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

Arjan Kortholt,**‡ Jason S. King,**‡ Ineke Keizer-Gunnink,* Adrian J. Harwood,**‡ and Peter J.M. Van Haastert*  

*Department of Molecular Cell Biology, University of Groningen, 9751 NN Haren, The Netherlands; and ‡School of Biosciences, Cardiff University, Cardiff CF10 3US, United Kingdom

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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 where 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-trisphosphate [PI(3,4,5)P3] at the leading edge (Parent et al., 1998; Funamoto et al., 2002; Huang et al., 2003). PI(3,4,5)P3 is produced by the phosphorylation of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] 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)P3 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)P3 at the leading edge results in recruitment of PI(3,4,5)P3 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)P2 by phospholipase C (PLC) to form two important second messengers, diacylglycer-
erol (DAG) and inositol-1,4,5-trisphosphate [I(1,4,5)P$_3$] (Katan, 1998). Dictyostelium contains a single plc gene, structurally similar to mammalian PLC-6 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 Go$_5$ and Go$_6$ 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 [1,3,4,5]P$_4$, 3,6-bisphosphatase 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 reinforced by mutation of the inositol triphosphate receptor iplA, which also had no apparent phenotype (Traynor et al., 2000). Here, because Pl(1,4,5)P$_3$ 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 Pl(3,4,5)P$_3$-mediated chemotaxis.

Although pten-null mutants have a relatively strong phenotype in which they form multiple pseudopodia, 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 Pl(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 Pl(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 plA 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 Pl(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 Pl(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 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 nematode dishes containing HC5 medium (14.3g/l peptone, 7.15 g/l glucose, 0.49 g/l KH$_2$PO$_4$ and 1.36 g/l Na$_2$HPO$_4$$\cdot$H$_2$O). To select for transformants with one of the extrachromosomal plasmids described below, cells were grown in HG-5 supplemented with 10 µg/ml Geneticin (G-418; Invitro- gen, Carlsbad, CA), hygromycin B (Invitrogen), or both. For development on filters, log-phase growing cells were washed twice in KK2 buffer (10 mM 16.5 M NaCl, 0.25 M K$_2$HPO$_4$, 0.01 M KH$_2$PO$_4$, 2.55 M Na$_2$CO$_3$, 0.025 M MgCl$_2$, 1 M CaCl$_2$, 1 M MgSO$_4$, 0.005 M glucose), and were resuspended in KK2 (10 mM KH$_2$PO$_4$, and 3.8 mM K$_2$HPO$_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'-GAA- GATCTAAAATGGATACCTTACAAATC-3' and reverse primer 5'-CT- CCTAGATGGCAAAAATGTTACAC-3'. The BgllI/XbaI fragment was cloned in the BgllII-Spel site of a modified vector that contains a hygromycin instead of a neomycin resistance (Boogaart et al., 2003). 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 pBLBPSR (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$_2$PO$_4$, 2.8 mM Na$_2$HPO$_4$, 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$_2$PO$_4$/Na$_2$HPO$_4$), 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 x 10$^5$ cells/ml. Droplets of ~0.1 µl of starved cells were placed on agar. Chemotaxis toward CAMP was tested after 30 min incubation by placing a second droplet of medium 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-apochromat 63x 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 x 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
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 (Nalgene 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 60x objectives 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 stimulations.

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 × 10^6 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 samples were removed and lysed directly into an equal volume of 2% LDS gel loading buffer (Invitrogen) supplemented with 300 mM NaF, 1.2 mM Na, 40 mM, and 12 mM EDTA. Samples were then fractionated on 3–8% NuPage Tris-aeate 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 chemotactant, 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 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.

PLC Regulates cAMP-dependent PI(3,4,5)P3 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)P3 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)P3 sensor PHCRA-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 PHCRA-GFP-expressing cells moved in random directions, and they showed an evenly distributed PHCRA-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 PHCRA-GFP from the cytosol to the membrane facing the cAMP source (Figure 2A, see Supplemental Movie 1). Pseudopodia then extended from PHCRA-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, PHCRA-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, PHCRA-GFP localization 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, PHCRA-GFP localization was dramatically reduced and most fluorescence remained cytosolic (Figure 2A, see Supplemental Movie 2).
PLC Regulation of P(3,4,5)P3-mediated Chemotaxis

Enhanced Levels of P(3,4,5)P3 in Cells Overexpressing PLC Lead to Chemotaxis Defects

Based on the hypothesis that PLC activity is required for cAMP-stimulated P(3,4,5)P3 accumulation, we would predict that cells overexpressing PLC may have an increased P(3,4,5)P3 response after cAMP stimulation. To investigate this hypothesis, we constructed a PLC expression plasmid containing a hygromycin-resistance cassette and transfected it into plc-/PHCRACGFP 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, PLCOE cells have a much broader and irregular front (Figure 2A). In plc-null cells, which maintain a persistently focused leading edge, this response was dramatically reduced, and it was undetectable in wild-type cells treated with LY294002 (Figure 3B). In plc-null cells, this response was substantially reduced accumulation of P(3,4,5)P3, and they support the second hypothesis that LY294002 resistance arises through a loss of P(3,4,5)P3 signaling and subsequent up-regulation of a compensatory signal pathway. Furthermore, they suggest a role for PLC as regulator of the P(3,4,5)P3 signaling in wild-type cells.

Figure 2. PLC regulates cAMP-mediated P(3,4,5)P3 formation during chemotaxis. To investigate the effect of PLC on P(3,4,5)P3 levels, the P(3,4,5)P3 detector PHcracGFP was expressed in wild-type, plc-null, and PLCOE 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 PLCOE is significant for chemotaxis, *p < 0.05 and for speed, ***p < 0.001, t test).

Figure 3. PLC regulates cAMP-mediated P(3,4,5)P3 signaling. (A) Membrane translocation of PHCRACGFP in AX2, HAD236 (plc-null), and PLCOE cells after uniform stimulation with 1 μM cAMP. Bar, 10 μm. Quantitation of PHCRACGFP 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 PLCOE is significant for ***p < 0.005). (B) PKB phosphorylation in wild-type and plc-null cells. Cells were cAMP pulsed, and then they stimulated 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.
of a cAMP gradient, many cells exhibit a much broader region along the leading edge that is associated with PHCRAC-GFP. In wild-type cells, an average of 16.5 ± 3.5% (n = 12) of the perimeter was covered by PHCRAC-GFP, whereas in PLCOE 29.3 ± 4.8% (n = 11) was occupied. Consistently, when PLCOE cells were globally stimulated with 1 μM cAMP, the amount of PHCRAC-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 PLCOE 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)P3 signaling and that the elevated levels of PI(3,4,5)P3 in PLCOE 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)P3 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)P3 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)P3 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 PHCRAC-GFP and presumably other PI(3,4,5)P3-binding proteins to the plasma membrane may still have significant effects during aggregation. Indeed, p3ki/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)P3 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.
LY294002 sensitivity in therefore, the lack of chemotaxis defects and loss of Vol. 18, December 2007 4777 et al mediates chemotaxis (Chen 2007). Furthermore, van Haastert et al (2007) showed that PTEN 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$_{21}$ and inhibitory G$_{2}$ (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$_{21}$ 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(3,4,5)P$_3$ 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 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 results reveal that PLC is an important regulator of PI3K-mediated chemotaxis. Examination of the distribution of the PI(3,4,5)P$_3$ sensor PHCRAC-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 sensitive 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 plc-null cells. Both plc-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 (Lijima 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$_2$-specific PH 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$_{21}$ and inhibitory G$_{2}$ (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$_{21}$ 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(3,4,5)P$_3$ 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

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**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.
PLC in PI(3,4,5)P3-mediated chemotaxis (Keizer-Gunnink et al., 2007). 8CPT-cAMP inhibits PLC through Go3. Consistent with our model, inhibition of PLC by SCPT-cAMP at the leading edge will lead to higher levels of PI(4,5)P2 and subsequently more membrane-bound PTEN upgradient. The resulting gradient of PI(4,5)P2 and PTEN will mediate an opposite gradient of PI(3,4,5)P3, therefore, the cells move downgradient and SCPT-cAMP is a repellent. PLC, PTEN, and PI3K are all essential components of this SCPT-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)P3 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)P3 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)P2 in this process. PLC controls cAMP-mediated PI(3,4,5)P3 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)P3-mediated chemotaxis in Dictyostellium and suggests a novel mechanism for the generation of PI(3,4,5)P3 gradients within the cell.

REFERENCES


