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Novel Mechanism of PTEN Regulation by Its Phosphatidylinositol 4,5-Bisphosphate Binding Motif Is Critical for Chemotaxis*

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In chemotaxing cells, localization of phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) to the leading edge of the cell sets the direction and regulates the formation of pseudopods at the anterior. We show that the lipid phosphatase activity of PTEN mediates chemotaxis and that the sharp localization of $PI(3,4,5)P_3$ requires localization of PTEN to the rear of the cell. Our data suggest that a phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) binding motif at the N terminus of PTEN serves the dual role of localizing the enzyme to the membrane and regulating its activity. Mutations in this motif enhance catalytic activity but render the enzyme inactive *in vivo* by preventing membrane association. The key role of this motif may explain the heretofore puzzling tumor-suppressing mutations occurring within the $PI(4,5)P_2$ binding motif. On the other hand, the localization of PTEN does not depend on its phosphatase activity, the actin cytoskeleton, or the intracellular level of $PI(3,4,5)P_3$, suggesting that events controlling localization are upstream of phosphoinositide signaling.

Cells sense external chemical gradients and respond by moving toward the higher concentration of chemoattractant. Chemotaxis is important for a wide variety of physiological and pathological events such as axon guidance, immune response, wound healing, tissue morphogenesis, and carcinoma invasion. The molecular mechanisms of chemotaxis are highly conserved among eukaryotes including mammalian neutrophils and *Dictyostelium discoideum* amoebae.

Chemotaxis plays an essential role in the developmental program of this social amoeba. Appropriately differentiated cells migrate toward aggregation centers in response to the chemoattractant, cAMP, and form multicellular structures. Several components involved in chemotaxis have been identified through genetic and biochemical studies. For example, cAR1, a seven-transmembrane cAMP receptor, binds cAMP and activates a heterotrimeric G-protein (1, 2). The activation of the G-protein eventually leads to polymerization of F-actin toward higher concentrations of cAMP and drives extension of pseudopods at the leading edge of cells. Thus, intracellular signaling events involved chemotaxis are activated locally at the leading edge. However, neither the receptors nor the receptor-coupled G-proteins are concentrated in this region but are found uniformly distributed along the plasma membrane (3–5).

In contrast, pleckstrin homology (PH)[1] domain-containing proteins, such as Crac, PKB, and PhdA, are highly localized at the front of chemotaxing cells (5, 6). The PH domain of Crac binds to phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) and phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$). Similar to Crac, in mammalian neutrophils and fibroblasts, several PH domain-containing proteins, such as PKB/AKT, are found to preferentially associate with the plasma membrane at the leading edge (4, 7). These findings suggest that these phosphoinositol lipids locally activate the signaling events that lead to actin polymerization at the leading edge of chemotaxing cells. Consistent with this idea, the addition of a membrane-permeable form of $PI(3,4,5)P_3$ to fibroblasts and neutrophils triggers the formation of membrane ruffles and increases their motility (8–10).

The production of $PI(3,4,5)P_3$ is regulated by phosphatidylinositol 3-kinase (PI3K) and PTEN. Recently, both PI3K and PTEN have been shown to be required for $PI(3,4,5)P_3$ production and chemotaxis toward cAMP (11, 12). In wild type *Dictyostelium* cells, the level of $PI(3,4,5)P_3$ transiently increases within 5 s upon cAMP stimulation and returns to basal levels within 60 s. However, in mutants lacking two of three PI3K genes, the production of $PI(3,4,5)P_3$ induced by cAMP is dramatically impaired, and the mutant cells are defective in chemotaxis and development (13, 14).

PTEN was originally identified as a tumor suppressor gene that is defective in a variety of human cancers (15, 16). PTEN is a lipid and protein-tyrosine phosphatase that specifically dephosphorylates phosphotyrosine as well as the D3 position of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (17–19). We have shown that a *Dictyostelium* homolog of PTEN is essential for efficient chemotaxis toward cAMP (12). The chemotaxis defects are due to hyperactivation of the actin cytoskeleton. *pten*$^-$ cells extend numerous pseudopodia in every direction and fail to restrict extension of pseudopodia to the leading edge in a chemoattractant gradient. Furthermore, in *pten*$^-$ cells, the PH domain, a sensor of local $PI(3,4,5)P_3$ levels, is strongly associated with the plasma membrane, and this localization is not restricted to the leading edge in a cAMP gradient. These observations indicate that the degradation of $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ is severely impaired in *pten*$^-$ cells. Supporting this idea, analysis of total cellular lipids directly demonstrated that excess $PI(3,4,5)P_3$ accumulates upon cAMP stimulation in *pten*$^-$ cells (13, 14).

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[1] The abbreviations used are: PH domain, pleckstrin homology domain; DB, development buffer; DTT, dithiothreitol; GFP, green fluorescent protein; HPLC, high pressure liquid chromatography; Ins, inositol; MES, 4-morpholinoethanesulfonic acid; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PTP, protein-tyrosine phosphatase.

Interestingly, *Dictyostelium* PTEN localization changes in response to chemoattractant. In resting cells a fraction of PTEN is uniformly associated with the plasma membrane. Upon uniform chemoattractant stimulation, PTEN transiently dissociates from the membrane and diffuses into the cytosol. When cells are exposed to a gradient of cAMP, PTEN accumulates at the rear of the chemotaxing cells. This distribution is opposite to that of PI3K, which localizes to the front of cells. The reciprocal localization of PI3K and PTEN have been proposed to spatially regulate the production of phosphoinositol lipids with synthesis occurring at the leading edge and degradation at the rear (11, 12).

Previously, we have shown that the membrane association of *Dictyostelium* PTEN requires its N-terminal $PI(4,5)P_2$ binding domain (12). In this paper, we extend this observation and show that even though only a very small fraction of the protein is bound to the plasma membrane, its function depends on this localization. Versions of PTEN with mutations in the $PI(4,5)P_2$ binding motif have enhanced catalytic activity but are defective in membrane binding and fail to complement the phenotypes seen in *pten*$^-$ cells. Furthermore, PTEN localization is regulated independently of $PI(3,4,5)P_3$-mediated signaling mechanisms and the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Cell Growth and Development—The *D. discoideum* wild type strain AX2 and *pten*$^-$ cells were axenically cultured in HL5 medium at 22 °C. Transformants carrying protein expression constructs were grown in HL5 containing 20 μg/ml G418. To assess developmental phenotypes, exponentially growing cells were washed twice in development buffer (DB; 5 mM Na_2HPO4, 5 mM NaH_2PO_4 (pH 6.5), 2 mM $MgSO_4$, 0.2 mM $CaCl_2$) and plated on 1.5% non-nutrient DB agar at 1×10^6 cells/cm^2.

Plasmid Construction and Transformation of Cells—pPTEN-GFP, a plasmid expressing *Dictyostelium* full-length PTEN fused to GFP at the C terminus from the actin 15 promoter, was described previously (12). To construct plasmids expressing truncated versions of PTEN fused to GFP from the actin 15 promoter, different regions of PTEN were PCR-amplified from its cDNA using the following primers, digested with XhoI and BglII, and subcloned into XhoI/BglII sites in pMIG containing the actin 15 promoter and GFP. PCR products were confirmed by DNA sequencing. For $PTEN_{0-445}$, primers P111 (5′-GGAGATCTGGAAACAAATAGAATGAG-3′) and P1462 (5′-CCCTCGAGGTAATATTATCGTGATGAGAG-3′) were used. For $PTEN_{16-515}$, primers P172 (5′-GGAGATCTTATCAAAAAAATGGTTACGAT-3′) and P1664 (5′-CCCTCGAGTCTGCTTCAACCTTTGGAGC-3′) were used. For $PTEN_{186-515}$, primers P682 (5′-GGAGATCTATCAAATATGTACCACGTAAT-3′) and P1664 were used. Point mutations in the phosphatase catalytic site and in the $PI(4,5)P_2$ binding motif were created using site-directed mutagenesis (QuikChange, Stratagene).

DNA encoding 600 amino acids from the N terminus of PI3K2 was PCR-amplified from the *Dictyostelium* genome using primers 5′-CGGGATCCATGAAAATGAGTGAAGGAATT-3′ and 5′-AGACGTCGACGCCATAGTTTGTCTCCAAAT-3′. PCR products were sequenced, digested with SalI and BamHI, and cloned into XhoI/BglII sites in pMIG.

Exponentially growing cells were transformed with expression vectors (10 μg of DNA) by electroporation and selected on plates for 7–10 days in HL5 containing 10 μg/ml G418. When foci were visible, cells were harvested and observed for expression by fluorescence microscopy and immunoblotting.

Chemotaxis Assay—Cells grown in HL5 were washed twice with DB and resuspended at 2×10^7 cells/ml. Cells were incubated for 1 h and then differentiated with 100 nM cAMP pulses at 6-min intervals for 4 h (41). Differentiated cells were plated on a chambered coverglass (Lab-Tek, Nalge Nunc) and allowed to adhere to the surface. A micropipette filled with 1 μM cAMP was positioned near the cells, and images of moving cells were recorded using an inverted Zeiss microscope (Axiovert 135 TV).

Radiolabeling and Detection of Phosphoinositol Lipid in Intact Cells—Cells were developed in phosphate-free MES-DB (20 mM MES (pH 6.5), 2 mM $MgSO_4$, 0.2 mM $CaCl_2$) for 5 h and labeled with 0.5 mCi of ^{32}P radionucleotide (PerkinElmer Life Sciences) for 1 h. Labeled cells were treated with 20 mM caffeine for 20 min and washed twice with MES-DB. Cells were resuspended at 8×10^7 cells/ml in MES-DB and stimulated with 1 μM cAMP. 150-μl samples were collected into 1 ml of cold 1 N HCl to stop the reaction. Lipids were extracted as described previously (13, 14), and phospholipids were separated and analyzed on a TLC plate (TLC aluminum sheet silica gel 60, EM Science).

Immunopurification of GFP Fusion Proteins—Cells expressing GFP fusion proteins were washed twice with DB, shaken for 2 h in DB, and resuspended into phosphate-buffered saline at 4×10^8 cells/ml. Cells were lysed in lysis buffer (50 mM Na_2HPO_4 (pH 7.6), 150 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40) containing protease inhibitors (2 μg/ml antipain, 4 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml chymostatin, 2 μg/ml pepstatin, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) for 15 min on ice. Lysates were clarified by centrifugation for 20 min at 4 °C. The lysate was incubated with protein A beads coupled to anti-GFP antibody for 2 h at 4 °C. Immune complexes were washed twice in lysis buffer, twice in Tris buffer (25 mM Tris-HCl (pH 7.2), 150 mM NaCl), and twice in reaction buffer (100 mM Tris-HCl (pH 8.0), 10 mM DTT). Proteins were separated by SDS-PAGE and analyzed by immunoblotting and silver staining.

Phosphatase Assays—The phosphatase activity of PTEN against phosphoinositol lipids was measured in 50 μl of reaction buffer (100 mM Tris-HCl (pH 8.0), 10 mM DTT) containing water-soluble D(+)-sn-1,2-di-*O*-octanoylglyceryl, 3-*O*-phospho-linked-phosphatidylinositol phosphate (Echelon) and 1 μg of immunoprecipitated proteins. The samples were incubated for 30 min at 22 °C with gentle shaking. Amounts of phosphate released from substrates were determined by a malachite green assay using a PhosFree Phosphate Assay Biochem Kit (Cytoskeleton).

The phosphatase activity against inositol phosphate was measured in 50 μl of reaction buffer containing 1 nM ^3H-labeled $Ins(1,3,4,5)P_4$ (PerkinElmer Life Sciences), 50 μM $Ins(1,3,4,5)P_4$ (Echelon), and 1 μg of immunoprecipitated proteins for 2 h at room template. Reactions were terminated by the addition of 400 μl of stop solution (2 M perchloric acid, 2 mM EDTA, 0.2 mg/ml IP6 (D-myo-inositol-1,2,3,4,5,6-hexakis-phosphate)) and then neutralized with 400 μl of neutralization buffer (1 M K_2CO_3, 5 mM EDTA). Inositol phosphates were separated by HPLC using anion exchange chromatography (partisphere SAX column, 12.5 cm × 4.6 mm) (Whatman). HPLC was run at a speed of 1 ml/min with a linear gradient of 0–1.3 M $(NH_4)_2PO_4$ (pH 3.8). Fractions were collected, and the radioactivity in each fraction was counted. Inositol phosphates were identified by their co-elution with radiolabeled standards of inositol phosphate.

RESULTS

PTEN Dephosphorylates $PI(3,4,5)P_3$ and $PI(3,4)P_2$ in Vitro—To determine the lipid phosphatase activity of *Dictyostelium* PTEN, we expressed PTEN fused to GFP in *pten*$^-$ cells and purified the protein by immunoprecipitation with protein A beads coupled to anti-GFP antibody. Immunopurified PTEN and a GFP control were incubated with $PI(3,4,5)P_3$ or $PI(3,4)P_2$ for 30 min. After incubation, released phosphate was measured using a malachite green assay (19, 20). PTEN released phosphate from both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ at a rate of 144.5 nmol/mg/min for $PI(3,4,5)P_3$ and 44.1 nmol/mg/min for $PI(3,4)P_2$ (Fig. 1*B*). These phosphatase activities of *Dictyostelium* PTEN are comparable with those of human PTEN, which exhibited phosphatase activity of 150.1 nmol/mg/min for $PI(3,4,5)P_3$ (Fig. 1*B*). In contrast, GFP showed no phosphatase activity.

Human and *Dictyostelium* PTEN sequences are co-linear with the protein-tyrosine phosphatase (PTP) domains in their N-terminal regions (Fig. 1*A*). We deleted N-terminal 185 amino acid residues, which removes PTP domains. This mutant, $PTEN_{186-515}$, did not show phosphatase activity toward $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Fig. 1*B*). In the middle of the PTP domain of mammalian PTEN, there are seven amino acids forming a catalytic site that binds directly to substrate. *Dictyostelium* PTEN contains the putative catalytic domain identical to mammalian PTEN. To confirm that this catalytic site is required for the lipid phosphatase activity of *Dictyostelium* PTEN, we introduced three point mutations in this region (C124S, K128M, G129E). Mutations in human PTEN equivalent to C124S and G129E have been shown to completely inhibit their lipid phosphatase activity against both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (18), whereas the K128M mutation reduced the

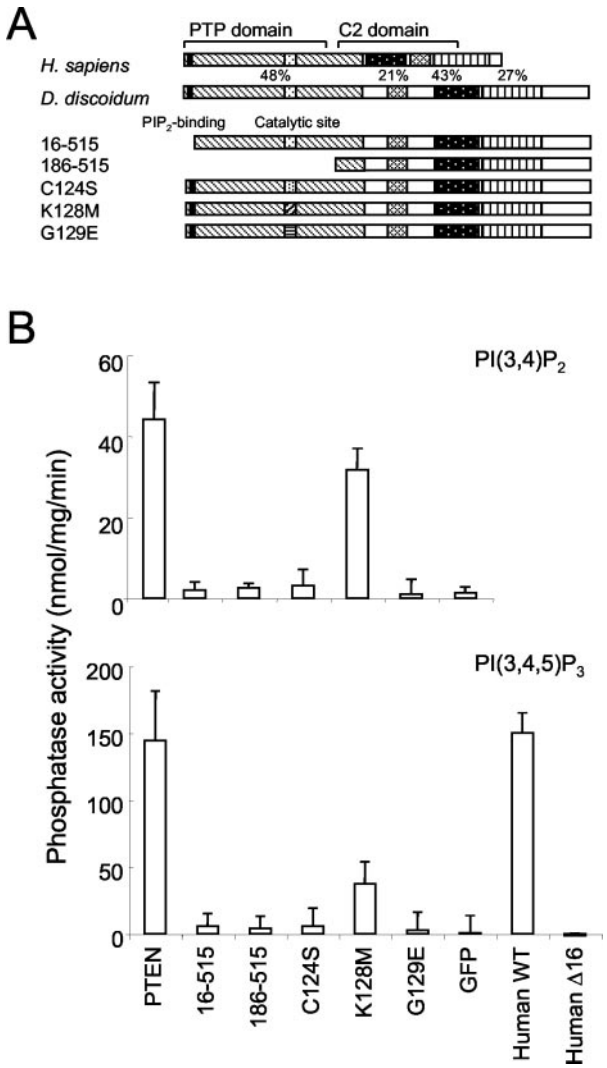

FIG. 1. **Dictyostelium PTEN dephosphorylates phosphoinositides *in vitro*.** *A*, constructs used in this study. Full-length PTEN comprises 515 amino acids. $PTEN_{16-515}$ and $PTEN_{186-515}$ lack 15 and 185 amino acids from the N terminus, respectively. To mutate the catalytic domain, three conserved residues were mutated; cysteine at residue 124 was changed to serine ($PTEN_{C124S}$), lysine at 128 to methionine ($PTEN_{K128M}$), and glycine at 129 to glutamic acid ($PTEN_{G129E}$). All constructs were tagged by GFP at the C terminus. Homologous regions between human and *Dictyostelium* PTENs are indicated by use of the *same patterns*, and amino acid sequence identities are shown. Both PTENs contain the $PI(4,5)P_2$ binding motif at their N terminus and putative catalytic site for lipid phosphatase activity within the PTP domain. The amino acid sequence of the catalytic site (HCKAGKGR) is fully conserved between human and *Dictyostelium*. *B*, lipid phosphatase activity. *pten*$^-$ cells expressing wild type and mutant forms of PTEN-GFP were lysed using Nonidet P-40-containing buffer, and the fusion proteins were immunoprecipitated using anti-GFP antibody. The immunoprecipitated proteins were quantitated using Western blotting and silver staining. 1 μg of PTEN proteins was incubated in 50 μl of 100 mM Tris-HCl, pH 8.0, 10 mM DTT for 30 min at room temperature in the presence of $PI(3,4)P_2$ or $PI(3,4,5)P_3$. Released phosphates were measured using a malachite green assay. Immunoprecipitated GFP from GFP expressing *pten*$^-$ cells was used as a control. Values represent the mean ± S.D. from four independent experiments.

activity only against $PI(3,4,5)P_3$ but not $PI(3,4)P_2$ (19). We found that $PTEN_{C124S}$ and $PTEN_{G129E}$ were defective in phosphatase activity against both $PI(3,4,5)P_3$ and $PI(3,4)P_2$. In contrast, $PTEN_{K128M}$ was partially defective in phosphatase activity against only $PI(3,4,5)P_3$ (Fig. 1*B*). The results demonstrated that the catalytic site in the PTP domain of human and *Dictyostelium* PTENs are orthologous.

Like human PTEN, *Dictyostelium* PTEN contains a putative

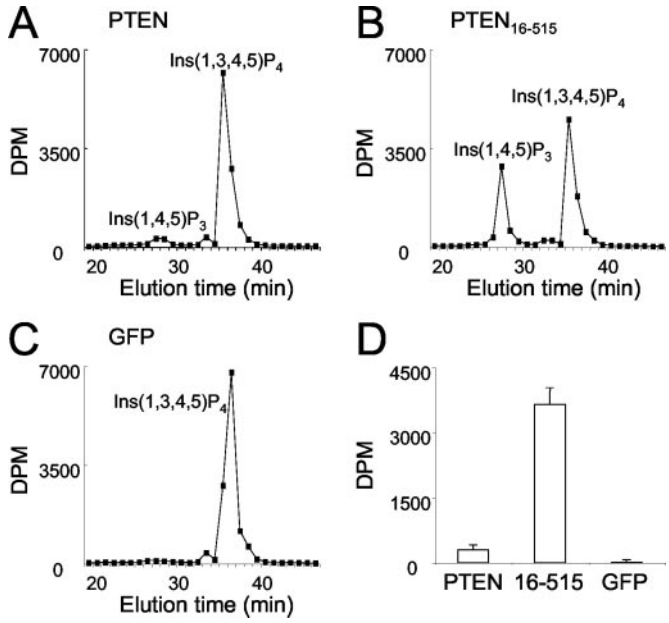

FIG. 2. **Inositol phosphate phosphatase activity of PTENs.** Wild type and mutant forms of PTEN-GFP were immunoprecipitated using anti-GFP antibody. The phosphatase activity against inositol phosphate was measured in 50 μl of reaction mixture containing 1 μg of immunoprecipitated protein, 100 mM Tris-HCl, pH8.0, 10 mM DTT, 50 μM $Ins(1,3,4,5)P_4$, and 1 nM [^{3}H]$Ins(1,3,4,5)P_4$ for 2 h at room temperature. Inositol phosphates were extracted and resolved by HPLC using a Partisphere SAX column (Whatman). GFP was used as a control. Four independent experiments were performed; representative elution patterns are shown for wild type PTEN (*A*), $PTEN_{16-515}$ mutant (*B*), and GFP control (*C*). Radioactivity in each fraction is shown. The identity of each peak was determined by using standards of [^{3}H]$Ins(1,4)P_2$, [^{3}H]$Ins(1,4,5)P_3$, and [^{3}H]$Ins(1,3,4,5)P_4$. *D*, total amounts of [^{3}H]$Ins(1,4,5)P_3$. Total radioactivity of [^{3}H]$Ins(1,4,5)P_3$ from each reaction was obtained by subtracting background radioactivity. Values represent the mean ± S.D. from four independent experiments.

$PI(4,5)P_2$ binding motif at its N-terminal end (Fig. 1*A*). We previously have shown that this sequence is essential for PTEN to associate with the plasma membrane. To determine whether the $PI(4,5)P_2$ binding domain is important for the phosphatase activity of PTEN, we truncated 15 amino acid residues from the N terminus ($PTEN_{16-515}$) and examined the phosphatase activity toward $PI(3,4,5)P_3$ and $PI(3,4)P_2$. $PTEN_{16-515}$ failed to dephosphorylate $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Fig. 1*B*). We also determined whether this $PI(4,5)P_2$ binding motif is important for phosphatase activity of human PTEN. The human PTENΔ16 lacking the $PI(4,5)P_2$ binding motif had no phosphatase activity against $PI(3,4,5)P_3$ (Fig. 1*B*). Therefore, the $PI(4,5)P_2$ binding motif is essential for $PI(3,4,5)P_3$ and $PI(3,4)P_2$-directed phosphatase activity of both *Dictyostelium* and human PTEN.

$PTEN_{16-515}$ Preferentially Dephosphorylates $Ins(1,3,4,5)P_4$—The observations that $PTEN_{16-515}$ is unable to dephosphorylate $PI(3,4,5)P_3$ and $PI(3,4)P_2$ suggest that the $PI(4,5)P_2$ binding motif regulates the specificity of the phosphatase activity of PTEN. We reasoned that the $PI(4,5)P_2$ binding motif is important for interaction with phosphatidylinositol lipids such as $PI(3,4,5)P_3$ and $PI(3,4)P_2$. If this is correct, $PTEN_{16-515}$ should dephosphorylate water-soluble substrates such as inositol phosphates. To verify our hypothesis, we examined the phosphatase activity of wild type PTEN and $PTEN_{16-515}$ toward inositol phosphate, $Ins(1,3,4,5)P_4$ (Fig. 2). Immunopurified wild type PTEN and $PTEN_{16-515}$ were incubated with [^{3}H]$Ins(1,3,4,5)P_4$. After incubation, inositol phosphates were separated using HPLC chromatography. We found that wild type PTEN, $PTEN_{16-515}$, and the GFP control converted 2.2, 34.6,

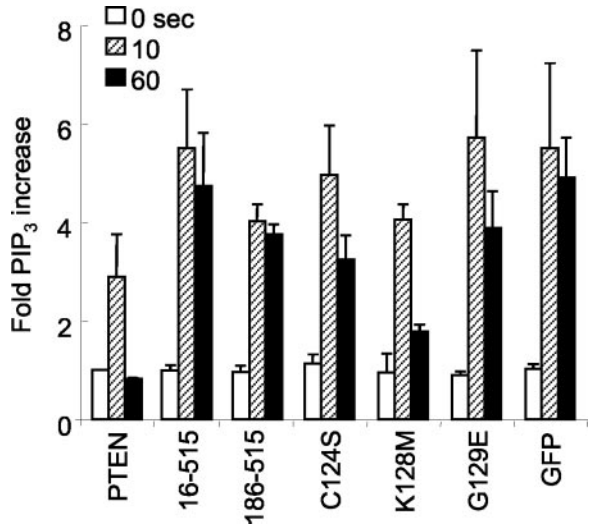

FIG. 3. **The lipid phosphatase activity of PTEN is required for regulating the level of PI(3,4,5)P3 *in vivo*.** The *pten*$^-$ cells expressing wild type and mutant forms of PTEN-GFP were starved for 5 h in MES-DB to induce development and then labeled with ^{32}P for 1 h. These cells were stimulated with 1 μM cAMP. Total cellular lipids were extracted at the indicated time points. Lipids were separated on TLC plates, and the intensity of bands corresponding to $PI(3,4,5)P_3$ was quantified using a phosphorimaging device. The levels of $PI(3,4,5)P_3$ were normalized relative to that seen in *pten*$^-$ cells expressing wild type PTEN at time 0 min. Values represent the mean ± S.D. from at least three independent experiments.

and 0% of $Ins(1,3,4,5)P_4$ to $Ins(1,4,5)P_3$ (Fig. 2). Thus, the $PI(4,5)P_2$ binding motif is not required for phosphatase activity against the soluble inositol phosphate head group of $PI(3,4,5)P_3$. The significantly enhanced activity of $PTEN_{16-515}$ toward $Ins(1,3,4,5)P_4$ indicates that the $PI(4,5)P_2$ binding motif actually inhibits catalytic activity. Taken together with the reduced activity toward $PI(3,4,5)P_3$ and $PI(3,4)P_2$, this result suggests that the $PI(4,5)P_2$ binding motif serves the dual role of associating the enzyme with a lipid substrate and regulating catalysis.

$PI(3,4,5)P_3$ Accumulates upon cAMP Stimulation in PTEN Mutants—To investigate the function of the lipid phosphatase activity of PTEN *in vivo*, we expressed mutant versions of PTEN in *pten*$^-$ cells and examined the production of $PI(3,4,5)P_3$ induced by chemoattractants (Fig. 3). Developed cells were metabolically labeled with [^{32}P]orthophosphate. After cAMP stimulation, total cellular lipids were extracted and resolved by TLC. In *pten*$^-$ cells expressing wild type PTEN, the level of $PI(3,4,5)P_3$ transiently increased ~3-fold at 10 s after cAMP stimulation, started to decline within 30 s, and returned to basal level in 60 s. In contrast, in the *pten*$^-$ cells expressing GFP, $PI(3,4,5)P_3$ level increased more than 5-fold and remained elevated even at 60 s after cAMP stimulation (12–14).

The *pten*$^-$ cells expressing $PTEN_{16-515}$ and $PTEN_{186-515}$ showed a pattern of $PI(3,4,5)P_3$ production similar to that observed in *pten*$^-$ cells expressing GFP. Cells expressing $PTEN_{C124S}$, $PTEN_{K128M}$, and $PTEN_{G129E}$ showed a partial restoration of $PI(3,4,5)P_3$ to wild type level. Stimulation with cAMP induced a 4–5-fold increase of $PI(3,4,5)P_3$ at peak, and then the lipids were reduced more rapidly. To quantify these data, we defined the "degradation index" by calculating the percentage of $PI(3,4,5)P_3$ remaining at 60 s relative to the amount of $PI(3,4,5)P_3$ at 10 s. For example, the degradation indices in *pten*$^-$ cells expressing wild type PTEN, GFP, $PTEN_{16-515}$, and $PTEN_{186-515}$ were −9.4, 86.4, 82.9, and 91.2%, respectively. In *pten*$^-$ cells expressing $PTEN_{C124S}$, $PTEN_{K128M}$, and $PTEN_{G129E}$, degradation indices were 55.1, 26.8, and 61.9%, respectively. These profiles of lipids production and degradation strongly suggest that PTEN is controlling lipid metabolism *in vivo*.

Mutations in the Catalytic Domain of PTEN Block Normal Development and Chemotaxis—We previously have shown that *pten*$^-$ cells have strong defects in development and chemotaxis (12). These developmental phenotypes are not simply because of defects in expression of early developmental genes such as the cAMP receptor and adenylyl cyclase (12). During the developmental stage, *Dictyostelium* amoeba chemotax toward higher concentrations of extracellular cAMP and aggregate to become multicellular organisms. Within 24 h they develop into a terminal structure called fruiting bodies consisting of spore and stalk cells (Fig. 4*A*). The expression of wild type PTEN completely reversed the developmental defects in the *pten*$^-$ cells. Interestingly, expression of $PTEN_{16-515}$, which lacks the $PI(4,5)P_2$ binding motif, did not alter the phenotype of the *pten*$^-$ cells. Cells expressing $PTEN_{16-515}$ remained as a smooth monolayer identical to control cells expressing GFP. The expression of $PTEN_{186-515}$ also did not rescue *pten*$^-$ cells. *pten*$^-$ cells expressing PTEN mutants with point mutations in the catalytic domain, $PTEN_{C124S}$ and $PTEN_{G129E}$, showed severe defects in development. Whereas wild type cells and *pten*$^-$ cells expressing PTEN aggregated within 6 h on the non-nutrient agar, the cells expressing $PTEN_{C124S}$ and $PTEN_{G129E}$ did not form a typical aggregation pattern. Eventually they developed into small structures that did not contain normal spore and stalk cells. The development of cells expressing $PTEN_{K128M}$ was also impaired. The aggregation territories were small, fruiting bodies were diminished, and many cells remained on the surface of the agar, suggesting that many cells could not enter the aggregation streams.

We next addressed whether these developmental defects resulted from the inability of the cells expressing mutant versions of PTEN to chemotax. We directly observed chemotaxis of wild type and *pten*$^-$ cells transformed with different constructs toward a micropipette releasing cAMP under a microscope (Fig. 4*B*). Consistent with previous results, the mean velocity toward attractant of wild type cells was 12.1 μm/min. This value for *pten*$^-$ was 2.5 μm/min, a 79% reduction in speed compared with wild type cells. The velocity for *pten*$^-$ cells expressing PTEN was almost restored to wild type levels (8.8 μm/min), and cells were nicely elongated and moved directly toward the needle. Two N-terminal truncation mutants did not rescue the chemotaxis defect of *pten*$^-$ cells. The velocities for $PTEN_{16-515}$ and $PTEN_{186-515}$ expressing cells were 2.4 and 2.0 μm/min and were inhibited 80 and 84%, respectively, compared with wild type cells. In chemotaxing cells, proteins of $PTEN_{16-515}$ and $PTEN_{186-515}$ were uniformly distributed in the cytosol, and changes of localization were not detected. Three cell lines expressing PTEN with point mutations in the phosphatase catalytic domain showed slightly faster velocity than *pten*$^-$ cells. The speed values were 3.1, 3.4, and 3.7 μm/min for $PTEN_{C124S}$, $PTEN_{K128M}$, and $PTEN_{G129E}$, respectively, about 70–74% slower than wild type cells. These results indicate that normal chemotaxis toward cAMP requires the phosphatase activity of PTEN, showing that the developmental defects of cells expressing PTEN mutants are highly correlated with their chemotaxis defects.

The Domain Required for Localization of PTEN on the Plasma Membrane—We have shown that PTEN is preferentially associated with the plasma membrane in *Dictyostelium* cells (12). To determine the region of PTEN required for its association with the plasma membrane, we made mutant versions of PTEN fused to GFP and expressed them in *pten*$^-$ cells (Fig. 5). We first tested whether the putative $PI(4,5)P_2$ binding motif at the N terminus of PTEN is required for plasma mem-

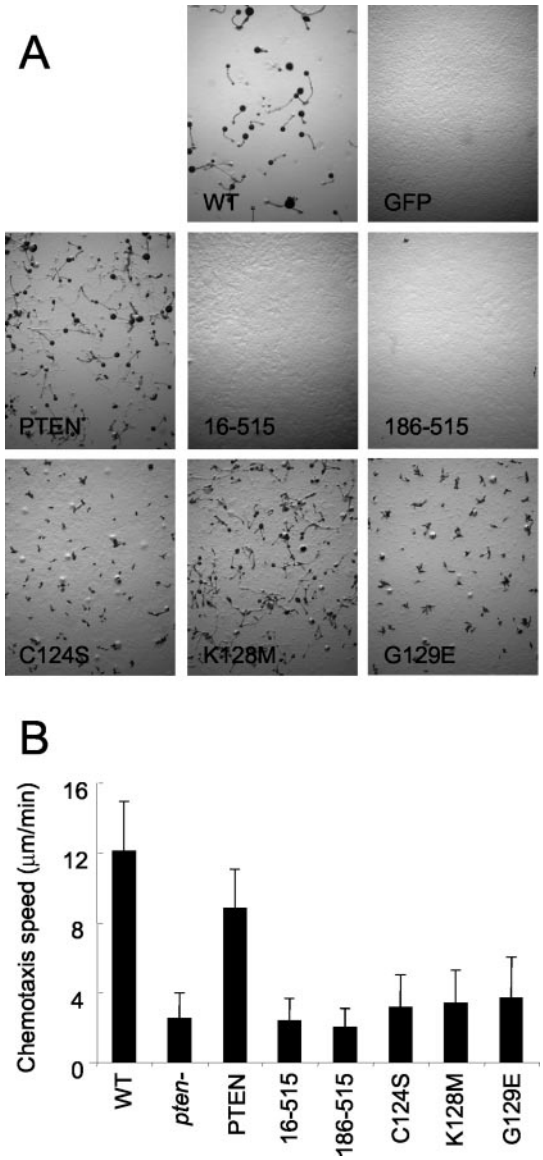

FIG. 4. **The phosphatase activity of PTEN is required for chemotaxis and development.** *A*, the phosphatase activity of PTEN is required for development. Wild type cells and *pten*$^-$ cells expressing wild type and mutant versions of PTEN were plated on non-nutrient agar at 1×10^6 cells/cm^2 to induce development. Images were taken after 24 h of development. *B*, the phosphatase activity of PTEN is required for chemotaxis. Wild type cells and *pten*$^-$ cells expressing wild type and mutant versions of PTEN were differentiated and placed in a gradient of cAMP. To generate cAMP gradient, a micropipette releasing 1 μM cAMP was used. Cells were observed for 10 min with 30-s intervals with fluorescence microscopy, and the rate of chemotaxis was determined using NIH Image software. At least 15 cells were examined for each mutant. Values represent the mean ± S.D.

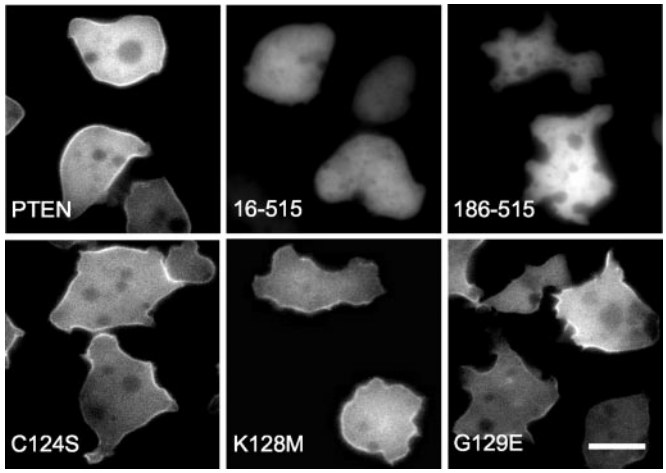

FIG. 5. **Subcellular localization of PTEN mutants.** Wild type and mutant forms of *Dictyostelium* PTEN were expressed in *pten*$^-$ cells. Cells were observed under fluorescence microscope. *Bar*, 10 μm.

brane localization. When 16 amino acid residues from the N-terminal containing the motif were truncated, the $PTEN_{16-515}$ protein localized in the cytosol. However, the $PI(4,5)P_2$ binding motif fused to GFP, $PTEN_{1-15}$, or $PTEN_{1-32}$ did not localize on the plasma membrane. This suggests that the $PI(4,5)P_2$ binding motif is necessary but not sufficient for PTEN localization.

The mammalian PTEN consists of two distinct domains, a PTP domain, and a C2 domain. The PTP domain is highly conserved between human and *Dictyostelium* PTENs. Although the C-terminal half contains well conserved blocks of sequences, the C2 domain is not defined by PSI-BLAST (22) and THREADER2 (23) in the *Dictyostelium* protein. The protein $PTEN_{186-515}$ in which PTP domain was removed did not bind to the plasma membrane, indicating that C-terminal half of PTEN, which has sequence similarity to C2 domain, was not sufficient for membrane localization. When the C-terminal 70 amino acid residues were truncated, this mutant version of $PTEN_{0-445}$ did bind to the plasma membrane and rescued the developmental and chemotaxis defects in *pten*$^-$ cells. However, further truncation of 140 amino acid residues from the C terminus resulted in a striking reduction of the steady-state level of PTEN, suggesting that C-terminal sequences are important for PTEN protein stability.

We next tested whether phosphatase activity is required for membrane localization. The mutations C124S and G129E caused loss of detectable activity against phosphoinositide, and the mutation K128M resulted in reduced phosphatase activity against $PI(3,4,5)P_3$. These three mutant versions, $PTEN_{C124S}$, $PTEN_{K128M}$, and $PTEN_{G129E}$, localized on the plasma membrane similarly to wild type PTEN in both wild type and *pten*$^-$ backgrounds. These results indicated that the phosphatase activity is not required for association of PTEN with plasma membrane.

PTEN Changes Its Localization in Response to Chemoattractant Stimulation—Previously we found that PTEN changes its localization in response to chemoattractant stimulation (12, 21). In response to a uniform increase in cAMP, wild type PTEN rapidly dissociated from the plasma membrane, diffused into the cytosol, and then returned to the plasma membrane within 1 min after stimulation (Fig. 6*A*). At this time, exogenous cAMP was still present around the cells, indicating that there are intracellular adaptation mechanisms that turn off activation. We investigated whether mutant proteins also alter the localization in response to the stimulation. The $PTEN_{0-445}$ responded to cAMP stimulation like the wild type protein. The two mutants $PTEN_{16-515}$ and $PTEN_{186-515}$ remained cytosolic during stimulation. These proteins are not able to bind to the plasma membrane, suggesting that there is no further mechanism to recruit the protein after cAMP stimulation. $PTEN_{C124S}$, $PTEN_{K128M}$, and $PTEN_{G129E}$, each of which contains single mutation in the phosphatase catalytic domain, dissociated and returned to the membrane with the same time course as wild type cells. This indicates that the changes in the localization of PTEN in response to stimulus are not regulated by its phosphatase activity and do not require PTPase activity. Next, we investigated whether the intracellular phosphatidylinositol levels affected the localization of PTEN. The cells expressing wild type PTEN were treated with 30 or 100 μM PI3K inhibitor LY294002. With LY294002 treatment, cells be-

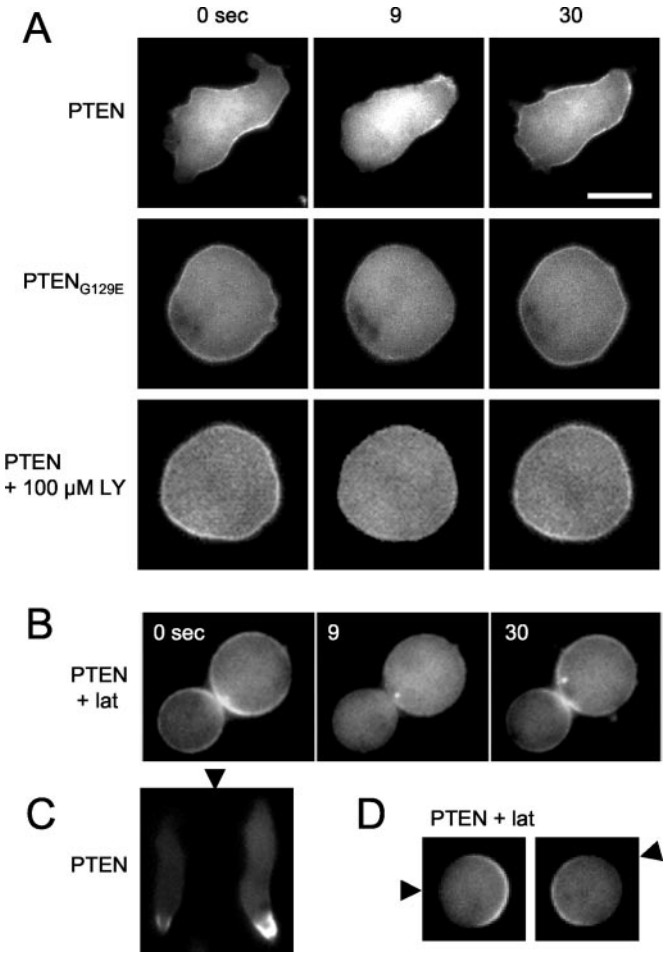

FIG. 6. **Subcellular localization of PTEN-GFP changes in response to stimulation with chemoattractant.** *A*, upon uniform stimulation with cAMP, PTEN transiently dissociates from the plasma membrane into cytosol independently of its phosphatase activity, PI3K, and the actin cytoskeleton. *pten*$^-$ cells expressing wild type PTEN-GFP or PTEN$_{G129E}$-GFP were differentiated for 5 h and stimulated with 1 μM cAMP. The *pten*$^-$ cells expressing wild type PTEN-GFP were treated with 100 μM LY294002 (*LY*) for 10 min before cAMP stimulation. *B*, *pten*$^-$ cells expressing wild type PTEN-GFP were treated with 5 μg/ml latrunculin A (*lat*) for 15 min before cAMP stimulation. *C* and *D*, PTEN responds to an extracellular cAMP gradient independently of the actin cytoskeleton. *pten*$^-$ cells expressing wild type PTEN-GFP were differentiated and placed in a cAMP gradient formed by micropipettes releasing 1 μM cAMP in the presence (*D*) or absence (*C*) of latrunculin A. *Arrowheads* show the position of micropipettes. A micropipette was positioned at the left corner of latrunculin A-treated cells and then was moved to the right corner. Images were taken at indicated time points under fluorescence microscope. *Scale bar*, 10 μm.

came rounded. PTEN in these cells behaved just like PTEN in wild type cells. These results showed that the distribution of PTEN is not regulated by intracellular amounts and changes in PI(3,4,5)P_3 levels. Therefore, the signal regulating PTEN localization lies upstream of the PI3K pathway.

We also tested whether PTEN localization requires the actin cytoskeleton (Fig. 6*B*). To determine the effect of the actin cytoskeleton on PTEN localization, we treated cells expressing wild type PTEN with an inhibitor of actin polymerization, latrunculin A. Cells exposed to latrunculin A became rounded and immobile; however, the PTEN was uniformly associated with plasma membrane and dissociated from plasma membrane in response to cAMP stimulation, similar to the cells without latrunculin A treatment. Furthermore, we examined the localization of PTEN in cells exposed to a gradient of chemoattractant. We have previously shown that PTEN localizes to the rear of chemotaxing cells. In the presence of latrunculin A, PTEN still accumulated at the "rear," which is opposite to the direction of the gradient. When the micropipette was repositioned to the other side, PTEN also redistributed accordingly away from the higher concentration. These results demonstrate that the movement of PTEN does not depend on the actin cytoskeleton. The localization of PTEN is likely regulated by plasma membrane components such as membrane protein and/or lipids.

Localization of PI3K Does Not Require PTEN—The amount of PI(3,4,5)P_3 is regulated by the activities of PTEN and PI3K. In *Dictyostelium* there are three PI3Ks similar to mammalian class I PI3K. Like PTEN, *Dictyostelium* PI3K1 and PI3K2 also change their localizations in response to cAMP (11), and a 600-amino acid N-terminal segment is sufficient for PI3K2 localization. The membrane localizations of PI3Ks correspond well with the activation of the enzymes (13). Recent studies have shown that delivery of exogenous PI(3,4,5)P_3 into neutrophils and fibroblasts triggers the activation of PI3K and accumulation of endogenous PI(3,4,5)P_3 (10, 24). If a similar positive feedback loop exists in *Dictyostelium*, PI3K should be constitutively activated in *pten*$^-$ cells.

To address whether localization of PI3K depends on PTEN, the N-terminal 600-amino acid segment from PI3K2 was fused to GFP (PI3K2$_{1-600}$) and expressed in wild type and *pten*$^-$ cells. Before cAMP stimulation, the majority of PI3K2$_{1-600}$ was found in the cytosol. Upon cAMP stimulation, PI3K2$_{1-600}$ was transiently translocated to the plasma membrane within 10 s and returned to the cytosol within 30 s after stimulation in both wild type and *pten*$^-$ cells. These results show that the localization of PI3K is not controlled by the intracellular level of PI(3,4,5)P_3.

DISCUSSION

In this study, we demonstrate that regulation of PTEN is essential for proper chemotaxis. Our previous studies showed that excess PI(3,4,5)P_3 accumulates in *pten*$^-$ cells and impairs chemotaxis (12). Here we prove that the lipid phosphatase activity of PTEN is critical for its role in chemotaxis. We show that proper binding of PTEN to the plasma membrane plays a key role in its regulation. Our evidence suggests that the N-terminal PI(4,5)P_2 binding motif both inhibits the enzyme and mediates its association with lipid substrates.

Although many studies have demonstrated that the regulation of lipid signaling is the physiological role of PTEN, some observations indicate that the PTEN directly dephosphorylate phosphotyrosine-containing polypeptides, focal adhesion kinase, and Shc (25–27). Protein phosphatase activity, not lipid phosphatase activity, is required for some PTEN functions including cell motility (28, 29). Even though PTEN has both activities, only the lipid phosphatase activity, which specifically dephosphorylates the D3 position of PI(3,4,5)P_3, is essential for in chemotaxis. Based on analogy with human PTEN, we introduced equivalent mutations in *Dictyostelium* PTEN that specifically block either the PTP activity (PTEN$_{G129E}$) or both protein and lipid phosphatase activities (PTEN$_{C124S}$) in human PTEN. The glycine at residue 129 is located at the bottom of the active site pocket. The G129E mutation reduces the size of PTEN pocket extension, which substantially blocks the lipids phosphatase activity without affecting protein phosphatase activity (18, 25). The cysteine at residue 124 is a catalytic residue for phosphatase activity. PTEN$_{C124S}$ tightly associates with its substrates and is defective in both lipid and protein phosphatase activity (18). Because we found that the phenotypes of *pten*$^-$ cells expressing PTEN$_{G129E}$ or PTEN$_{C124S}$ are essentially identical, the PTP activity is not important for the function of PTEN. These results suggest that normal growth, de-

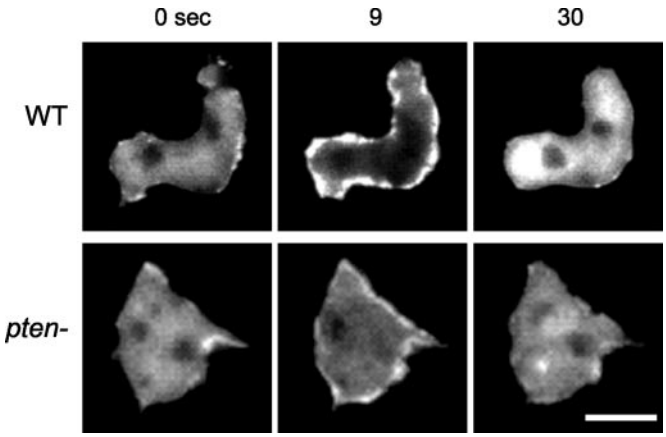

FIG. 7. **PI3K localization does not require PTEN.** Wild type and *pten*− cells expressing $PI3K_{0-600}$ were differentiated and uniformly stimulated with 1 μM cAMP. Upon stimulation with cAMP, $PI3K2_{0-600}$-GFP transiently associated with the plasma membrane in both wild type and *pten*$^-$ cells. Pictures were taken every 3 s under fluorescence microscope. *Bar*, 10 μm.

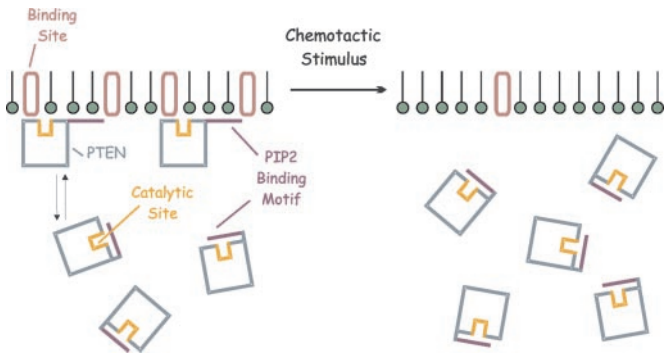

FIG. 8. **Membrane localization and activity of PTEN.** Diagram shows equilibration between membrane-bound and cytosolic PTEN in the presence and absence of chemoattractant. Resting membrane contains binding sites (*pink*) for PTEN (*blue*). PTEN in the cytosol is depicted in a "closed" conformation where the $PI(4,5)P_2$ binding motif (*purple*) masks the catalytic domain (*orange*). Bound PTEN is depicted in an open conformation where the catalytic domain is exposed. Membrane binding requires portions of PTEN besides the $PI(4,5)P_2$ binding motif. Chemoattractant reduces the binding sites for PTEN, and more protein is found in the cytosol.

velopment, and chemotaxis require the lipid phosphatase activity in *Dictyostelium*.

There was not a complete correspondence between the development and chemotaxis assays. For example, $PTEN_{K128M}$, which showed partial phosphatase activity, supported aggregation in the developmental assay but did not support chemotaxis. During development, which takes more than 24 h, the residual phosphatase activity of this mutant may be sufficient to induce partial aggregation of *pten*$^-$ cells. In our chemotaxis assay, which took only 10 min, we clearly saw that this mutant was defective in moving along the cAMP gradient. Therefore, the chemotaxis assay proved a more sensitive assay for defects in PTEN than the developmental assay. Although $PTEN_{G129E}$ and $PTEN_{C124S}$ have no detectable lipid phosphatase activity *in vitro*, it is possible that they have very low residual activities, which can partially rescue the phenotype of *pten*$^-$ cells in the developmental assay. However, we cannot exclude the possibility of unknown effects from other domains in PTEN.

In *pten*$^-$ cells, the basal level of $PI(3,4,5)P_3$ was similar to that seen in wild type cells (Fig. 3) (13). This observation suggests that there is another mechanism that controls the level of $PI(3,4,5)P_3$ in addition to PTEN. Supporting this idea, in PTEN mutants, although it takes longer than in wild type cells, $PI(3,4,5)P_3$ levels do finally return to the prestimulus level (12, 13). We have found another PTEN homolog in *Dictyostelium*.[2] This PTEN homolog may regulate the basal level of $PI(3,4,5)P_3$ in unstimulated cells. Furthermore, we show that PI3K is not activated without cAMP stimulation (Fig. 7). This could maintain a low basal level of $PI(3,4,5)P_3$ in the absence of PTEN.

PTEN associates with the plasma membrane in *Dictyostelium* cells in the absence of cAMP stimulation. In mammalian cells, although PTEN is mostly in the cytosol, several studies have reported a membrane localization (30–33), and several possible mechanisms for their localization are proposed. For example, the Ca^{2+}-independent C2 domain at the C terminus of mammalian PTEN binds to phospholipid vesicles consisting of phosphatidylcholine and phosphatidylserine (19). However, *Dictyostelium* $PTEN_{186-515}$, containing a sequence corresponding to the mammalian C2 domain, did not associate with the plasma membrane *in vivo*. Therefore, the C-terminal region of PTEN is not sufficient for its localization. In addition, it has been suggested that the PDZ-binding domain at the C terminus, (T/S)*X*V-COOH, in mammalian PTEN mediates its association with the plasma membrane through interactions with PDZ-containing proteins (30, 34, 35). However, *Dictyostelium* PTEN does not have the PDZ-binding domain. It is unlikely that the protein-protein interaction with PDZ-containing proteins regulates the membrane localization of PTEN in *Dictyostelium*.

A $PI(4,5)P_2$ binding motif, (K/R)X_4(K/R)*X*(K/R)(K/R), which is found in mammalian, *Caenorhabditis elegans*, *Saccharomyces pombe*, and *Dictyostelium* PTEN, is located at the N terminus of PTEN and is required for PTEN membrane localization in *Dictyostelium* cells. The removal of this motif, or point mutations of conserved residues in this motif, results in loss of function *in vivo* and loss of phosphatase activity against phosphoinositol lipids. When amino acid residues at Arg-6 and Lys-11, Lys-13, and Arg-14 or Arg-15 were changed to Ala, these three mutant versions of PTEN did not localize to the plasma membrane and lost lipid phosphatase activity against $PI(3,4)P_2$ and $PI(3,4,5)P_3$.[2] Interestingly, the missense mutation at amino acid residue Arg-15 is one of the most frequent mutations found in glioblastoma, and this mutation causes a loss of phosphatase activity against lipids (36). The cytosolic localization of PTEN, which contains mutations in the $PI(4,5)P_2$ binding motif, does not result from defects in phosphatase activity, and because the phosphatase-inactive mutants $PTEN_{C124S}$ and $PTEN_{G129E}$ associate with plasma membrane as wild type PTEN, phosphatase activity is not required for membrane localization.

These results indicate that the $PI(4,5)P_2$ binding motif is important for both localization and phosphatase activity. Interestingly, whereas the phosphatase activity of $PTEN_{16-515}$ is decreased against $PI(3,4,5)P_3$, it is preferentially increased against the water-soluble head group, $Ins(1,3,4,5)P_4$. These observations suggest that the $PI(4,5)P_2$ binding motif determines the substrate specificity for PTEN. We propose that the $PI(4,5)P_2$ binding motif masks the catalytic domain when the protein is in the cytosol (Fig. 8). Upon membrane binding, the constraint by the $PI(4,5)P_2$ binding motif is relieved, and the membrane-associated enzyme attains full activity. We presume that similar events occur *in vitro* when micellar substrates such as $PI(3,4,5)P_3$ and $PI(3,4)P_2$ are used. Recently two groups reported very interesting results demonstrating that PTEN binds to $PI(4,5)P_2$-containing vesicles through the $PI(4,5)P_2$ binding motif, and $PI(4,5)P_2$ enhances PTEN lipid phosphatase activity (37, 38). However, despite its importance,

[2] M. Iijima and P. N. Devreotes, unpublished observations.

the $PI(4,5)P_2$ binding motif is not sufficient for membrane localization, and additional domains contribute to the localization of PTEN.

PTEN transiently dissociates from the plasma membrane in response to cAMP stimulation (11, 12). In this study, we demonstrate that the transient dissociation of PTEN from the plasma membrane is independent of intracellular level of $PI(3,4,5)P_3$ and the actin cytoskeleton. Three phosphatase inactive mutants of PTEN, $PTEN_{C124E}$, $PTEN_{K128M}$, and $PTEN_{G129E}$, normally associate with the plasma membrane and transiently relocate into the cytosol in response to cAMP in *pten*$^-$ cells. In a gradient of cAMP, these PTEN mutants are normally located at the rear of chemotaxing cells. Similarly, PTEN shows normal distribution in the presence of the PI3K inhibitor LY294002 (~100 μM), which almost completely inhibits its $PI(3,4,5)P_3$ production and the redistribution of the PH domain derived from Crac upon cAMP stimulation in *Dictyostelium* cells (39). These results clearly indicate that PTEN localization is regulated by a signaling pathway distinct from $PI(3,4,5)P_3$-mediated mechanisms. Furthermore, the localization of PTEN does not depend on the actin cytoskeleton. PTEN is normally distributed in cells in the presence of latrunculin A. A recent study has shown that mammalian PTEN localizes at the rear of chemotaxing neutrophils lacking PI3Kγ (33), although another report found that PTEN-GFP does not bind to the membrane in neutrophils (40).

The localization of PI3K is also regulated independently of $PI(3,4,5)P_3$. PI3K is located in the cytosol in the absence of cAMP and transiently binds to the plasma membrane upon cAMP stimulation. We found that the transient translocation of PI3K to the plasma membrane normally occurs in *pten*$^-$ cells. Similarly, PI3K also normally distributed in the presence of LY294002.[2] We have previously shown that the activation of PI3K induced by cAMP stimulation is indistinguishable between wild type and *pten*$^-$ cells (13). Thus, both the recruitment of PI3K to the plasma membrane and its activation take place independently of $PI(3,4,5)P_3$. We speculate that the $PI(3,4,5)P_3$-independent membrane association of PI3K may be a mechanism for the activation (13). In this scenario, the recruitment of PI3K could increase local concentration of PI3K and its substrate. Alternatively, PI3K could be activated by other proteins located in the plasma membrane. Consistent with this model, Ras GTPase is proposed to activate PI3K at the plasma membrane (11).

In summary, we demonstrate that *Dictyostelium* PTEN directly controls the sensing of chemoattractant gradients. The localization of PI3K and PTEN is regulated upstream of $PI(3,4,5)P_3$-mediated lipid signaling and is critical for their function in the directional sensing of extracellular chemical gradient. It will be of interest to find binding sites for PTEN and PI3K in the plasma membrane and to determine how these sites are created by chemoattractant.

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