4,5)P_3 generated by a second route of synthesis via a I(1,3,4,5,6)P_5 3/6-biphosphatase that is up-regulated in *plc*-null cells (Van Dijken *et al*., 1997). Previous work has generally concentrated on the roles of the produced second messengers I(1,4,5)P_3 and DAG and the observation that PLC seemed to play no role in chemotaxis was reenforced by mutation of the inositol triphosphate receptor iplA, which also had no apparent phenotype (Traynor *et al*., 2000). Here, because PI(4,5)P_2 is also the substrate of PI3K at the leading edge and it is important for the membrane localization of PTEN in the rear of the cell, we investigate an alternative role for PLC in the regulation of PI(3,4,5)P_3-mediated chemotaxis.

Although *pten*-null mutants have a relatively strong phenotype in which they form multiple pseudopdia, and they are defective in chemotaxis (Iijima and Devreotes, 2002), loss of PI3K activity through gene knockout or use of the PI3K inhibitor LY294002 has only mild phenotypic effects, demonstrating that the PI(3,4,5)P_3 is not essential for direction sensing, or cell movement (Loovers *et al*., 2006; Hoeller and Kay, 2007; Takeda *et al*., 2007). This suggests that PI(3,4,5)P_3-mediated signaling works in conjunction with one or more other pathways to mediate chemotaxis and that these pathways can compensate for each other under standard conditions. Recent studies have suggested that this second, parallel pathway may be mediated by phospholipase A_2 (Chen *et al*., 2007; van Haastert *et al*., 2007). Chen *et al*. (2007) described that loss of the Ca^{2+}-independent phospholipase A_2 (iPLA2) homologue *plaA* results in cells that are hypersensitive to LY294002 and that show dramatically reduced chemotaxis (Chen *et al*., 2007). Van Haastert *et al*. (2007) showed that inhibition of either PI3K or PLA2 has minor effects on chemotaxis, whereas inhibition of both enzymes inhibits chemotaxis nearly completely (van Haastert *et al*., 2007). Furthermore, they showed that inhibition of PLA2 completely blocks chemotaxis in *plc*-null cells, whereas inhibition of PI3K has no effect (van Haastert *et al*., 2007). To investigate whether the presence of compensatory signaling processes may explain the apparent lack of phenotype in the *plc*-null mutants, we investigated the role of PLC in PI(3,4,5)P_3-mediated signaling. We show here that although *plc*-null cells have normal chemotaxis, they are resistant to the effects of the PI3K inhibitor LY294002. We further show that this does not arise through up-regulation of PI3K signaling, but in fact it is accompanied by a dramatic reduction. We investigated the mechanism for this change, and we demonstrate that PLC activity regulates the localization of PTEN, and it is important for the control of the PI(3,4,5)P_3 gradient along the plasma membrane.

MATERIALS AND METHODS

Cell Culture

To avoid effects due to specific parental strain backgrounds different *plc*-null mutant strains were used in subsequent studies. The first strain 1.19 was that described previously (Drayer *et al*., 1994), and it is in the DH1 strain background. The other strains, referred to as HAD236 and HAD237, were newly generated in the AX2 strain background as described below. All experiments were carried out with the appropriate strain controls. All cell lines were grown in 9-cm dishes containing HG5 medium (14.3g/l peptone, 7.15 g/l glucose, 0.49 g/l KH_2PO_4, and 1.36 g/l $Na_2HPO_4{\cdot}H_2O$). To select for transformants with one of the extrachromosomal plasmids described below, cells were grown in HG-5 supplemented with 10 μg/ml Geneticin (G-418; Invitrogen, Carlsbad, CA), hygromycin B (Invitrogen), or both. For development on filters, log-phase growing cells were washed twice in KK2 buffer (10 mM 16.5 mM KH_2PO_4, and 3.8 mM K_2HPO_4 pH 6.2), serially diluted to the appropriate densities, and then spread onto 47 mm nitrocellulose filters (Millipore, Billerica, MA) presoaked in KK2. Images were taken at 20 h.

Plasmid Construction

The plasmid pWF38 expressing the pleckstrin homology (PH) domain of CRAC fused to enhanced green fluorescent protein (GFP) (Parent *et al*., 1998), and the plasmid expressing PTEN-GFP were kindly provided by Dr. Peter Devreotes (Iijima and Devreotes, 2002). To express PLC in *Dictyostelium*, the encoding DNA fragment was amplified using the forward primer: 5′-GAAGATCTAAAATGGATACTTTAACAAATTC-3′ and reverse primer 5′-CTTCTAGATTCAACAAATGTAAATTTAC-3′. The BglII/XbaI fragment was cloned in the BglII-SpeI site of a modified vector that contains a hygromycin instead of a neomycin resistance (Bosgraaf *et al*., 2005). This yielded the plasmid pDm133PLC.

Generation of an AX2-derived plc Knockout

A new *plc* knockout construct was made by polymerase chain reaction (PCR), and it was used to generate AX2 cells containing a 100-bp deletion (from 199 to 298 base pairs of the genomic sequence) at the *plc* locus, with insertion of the loxP-flanked blasticidin cassette from pBLPBSR (Faix *et al*., 2001). Successful recombination was screened by PCR, and the loss of protein was confirmed by Western blot by using a PLC-specific antibody (Drayer *et al*., 1994; data not shown).

Chemotaxis

Chemotaxis was measured with the small population assay (Konijn, 1970). Experiments were performed in a six-well plate containing 1 ml of nonnutrient hydrophobic agar (11 mM KH_2PO_4, 2.8 mM Na_2HPO_4, 7 g/l hydrophobic agar). AX3 and *plc*-null cells were harvested by centrifugation for 3 min at 300 g, washed in phosphate buffer (PB) (10 mM KH_2PO_4/Na_2HPO_4), and starved in PB buffer for 5 h. Starved cells were resuspended in PB, washed once in PB, and resuspended in PB to a final concentration of 6×10^6 cells/ml. Droplets of ~0.1 μl of starved cells were placed on agar. Chemotaxis toward cAMP was tested after 30 min by placing a second 0.1-μl droplet, with the indicated amount of cAMP, next to the droplet of cells. The distribution of the cells in the droplet was observed about every 10 min during 90 min, and they were scored positive when at least twice as many cells are pressed against the side of the population closer to higher cAMP concentration as against the other side of the droplet. The fraction of droplets scored positive, averaged over three successive observations at and around the moment of the maximal response, is recorded here. The data presented are the means and SE of the means of at least three independent measurements on different days.

Chemotaxis was also measured with micropipettes containing 10^{-4} M cAMP with a Zeiss LSM 510 META NLO confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss plan-apochromatic 63× numerical aperture 1.4 objective. The chemotaxis index defined as the ratio of the cell displacement in the direction of the gradient, and its total traveled distance was determined for ~25 cells in a movie as follows. First, the position of the centroid was determined with ImageJ (rsb.info.nih.gov/ij) for frames at 30-s interval, yielding a series of coordinates for that cell. Using these coordinates, the chemotaxis index of each 30-s step was calculated and averaged, yielding the chemotaxis index for that cell in the movie. The data shown are the average and SD of the mean of the chemotaxis indices from at least three independent experiments with ~25 cells per experiment.

Localization of GFP-tagged Proteins during Chemotactic Stimulation

The same experimental setup with micropipettes was used for analyses of cells expressing PHcrac-GFP, PI3K-GFP, and PTEN-GFP. For excitation of the fluorochrome GFP (S65T variant) a 488-nm argon/krypton laser was used, and the fluorescence was detected by a BP500-530 IR LP560 photo multiplier tube. The field of observation is 206 × 206 μm.

To determine the localization of marker proteins during chemotaxis, we define the front of the cell as the foremost point of the cell in the direction of

movement toward the pipette. Then, the cell was divided into three equal regions (front, middle, and rear region) by using two lines perpendicular to the direction of movement. For analysis of the fluorescence intensity of cytosolic PI3K-GFP, the mean fluorescence intensity of the cytosol in the front one-third region was compared with the fluorescence intensity of the cytosol in the middle-third region of the same cell, taking care to exclude the nucleus (Postma *et al*., 2003). For analysis of the fluorescence intensity of membrane-associated PTEN-GFP, the fluorescence intensity at the boundary of the cell was determined using a three-pixel wide boundary essentially as described previously (Postma *et al*., 2003). The mean fluorescence intensity of the boundary in front one-third region of the cell was compared with the mean fluorescence intensity of the boundary in the remaining third thirds (middle and rear part) of the cell. Data were averaged over ~20 images of the same cell moving toward a micropipette with cAMP. The data presented are the means and standard deviations of this value for 10 cells.

Localization of GFP-tagged Proteins during Uniform Stimulation

Before stimulation, cells were washed and resuspended at 5×10^6 cells/ml in KK2 buffer, and they were given 6-min pulses of 100 nM cAMP for 5 h. Cells were then transferred into a final volume of 1 ml in four-well Lab-Tek chambered coverslips (Nalge Nunc International, Rochester, NY) and left 5 min to adhere. Cells were then stimulated with 1 μM cAMP by the addition of 50 μl of a 20 μM stock solution. For analysis of the translocation of GFP-fusion proteins, movies were taken using an IX71 inverted fluorescence microscope (Olympus, Tokyo, Japan). Images were taken at 2-s intervals by using a 60× objective. Membrane association of GFP-fusion proteins was measured indirectly by measuring changes in the fluorescence intensity of the cytoplasm relative to prestimulated levels in that cell. This was done using the ImageJ software, by selecting oval cytosolic sections within each cell and calculating the mean intensity at each frame. For each experiment, a minimum of 20 cells was measured in this way, over four independent simulations.

Protein Kinase B (PKB)/Akt Phosphorylation Assays

Before stimulation, cells were pulsed with cAMP for 5 h, with 100 μM LY294002 added for the last hour when required. Cells were then washed twice, resuspended at 2×10^7 cells/ml in KK2 (including LY294002 if appropriate), and left for 10 min with gentle agitation. Cell suspensions were then stimulated with a final concentration of 1 μM cAMP, and 100-μl were samples removed and lysed directly into an equal volume of 2× LDS gel loading buffer (Invitrogen) supplemented with 300 mM NaF, 1.2 mM Na_3VO_4, and 12 mM EDTA. Samples were then fractionated on 3–8% NuPage Tris-acetate gels (Invitrogen), blotted onto nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, United Kingdom), and probed using an anti-phosphothreonine antibody (Cell Signaling Technologies, Beverly, MA; catalog no. 9381).

RESULTS

Chemotaxis of PLC-Null Cells Is Resistant to the PI3K Inhibitor LY294002

Previously, we used the small population assay (Konijn, 1970; van Haastert *et al*., 2007) to measure the effect of different concentrations of LY294002 on the chemotactic activity of several mutants toward different concentrations of cAMP (Postma *et al*., 2004; Loovers *et al*., 2006). In this assay, small droplets containing wild-type cells were placed on hydrophobic agar very close to another small drop of chemoattractant, setting up a gradient. Cells can freely move within the boundary of the droplet, but they cannot move out of the droplet. Therefore, any directional movement of the cells leads to the accumulation of cells at the boundary of the small population, which is easily and rapidly scored. Any defects in the ability of the cells to either detect, or respond to, the chemoattractant will therefore result in less cells accumulating at the boundary. Figure 1 shows that in wild-type cells, low concentrations of cAMP induce only a weak chemotactic response in a small fraction of the populations, whereas 1000 nM cAMP induces a strong chemotaxis response in 90–100% of the populations. Disruption of the *plc* gene does not influence chemotaxis in this assay, at any cAMP concentration, consistent with previously described experiments (Drayer *et al*., 1994). To investigate PI3K signaling in these cells, both wild-type and *plc*-null cells were incubated with the PI3K inhibitor LY294002 at a concentration of 50 μM. The concentration of drugs used was obtained from published dose–response curves (Loovers *et al*., 2006; van Haastert *et al*., 2007). In wild-type cells, inhibition of PI3K results in a strong reduction of chemotaxis at low cAMP concentrations, whereas at high concentrations of cAMP this effect is greatly reduced, indicating that the effect of LY294002 is specific and not harmful to the cells. Furthermore, it shows that PI3K activity is important but not essential for chemotaxis of wild-type cells (Iijima and Devreotes, 2002; Funamoto *et al*., 2002; Postma *et al*., 2004; Loovers *et al*., 2006). In contrast, the chemotaxis of *plc*-null cells was resistant to LY294002 treatment at all cAMP concentrations.

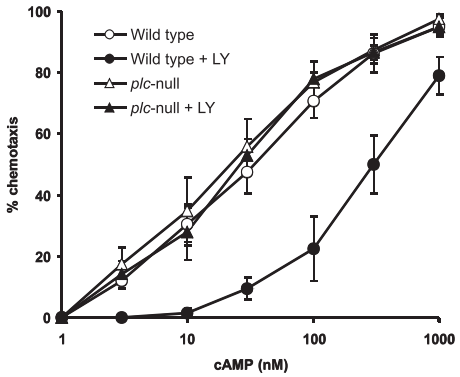

Figure 1. Effect of the PI3K inhibitor LY294002 on chemotaxis of AX3 and plc-null cells. Chemotaxis of wild-type (open circle) and plc-null (open triangle) cells was measured to different concentration of cAMP. To investigate the role of PI3K in chemotaxis, both wild-type (closed circle) and *plc*-null (closed triangle) cells were incubated with the PI3K inhibitor LY294002 at a concentration of 50 μM. The results show the means and SE of the means of three independent experiments.

PLC Regulates cAMP-dependent PI(3,4,5)P_3 Formation

The loss of LY294002 sensitivity in the *plc*-null cells could arise through two alternative mechanisms: either a large up-regulation of PI(3,4,5)P_3 synthesis or the up-regulation of a compensatory pathway. To test these alternatives, both wild-type and *plc*-null cells were transformed with the PI(3,4,5)P_3 sensor PH_{CRAC}GFP, and cells were stimulated with cAMP (Parent *et al*., 1998). To avoid effects due to specific parental strain backgrounds, different *plc*-null mutant strains were used in subsequent studies. The first strain 1.19 was that described previously (Drayer *et al*., 1994), and it is in the DH1 strain background. The other strains, referred to as HAD236 and HAD237, were newly generated in the AX2 strain background. All experiments were carried out with the appropriate strain controls. Without cAMP stimulation, both wild-type– and *plc*-null PH_{CRAC}GFP-expressing cells moved in random directions, and they showed an evenly distributed PH_{CRAC}GFP localization in the cytosol. As seen in previous investigations (Parent *et al*., 1998; Funamoto *et al*., 2002; Huang *et al*., 2003), introduction of a cAMP-filled pipette to one side of wild-type cells induced a strong translocation of PH_{CRAC}GFP from the cytosol to the membrane facing the cAMP source (Figure 2A, see Supplemental Movie 1). Pseudopodia then extended from PH_{CRAC}GFP-containing areas of the plasma membrane, and cells moved persistently toward the pipette. Although *plc*-null cells extend pseudopodia and move toward the pipette, PH_{CRAC}GFP localization at the leading edge was dramatically reduced and most fluorescence remained cytosolic (Figure 2A, see Supplemental Movie 2). In addition, when cells were globally stimulated with 1 μM cAMP, PH_{CRAC}GFP

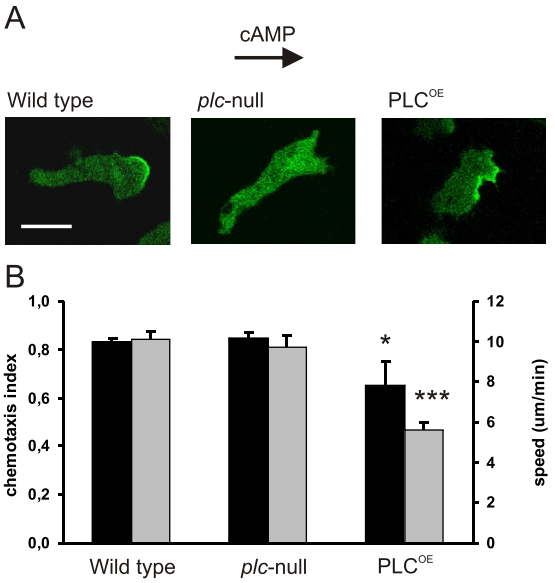

Figure 2. PLC regulates cAMP-mediated PI(3,4,5)P_3 formation during chemotaxis. To investigate the effect of PLC on PI(3,4,5)P_3 levels, the PI(3,4,5)P_3 detector PHcracGFP was expressed in wild-type, *plc*-null, and PLC^{OE} cells. (A) Confocal images are shown for cells stimulated with a micropipette, containing 10^{-4} cAMP from the right. The figures show a representative cell for each case. Bar, 10 μm. (B) Chemotactic properties of cells stimulated with a micropipette, containing 10^{-4} cAMP. The chemotaxis index (black bar) and speed (gray bar) were calculated from three independent movies; data shown are the mean ± SD of the mean (the difference between control and PLC^{OE} is significant for chemotaxis, *$p < 0.05$ and for speed, ***$p < 0.001$, *t* test).

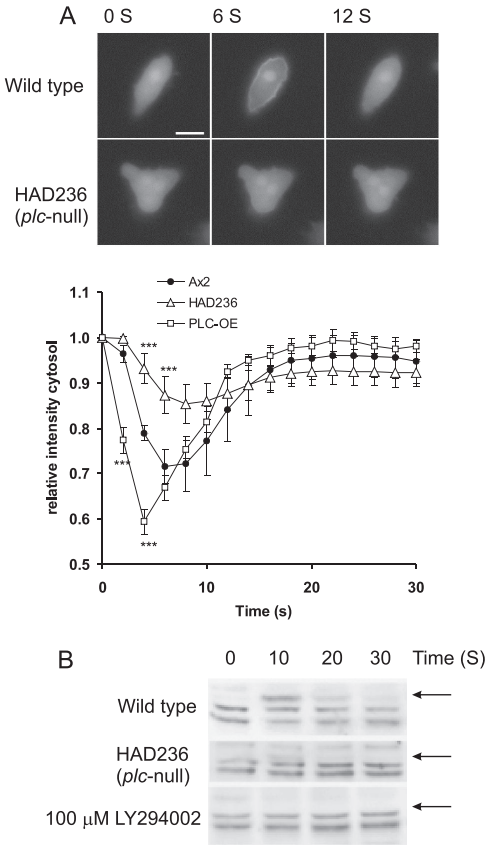

Figure 3. PLC regulates cAMP-mediated PI(3,4,5)P_3 signaling. (A) Membrane translocation of PH_{CRAC}GFP in AX2, HAD236 (*plc*-null), and PLC^{OE} cells after uniform stimulation with 1 μM cAMP. Bar, 10 μm. Quantitation of PH_{CRAC}GFP in the cytosol is shown in the bottom. Values are the mean of three independent experiments. Error bars represent SD (the difference between AX2 and *plc*-null or between AX2 and PLC^{OE} is significant for ***$p < 0.005$). (B) PKB phosphorylation in wild-type and *plc*-null cells. Cells were cAMP pulsed, and then they simulated with 1 μM cAMP. Samples were removed at the times indicated and lysed directly into SDS gel loading buffer. Extracts were then fractionated by SDS-PAGE and analyzed by Western blotting probing with a phospho-threonine–specific antibody. The arrows indicate a protein of ~60 kDa that in wild type was rapidly induced in response to stimulation, indicating, corresponding to the phosphorylation of PKB/Akt, described by others (Lim *et al.*, 2005). This phosphorylation was blocked by pretreatment with the PI3K inhibitor LY294002, and it was strongly reduced in *plc*-null cells. The blot shown is representative of three independent experiments.

was rapidly recruited to the plasma membrane in wild-type cells, an effect that was again significantly reduced in *plc*-null cells (Figure 3A; $p < 0.005$, *t* test).

In *Dictyostelium*, the activity of Akt/PKB is transiently stimulated in response to cAMP, in a PI(3,4,5)P_3-dependent manner, peaking at 10 s after simulation (Meili *et al.*, 1999; Lim *et al.*, 2005). *Dictyostelium* Akt/PKB is activated by phosphorylation at conserved threonine residues in the kinase domain and C terminus (Meili *et al.*, 1999), which can be detected as a 51-kDa protein on a Western blot by using a phospho-threonine–specific antibody (Lim *et al.*, 2005). Wild-type and *plc*-null cells were stimulated with cAMP, and cell lysates were analyzed by Western blotting. In lysates from wild-type cells, a protein of 51 kDa was transiently phosphorylated, peaking at 10 s after cAMP stimulation (Figure 3B). In *plc*-null cells, this response was dramatically reduced, and it was undetectable in wild-type cells treated with LY294002 (Figure 3B).

These two experiments both indicate that *plc*-null cells have substantially reduced accumulation of PI(3,4,5)P_3, and they support the second hypothesis that LY294002 resistance arises through a loss of PI(3,4,5)P_3 signaling and subsequent up-regulation of a compensatory signal pathway. Furthermore, they suggest a role for PLC as regulator of the PI(3,4,5)P_3 signaling in wild-type cells.

Enhanced Levels of PI(3,4,5)P_3 in Cells Overexpressing PLC Lead to Chemotaxis Defects

Based on the hypothesis that PLC activity is required for cAMP-stimulated PI(3,4,5)P_3 accumulation, we would predict that cells overexpressing PLC may have an increased PI(3,4,5)P_3 response after cAMP stimulation. To investigate this hypothesis, we constructed a PLC expression plasmid containing a hygromycin-resistance cassette and transfected it into *plc*$^-$/PH_{CRAC}GFP cells. In contrast to wild-type and *plc*-null cells, which maintain a persistently focused leading edge in the direction of a micropipette filled with cAMP, PLC^{OE} cells have a much broader and irregular front (Figure 2A), a significantly decreased cell speed ($p < 0.001$, *t* test), and they follow a less directional path toward the pipette ($p < 0.05$, *t* test; Figure 2B).

To examine whether the chemotaxis defect of PLC^{OE} cells is due to altered PI(3,4,5)P_3 signaling, we examined the distribution of PH_{CRAC}GFP in PLC^{OE} cells. Nonstimulated, randomly moving PLC^{OE} cells have an evenly distributed cytosolic PH_{CRAC}GFP localization, similar to that of wild-type and *plc*-null cells (Figure 2A). However, in the presence

of a cAMP gradient, many cells exhibit a much broader region along the leading edge that is associated with $PH_{CRAC}GFP$. In wild-type cells, an average of 16.5 ± 3.5% (n = 12) of the perimeter was covered by $PH_{CRAC}GFP$, whereas in PLC^{OE} 29.3 ± 4.8% (n = 11) was occupied. Consistently, when PLC^{OE} cells were globally stimulated with 1 μM cAMP, the amount of $PH_{CRAC}GFP$ recruited to the plasma membrane occurred much faster, and it was significantly more compared with that in wild-type and *plc*-null cells (Figure 3A). The depletion was 15, 28, and 41% in *plc*-null, wild-type, and PLC^{OE} cells, and it was maximal at 8, 6, and 4 s after stimulation, respectively. From these data, we conclude that PLC activation regulates $PI(3,4,5)P_3$ signaling and that the elevated levels of $PI(3,4,5)P_3$ in PLC^{OE} cells lead to chemotactic defects.

PLC Regulates the Localization of PTEN

To investigate the mechanism by which PLC regulates cAMP-mediated $PI(3,4,5)P_3$ accumulation, the distribution of PI3K-GFP and PTEN-GFP was analyzed during chemotactic stimulation. In nonstimulated, wild-type cells, PI3K is mainly localized in the cytosol, whereas introduction of a pipette filled with cAMP results in the transient localization of PI3K-GFP at the front of the cell (Figure 4A; Funamoto *et al*., 2002). As shown in Figure 4A, the distribution of PI3K-GFP in *plc*-null during chemotaxis is similar to that of wild-type cells. We have quantified the fluorescence intensity of PI3K-GFP in the cytoplasm in the front of the cell relative to the middle of the cell, which is 192 ± 21% in wild-type and 172 ± 12% in *plc*-null cells (mean and SD of 10 cells; the difference between wild-type and *plc*-null cells is not significant by *t* test). These results show that although *plc*-null cells have defective $PI(3,4,5)P_3$ signaling, PI3K still localizes at the leading edge of chemotaxing cells.

We next examined the distribution of PTEN-GFP in wild-type and *plc*-null cells. Consistent with previously described results (Funamoto *et al*., 2002; Iijima and Devreotes, 2002), in chemotaxing wild-type cells PTEN localizes at the plasma membrane along the sides and posterior of the cell, but it is absent at the leading edge (Figure 4A). In contrast, chemotaxing *plc*-null cells show a more uniformly distributed PTEN-GFP localization at the membrane (Figure 3A). The fluorescence intensity of PTEN-GFP at the boundary of the cell was quantified and expressed as the mean value of the front one third of the cell relative to the rear two thirds of the cell, which is 77.4 ± 3.4% in wild-type and 96.5 ± 3.6% in *plc*-null cells.

When wild-type cells were uniformly stimulated with cAMP, PTEN-GFP was rapidly dissociated from the plasma membrane. However, when *plc*-null cells were stimulated with uniform cAMP, PTEN translocation was dramatically reduced (Figure 4B; $p < 0.01$). These results indicate a mislocalization of PTEN, and they suggest that this may explain the reduced $PI(3,4,5)P_3$ signaling in *plc*-null cells and the regulatory role for PLC in the localization of PTEN.

plc-Null Cells Are Unable to Aggregate at Low Density

Although *plc*-null cells have no detectable deficiency in general chemotaxis assays by using a static, single cAMP signal, the impaired Akt/PKB phosphorylation and inability of these cells to effectively induce the recruitment of $PH_{CRAC}GFP$ and presumably other $PI(3,4,5)P_3$-binding proteins to the plasma membrane may still have significant effects during aggregation. Indeed, *pi3k1/2*-null and *pkbA*-null cells exhibit aggregation defects that can be detected at low cell densities (Zhou *et al*., 1998; Meili *et al*., 1999). We therefore investigated whether *plc*-null cells posses a defect in cell aggregation by plating cells at different cell densities. When cells were allowed to develop naturally on nitrocellulose filters, although they were identical to wild-type at high density, *plc*-null cells were unable to aggregate at low density, when wild-type controls could (Fig. 5). Thus, *plc*-null and *pkbA*-null cells exhibit aggregation defects despite having normal cAMP relay (Drayer *et al*., 1994; Meili *et al*., 1999), suggesting that although intact $PI(3,4,5)P_3$ signaling may not be essential for chemotaxis in a static gradient, it plays a physiological role in the more complex conditions that occur during aggregation.

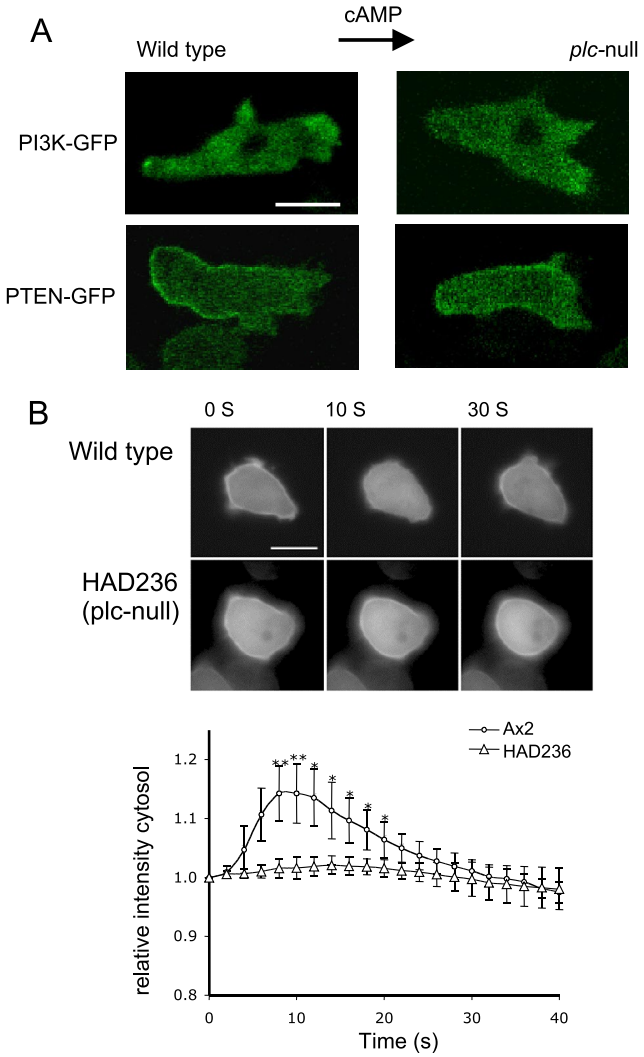

Figure 4. Distribution of PI3K-GFP and PTEN-GFP in AX3 and plc-*null cells*. To investigate the mechanism by which PLC regulates cAMP mediated $PI(3,4,5)P_3$ accumulation, the distribution of PI3K-GFP and PTEN-GFP were analyzed during chemotactic stimulation. (A) Confocal images are shown for AX3 and *plc*-null cells expressing PI3K-GFP (top) and PTEN-GFP (bottom). Cells were stimulated with cAMP by a pipette that is positioned at the right. Bar, 10 μm. (B) Translocation of PTEN-GFP in Ax2 and HAD236 (*plc*-null) cells after uniform stimulation with 1 μM cAMP. Quantitation of the translocation of PTEN-GFP into the cytosol upon uniform cAMP stimulation is shown (bottom). Values are the mean of three independent experiments, and error bars represent SD (*$p < 0.05$, **$p < 0.01$, *t* test).

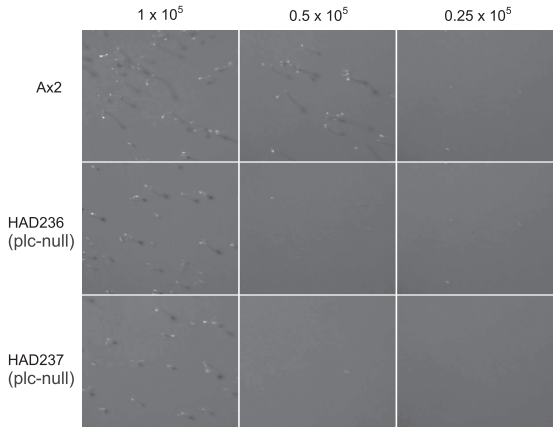

Figure 5. *plc*-null cells are defective in aggregation at low cell density. Wild-type (Ax2) and two independent *plc*-null clones were allowed to develop on nitrocellulose filters at different cell densities (cells per square centimeter). Images were taken after 20 h, and they are representative of multiple experiments.

DISCUSSION

A Model for PLC-regulated PI(3,4,5)P_3 Chemotaxis in Dictyostelium

These experiments reveal that PLC is an important regulator of PI3K-mediated chemotaxis. Examination of the distribution of the PI(3,4,5)P_3 sensor PH_{CRAC}GFP revealed that the cAMP-mediated PI(3,4,5)P_3 response is strongly decreased in *plc*-null cells and enhanced in PLC^{OE} cells. Consistently, the PKB/Akt response in *plc*-null cells is substantially reduced compared with wild type. However, both the PHcrac and PKB responses are still detectable at a low level, suggesting that the effect of *plc*-deletion is only partial, and this may explain why *plc*-null cells do not share the defects in growth, development, pinocytosis, and chemotaxis observed in *pi3k*-null cells (Zhou *et al*., 1995; Zhou *et al*., 1998; Funamoto *et al*., 2001). Consistently, a recent study showed that upon decreasing level of functional PI3K in the cell, the chemotaxis phenotypes become more severe (Takeda *et al*., 2007).

Another explanation for the apparent lack of chemotactic defects in *plc*-null mutants would be the up-regulation of a second compensatory pathway. Previously, it has been shown that *plc*-null cells contain normal levels of I(1,4,5)P_3 generated by the up-regulation of a second route of synthesis via a I(1,3,4,5,6)P_5 3/6-biphosphatase (Van Dijken *et al*., 1997). Recent studies have suggested that besides the PI(3,4,5)P_3 pathway, a second, parallel pathway exists that mediates chemotaxis (Chen *et al*., 2007; van Haastert *et al*., 2007). Furthermore, van Haastert *et al*. (2007) showed that chemotaxis in *plc*-null cells is sensitive to PLA2 inhibitors; therefore, the lack of chemotaxis defects and loss of LY294002 sensitivity in *plc*-null cells could be explained by the down-regulation of PI(3,4,5)P_3 synthesis, accompanied by the up-regulation of compensatory pathways.

Generation of a spatial PI(3,4,5)P_3 signal is regulated by both its synthesis by PI3K and degradation by PTEN. In wild-type cells, upon stimulation, PTEN is removed from the plasma membrane at the leading edge, and it is permissive for the accumulation of PI(3,4,5)P_3 from cAMP-activated PI3K there (Iijima and Devreotes, 2002). Here, we show that in *plc*-null cells, this dissociation is strongly reduced in cells stimulated with either uniform cAMP or within a gradient. Because PI3K is still able to localize to the leading edge in these cells, we propose that the reduced PI(3,4,5)P_3 accumulation in *plc*-null cells is due to retention of PTEN at the plasma membrane rather than reduced PI(3,4,5)P_3 synthesis. This mechanism also would explain the chemotaxis defects seen when PLC is overexpressed, because extended delocalization of PTEN would give a phenotype reminiscent of *pten*-null cells. Both *pten*-null and PLC-overexpressing cells exhibit reduced chemotaxis and elevated phosphatidylinositol (3,4,5)-trisphosphate levels at a broad front with many protrusions.

The exact mechanism of PTEN binding to the plasma membrane remains unknown, but it involves a number of protein–lipid and protein–protein interactions, and the translocation from the cytosol to the plasma membrane is regulated by several mechanisms, including C-terminal phosphorylation (Vazquez *et al*., 2000; Vazquez and Devreotes, 2006). It has been demonstrated that PTEN binds PI(4,5)P_2 and that this binding is essential for membrane localization and PI(3,4,5)P_3-degrading activity (Iijima *et al*., 2004). Studies in mammalian cells have indeed indicated that it is possible to translocate PI(4,5)P_2-specific PH domains into the cytosol upon stimulation and that this is blocked by PLC inhibitors, but not by PI3K inhibitors (Stauffer *et al*., 1998). Others have shown that calcium-activation of PLCs is also able to significantly deplete PI(4,5)P_2 (Varnai and Balla, 1998), and in *Dictyostelium*, the PI(4,5)P_2-binding domain of Phg2 is displaced during macropinocytosis and phagocytosis (Blanc *et al*., 2005). Therefore, the most direct explanation for the regulation of PTEN localization by PLC is by the depletion of PI(4,5,)P_2, and we propose that cAMP activation not only stimulates PI3K but also PLC, leading to a local decrease in PI(4,5)P_2 and dissociation of PTEN. Indeed, recent work by Vazquez and Devreotes (2006) has suggested that PTEN has a low affinity for the membrane; therefore, the combined actions of PLC and PI3K may be able to reduce PI(4,5)P_2 sufficiently to stop PTEN binding, while retaining enough substrate for PI3K to generate PI(3,4,5)P_3. For the detailed understanding of our model and the role for PI(4,5)P_2 as a local signal, techniques to study PI(4,5)P_2 levels with spatial and temporal resolution in single cells are required. Unfortunately, approaches to visualize PI(4,5)P_2 in *Dictyostelium* cells by expression of different constructs of known PI(4,5)P_2-binding proteins have failed (data not shown).

Our data provide new insight into the regulation of PTEN localization, and we propose a simple model for the PLC regulation of PI(3,4,5)P_3-mediated chemotaxis (Figure 6); PLC is regulated via the activating $G\alpha_2$ and inhibitory $G\alpha_1$ (Bominaar and van Haastert, 1994; Bominaar *et al*., 1994; Drayer *et al*., 1995; Keizer-Gunnink *et al*., 2007). cAMP binding to the cAR1 receptor leads to the activation of $G\alpha_2$ and then PLC upgradient of the cell. Activation of PLC results in the degradation of PI(4,5)P_2 at the leading edge and removal of PTEN from the membrane. The resulting gradient of PTEN localization and activation mediates an inverse PI(3,4,5)P_3 gradient, which leads to localized actin polymerization and pseudopod extension from the leading edge. The PI(4,5)P_2/PI(3,4,5)P_3 gradient is stabilized, because both PI3K and PTEN are localized at sites of their products [PI3K at PI(3,4,5)P_3-induced F-actin, and PTEN at PI(4,5)P_2, respectively]. This mutually spatial exclusion of PI3K and PTEN will result in symmetry breaking, by which small spatial differences in the underlying polarity gradient can be amplified to the observed strong PI(3,4,5)P_3 gradient. In accordance with our model, *plc*-null cells, which have stabilized PI(4,5)P_2 levels, have more PTEN at the membrane and therefore less PI(3,4,5)P_3 formation.

Recent observations in *Dictyostelium* with the antagonist 8CPT-cAMP show the importance of the regulatory role of

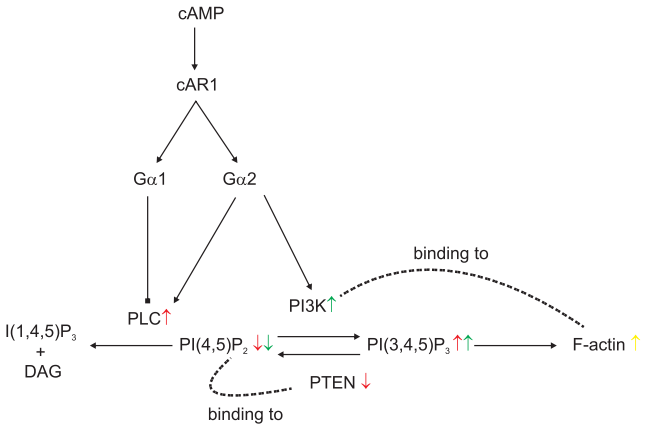

Figure 6. Model of PLC-mediated PI(3,4,5)P_3 formation at the leading edge of chemotaxing cells. The model contains of two regulatory loops: first, a PLC-regulated PI(4,5)P_2/PTEN loop (indicated in red) inhibiting PI(3,4,5)P_3 degradation; and second, a PI3K/F-actin loop (indicated in green) providing PI(3,4,5)P_3 formation and pseudopod extension. cAMP binding to the cAR1 receptor leads to the activation of $G\alpha_2$ and subsequently in activation of PLC at the leading edge of the cell. Activation of PLC results in the degradation of PI(4,5)P_2 at the leading edge and translocation of PTEN to the rear of the cell. The resulting gradient of PI(4,5)P_2/PTEN mediates an opposite PI(3,4,5)P_3 gradient. PI3K and PTEN are localized at sites of their effector; hence, PI(3,4,5)P_3 induced F-actin and PI(4,5)P_2, providing stabilization of the gradient and pseudopod extension from the leading edge.

PLC in PI(3,4,5)P_3-mediated chemotaxis (Keizer-Gunnink *et al*., 2007). 8CPT-cAMP inhibits PLC through $G\alpha_1$. Consistent with our model, inhibition of PLC by 8CPT-cAMP at the leading edge will lead to higher levels of PI(4,5)P_2 and subsequently more membrane-bound PTEN upgradient. The resulting gradient of PI(4,5)P_2 and PTEN will mediate an opposite gradient of PI(3,4,5)P_3; therefore, the cells move downgradient and 8CPT-cAMP is a repellent. PLC, PTEN, and PI3K are all essential components of this 8CPT-cAMP–induced polarity switch. Although PI3K and PLC are not essential for chemotaxis (Drayer *et al*., 1994; Loovers *et al*., 2006; Takeda *et al*., 2007) these and previously described results (Keizer-Gunnink *et al*., 2007; Van Haastert *et al*., 2007) show they are key components of the directional sensing pathway, especially at low cAMP concentrations. Indeed, the localized production of PI(3,4,5)P_3 may be sufficient to orientate the cell. Moreover, the defects in PKB/Akt activation described here clearly indicate a further role for PLC and PI(3,4,5)P_3 in the more complex and physiological process of aggregation.

In summary, this study shows that PLC plays an important role in the regulation of PI3K-mediated chemotaxis and the role of PI(4,5)P_2 in this process. PLC controls cAMP-mediated PI(3,4,5)P_3 formation by repressing the association of PTEN with the plasma membrane. This provides an additional regulatory layer to the signaling pathways regulating PI(3,4,5)P_3-mediated chemotaxis in *Dictyostelium* and suggests a novel mechanism for the generation of PI(3,4,5)P_3 gradients within the cell.

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