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A PTEN-related 5-Phosphatidylinositol Phosphatase Localized in the Golgi*

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Sylvain Merlot‡, Ruedi Meili‡, David J. Pagliarini§, Tomohiko Maehama¶‖, Jack E. Dixon§¶, and Richard A. Firtel‡

From the ‡Section of Cell and Developmental Biology, Division of Biological Sciences, Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0634, the ¶Department of Biological Chemistry, Medical School and Life Sciences Institute, University of Michigan, Ann Arbor, Michigan 48109, and the §Departments of Pharmacology, Cellular and Molecular Medicine, and Chemistry and Biochemistry, School of Medicine, University of California, San Diego, La Jolla, California 92093-0602

Phosphoinositides play important roles as signaling molecules in different cell compartments by regulating the localization and activity of proteins through their interaction with specific domains. The activity of these lipids depends on which sites on the inositol ring are phosphorylated. Signaling pathways dependent on phosphoinositides phosphorylated at the D3 position of this ring (3-phosphoinositides) are negatively regulated by 3-phosphoinositide-specific phosphatases that include PTEN and myotubularin. Using the conserved PTEN catalytic core motif, we have identified a new protein in the *Dictyostelium* genome called phospholipid-inositol phosphatase (PLIP), which defines a new subfamily of phosphoinositide phosphatases clearly distinct from PTEN or other closely related proteins. We show that PLIP is able to dephosphorylate a broad spectrum of phosphoinositides, including 3-phosphoinositides. In contrast to previously characterized phosphoinositide phosphatases, PLIP has a preference for phosphatidylinositol 5-phosphate, a newly discovered phosphoinositide. We found that PLIP is localized in the Golgi, with its phosphatase domain facing the cytoplasmic compartment. PLIP null cells created via homologous recombination are unable to effectively aggregate to form multicellular organisms at low cell densities. The presence of PLIP in the Golgi suggests that it may be involved in membrane trafficking.

A significant portion of our knowledge on the role of phosphoinositides as signaling molecules comes from the analysis of 3-phosphoinositides that regulate the localization and activity of proteins through interaction with their PH,[1] FYVE, or Phox homology domains (1, 2). A subfamily of PH domains, including the PH domain of protein kinase B (Akt), specifically bind $PI(3,4,5)P_3$ and $PI(3,4)P_2$, the products of Class I phosphatidylinositol 3-kinases (3, 4). In response to growth factor or chemoattractant stimulation, protein kinase B translocates transiently to the plasma membrane through the binding of the PH domain to $PI(3,4,5)P_3$/$PI(3,4,)P_2$ (5–11). In contrast, PI(3)P, the product of Class III phosphatidylinositol 3-kinases, is primarily found in endosomal membranes, where it recruits FYVE domain-containing proteins such as EAA1 or Vac1p to regulate membrane trafficking and fusion (12–15). Furthermore, PI(5)P and $PI(3,5)P_2$ were identified more recently as naturally occurring phosphoinositides and have been proposed to act as signaling molecules through a mechanism yet to be discovered (16–18).

Although most of the efforts have been dedicated to the identification of kinases involved in the synthesis of these phospholipids, the identity and regulation of the lipid phosphatases involved in their turnover are not well understood. Two families of phosphatases have been implicated in the dephosphorylation of 3-phosphoinositides: the tumor suppressor PTEN and myotubularin (MTMR) phosphatases display the signature motif CX_5R characteristic of the dual specificity protein phosphatase family, but *in vivo* dephosphorylate $PI(3,4,5)P_3$ and PI(3)P/$PI(3,5)P_2$, respectively (16, 19–21). The catalytic domain of the PTEN and myotubularin (MTMR) phosphatases is unrelated to one of the characterized 5-phosphoinositide phosphatases that is composed of two signature motifs: (F/I)W*X*GD*X*N(F/Y)R and (R/N)*X*P(S/A)(W/Y)(C/T) DR(I/V)(L/I) (22). Mutations in *PTEN* are associated with a wide variety of human tumors and are linked to the development of Cowden's disease and Bannayan-Zonana syndrome (23–25). Mutations in the myotubularin *MTM1* gene cause myotubular myopathy, whereas mutations in the related gene *MTMR2* are linked to the neuromuscular disorder Type 4B Charcot-Marie-Tooth disease (21, 23, 26).

The crystal structure of PTEN indicates that the conserved basic amino acids (Lys^{125} and Lys^{128}) present in the PTEN catalytic loop (CK*XXKX*R) define the specificity of PTEN for $PI(3,4,5)P_3$ (27). Using this information, we screened the *Dictyostelium* genomic data base for open reading frames containing a catalytic loop similar to that of PTEN. We identified a new phosphoinositide phosphatase we call phospholipid-inositol phosphatase (PLIP), which, along with related proteins in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY347275.

‖ Present address: Dept. of Pharmacology, Tokyo Metropolitan Inst. of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8623, Japan.

** To whom correspondence should be addressed: Center for Molecular Genetics, Rm. 225, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0634. Tel.: 858-534-2788; Fax: 858-822-5900; E-mail: rafirtel@ucsd.edu.

[1] The abbreviations used are: PH, pleckstrin homology; PI, phosphatidylinositol; PLIP, phospholipid-inositol phosphatase; HA, hemagglutinin; GFP, green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate.

This paper is available on line at http://www.jbc.org

metazoans, define a new family of phosphoinositide phosphatases distinct from PTEN and conserved in eukaryotes. Unexpectedly, PLIP prefers PI(5)P as substrate *in vitro*. PLIP has a transmembrane domain and localizes to the Golgi, suggesting that it may have a role in protein sorting or membrane trafficking.

EXPERIMENTAL PROCEDURES

Cloning and Sequence Analysis—PLIP cDNA was cloned by screening 1×10^6 plaque-forming units of a *Dictyostelium* cDNA library using, as a probe, a PCR fragment corresponding to the catalytic domain of PLIP. Among the five clones identified, the one containing the longest insert (900 bp) was converted to a plasmid (pBS-PLIP) and sequenced. Sequence analysis and alignment were done using the BLAST and ClustalW programs.

PLIP tagged by the HA epitope was obtained by PCR amplification of the PLIP coding sequence from pBS-PLIP using primers 5′-TTTGGATCCATGAATGAAAATGAAGGAATA-3′ and 5′-TTTTTTCTCGAGTTTTTATTAAGCATAATCTGGAACATCATATGGATAGATCTCAATTGATGTTGTTGAATTAATATTAC-3′. The PLIP(D136A)-HA mutant was obtained by PCR using mutagenic primers 5′-TGGTTCGAAATGAGCAACCACTGGCAC-3′ and 5′-GTGCCAGTGGTTGCTCATTTCGAACCA-3′. PCR products were cloned as *Bam*HI/*Xho*I fragments into the DIPJ vector (28).

GFP was fused at the C terminus of PLIP (PLIP-GFP) by PCR using primers 5′-TTTGGATCCATGATTGAAAATGAAGGAATA-3′ and 5′-CCGGAATTCAGATCTAATTGATGTTGTTGAATTAAT-3′. The PCR product was then digested with *Bam*HI and *Bgl*II and fused to GFP to make a translation fusion into the DIPJ vector. The sequences of the constructs were verified by sequencing. N-terminally GFP-tagged golvesin (GFP-golvesin) and C-terminally GFP-tagged golvesin (golvesin-GFP) constructs (29) were provided by Günther Gerisch (Max Planck Institute for Biochemistry, Martinsreid, Germany). These constructs were introduced into *Dictyostelium* by electroporation, and stable transformants were selected on G418.

Immunofluorescence Study—For immunofluorescence localization of the proteins, cells attached to a coverslip were fixed with 3.7% formaldehyde for 20 min. Cells were then permeabilized with 0.5% Triton X-100 for 1 min and washed with phosphate-buffered saline (pH 7.4). The coverslips were incubated with the primary antibody in phosphate-buffered saline (pH 7.4) containing 1 mg/ml bovine serum albumin and 0.2% Tween 20 for 2 h at room temperature. Anti-HA polyclonal antibody (Santa Cruz Biotechnology) and anti-α-tubulin antibody DM1A (Sigma) were diluted 200 times. Antibody 1/39-16 was obtained from P. Cosson (University of Geneva, Geneva, Switzerland) and was diluted 10 times. Coverslips were washed with phosphate-buffered saline and incubated for 1 h under the conditions described above with either anti-rabbit or anti-mouse antibody coupled to fluorescein isothiocyanate or TRITC (Sigma). During the second incubation, Hoechst dye was added (0.001% final concentration) to stain the nuclei. Slides were observed using a Nikon fluorescent microscope equipped with a ×60 objective. Pictures were captured with a CDD digital camera and processed with Metamorph software (Universal Imaging Corp., Downingtown, PA).

Protein Extraction, Fractionation, and Trypsin Digest—Log phase cells ($2–4 \times 10^6$ cells/ml) were washed once with 12 mM phosphate buffer (pH 6.1) and resuspended in lysis buffer (50 mM Hepes (pH 8), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mg/ml aprotinin, and 2 mg/ml leupeptin). Cells were disrupted by passage through two layers of 3-μm Nuclepore membrane. The lysate was precleared by centrifugation at $1000 \times g$ for 5 min and then fractionated into soluble and insoluble (pellet) fractions by 1 h of centrifugation at $100{,}000 \times g$. The precleared lysate was incubated on ice for 15 min in the presence of NaCl, Na_2CO_3, or Triton X-100 before fractionation.

For the trypsin digest, the precleared cell lysate was incubated on ice for 20 min with trypsin at a protein/trypsin ratio from 1:5 to 1:20 (w/w). The reaction was stopped by the addition of Laemmli loading buffer.

Phosphatase Assay—Dictyostelium PLIP was cloned into pGEX-6P-1 (Amersham Biosciences) and expressed in *Escherichia coli*. The glutathione *S*-transferase fusion protein was purified on glutathione-agarose beads, followed by separation on a Superdex 75 gel filtration column (Amersham Biosciences). The phosphatase assays were conducted using the malachite green reagent as previously reported (24) with minor modifications. The assays were carried out in a final concentration of 0.5 mM phosphatidylserine (C_{18}; Sigma) and 0.1 mM D-*myo*-phosphatidylinositol substrate (Echelon Biosciences). Ten μg of partially purified PLIP was used in each assay for various time points at 37 °C and pH 5.5. *p*-Nitrophenyl phosphate activities were examined as previously reported (30).

Creation of the PLIP Null Mutant—A 1.6-kb genomic sequence emphasizing the PLIP coding sequence was amplified with oligonucleotides 5′-AAAAACTCGAGAAAAATCTATTTCTCTCGC-3′ and 5′-CGACTTAAGCTTCTAAAGCACCTGATCCACC-3′ from wild-type KAx-3 DNA and cloned as a *Hin*dIII/*Xho*I fragment into the pSP71 vector (Promega). The *Bsa*BI restriction site located in the PLIP coding sequence was used to insert the blasticidin resistance cassette (31). This construct was transformed into KAx-3 cells, and clones resistant to blasticidin were randomly selected. PLIP knockout mutants were selected by Southern blot analysis.

PLIP expression was analyzed by Northern blot analysis using total RNA (6 μg) extracted from vegetative cells (no starvation) and from cells starved for 4, 8, 12, 16, 20, and 24 h on phosphate buffer plates to induce development. For PLIP knockout cell lines, we used RNA extracted from vegetative cells.

α-Mannosidase and β-Glucosidase Processing Assay—[^{35}S]Met labeling of *Dictyostelium* cells and immunoprecipitation of α-mannosidase and β-glucosidase were done as described previously (32) with the following minor modifications. Cells were pulsed for 20 min with 750 μCi/ml [^{35}S]Met. Cells were spun down and resuspended in HL5 medium containing 10 and 8.5 mM unlabeled Met and Cys, respectively. Monoclonal antibodies against α-mannosidase (clone 2H9) and β-glucosidase (clone 2F5) were a gift from James A. Cardelli.

RESULTS

Identification of a PTEN-related Gene in D. discoideum—To identify potential homologs of the phosphatidylinositol 3-phosphatase PTEN in *Dictyostelium*, we screened the *Dictyostelium* genome using the BLAST program with the human PTEN P-loop as bait. In addition to the previously identified *Dictyostelium* homolog of mammalian PTEN (33, 34), we identified a predicted protein (GenBank™ accession number AA052139) containing a domain with high sequence homology to the human PTEN P-loop catalytic domain. Because of this homology to PTEN in the catalytic domain, we designated this protein PLIP for phospholipid-inositol phosphatase. A PCR product containing this domain was used to screen a cDNA library. The sequence of the longest clone (pBS-PLIP) revealed an open reading frame of 232 amino acids starting with an ATG codon in a good context for translation initiation in *Dictyostelium*. The deduced sequence of PLIP was identical to the predicted protein AA052139.

The PLIP phosphatase signature motif is more closely related to the P-loop of PTEN (Fig. 1*A*) than to other members of this family of phosphatases, which includes protein-tyrosine phosphatases. This domain includes two conserved basic amino acids, Lys^{167} and Arg^{170}, in PLIP, corresponding to Lys^{125} and Lys^{128} in human PTEN. These two lysines give to the catalytic pocket a basic character compatible with the negative charge of the phospholipids, and Lys^{128} makes contact with the phosphate group at the 5′-position of the inositol ring and is therefore a key determinant of the PTEN specificity for PI(3,4,5)P_3. Substitution of Arg for Lys^{128} in human PTEN does not affect the catalytic activity of PTEN for PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3 *in vitro* (27), suggesting that the Lys-to-Arg substitution in PLIP may not affect substrate specificity of the enzyme. Asp^{136} and His^{137} in PLIP correspond to Asp^{92} and His^{93} in human PTEN, which are also important for human PTEN catalytic activity and substrate specificity. These features suggest that PLIP could be a phosphatidylinositol 3-phosphatase.

Besides this conserved catalytic loop, the catalytic domain of PLIP is clearly distinct from that of PTEN (Fig. 1*B*). Furthermore, PTEN and the closely related proteins PTEN2, TPIP, and TPEP (35–37) have a C2 domain in their C termini that it supposed to bind lipids. PLIP does not contain a C2 lipid-interacting domain. However, PLIP has a hydrophobic stretch of 23 amino acids at its N terminus (Fig. 2*A*) that is predicted

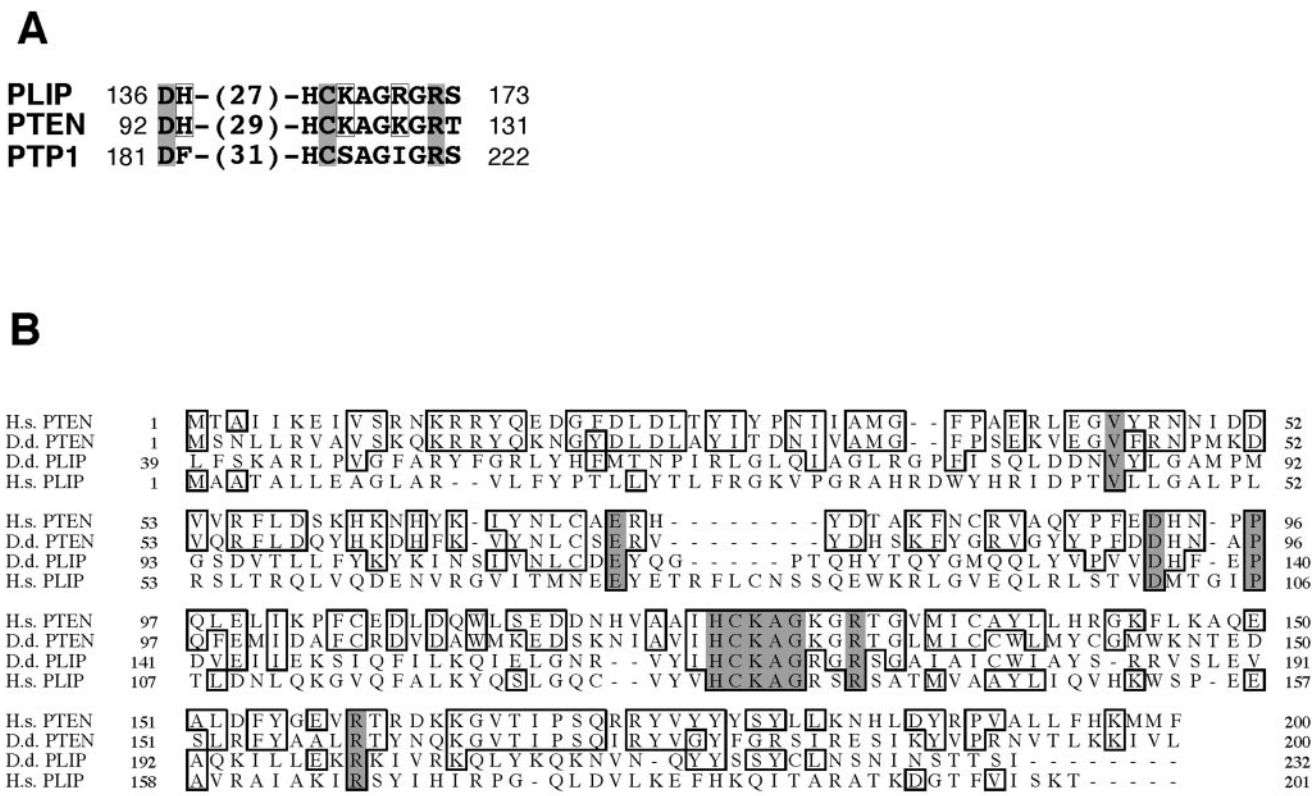

FIG. 1. **Comparison between PLIP and PTEN phosphatases.** *A*, sequence alignment of the catalytic sites of PLIP (GenBank™ accession number AA052139), human PTEN (GenBank™ accession number gi 14740594), and human protein tyrosine phosphatase-1 (*PTP1*). This alignment shows the conserved amino acids necessary for the dephosphorylation reaction (*shaded*) and the basic amino acids conserved between PTEN and PLIP (*boxed*). *B*, comparison of the phosphatase domain of PTEN from both *Homo sapiens* (*H.s.*; gi 14740594) and *Dictyostelium discoideum* (*D.d.*; gi 18542385) and the phosphatase domain from *Dictyostelium* PLIP (AA052139) and its human counterpart (gi 18044254).

to be a transmembrane helical domain (TMHMM Version 2.0), suggesting that PLIP may be an integral membrane protein. This analysis of the PLIP sequence indicates that PLIP is only weakly related to PTEN and therefore is not a *Dictyostelium* homolog of PTEN2, TPIP, or TPEP.

Screening of available data bases has identified PLIP homologs in plants and metazoans, including humans, but no clear homolog has been found in yeast (Fig. 1*B*). We propose that PLIP defines a previously unidentified family of phosphatidylinositol phosphatases (see below). These proteins share a catalytic loop very homologous to that of PLIP and homology throughout their sequence, including an invariant LGA*X*P motif upstream of the CX_5R active site (Fig. 2), the function of which is not known. The putative N-terminal transmembrane domain is not a conserved feature of this family because human and mouse members of this family do not contain any putative transmembrane domain. Although a putative N-terminal transmembrane domain was identified in the homologs from *Arabidopsis* and zebrafish, its precise location within these proteins is not conserved, and there are no data to demonstrate that this is a transmembrane domain.

PLIP Localizes in the Golgi System—In unstimulated cells, PTEN localizes to the plasma membrane, where it is involved in the dephosphorylation of PI(3,4,5)P_3/PI(3,4)P_2 (33, 34), whereas PTEN2 localizes to the Golgi, and TPIPα localizes to the endoplasmic reticulum (35, 36). We investigated the intracellular localization of PLIP by indirect immunofluorescence using cells expressing HA-tagged PLIP. Fig. 3 shows that PLIP-HA localized in patch-like structures in the cell. We did not observe any plasma membrane staining in growing or chemotaxis-competent cells before or after chemoattractant stimulation (data not shown). The same subcellular localization was observed using C-terminally GFP-tagged PLIP (PLIP-GFP) (data not shown). Co-staining of PLIP-HA and nuclei revealed that the region of PLIP staining was always closely associated with the nucleus. In multinucleate cells, which comprise a low percentage of cells in axenically grown *Dictyostelium* cell populations, each nucleus was associated with a similar PLIP-staining region. These observations suggest that PLIP localizes to the Golgi system. As shown in Fig. 3, this conclusion was confirmed by co-immunostaining of PLIP-HA and the Golgi marker antibody 1/39 (38) or golvesin-GFP (29).

In *Dictyostelium*, as in mammalian cells, the Golgi system is closely linked to the microtubule organization center (39–42); and the drug nocodazole, which induces microtubule depolymerization, promotes the disassembly of the Golgi apparatus into smaller, scattered structures (39, 40, 43). Co-labeling experiments with anti-α-tubulin antibody indicated that PLIP-HA co-localized with the microtubule organization center (Fig. 3). After a 1-h treatment with 10 μM nocodazole, the microtubule network had disappeared, whereas the microtubule organization center remained intact. In these nocodazole-treated cells, PLIP-HA associated with smaller, patched structures linked to the microtubule organization center that scattered throughout the cytoplasm. These structures probably represent smaller, dispersed Golgi vesicles. These data are consistent with PLIP being a Golgi protein.

PLIP Topology—The presence of a putative transmembrane domain suggests that PLIP is an integral membrane protein of the Golgi system. To test this model, cells expressing PLIP-HA and PLIP-GFP were mechanically lysed, and the particulate fraction was isolated by high speed centrifugation. Western blot analyses demonstrated that PLIP-HA was detected only in the particulate fraction (Fig. 4*A*). It was not solubilized by 1 M NaCl, 100 mM Na_2CO_3 (pH 10.5–11), or 5% Triton X-100. Treat-

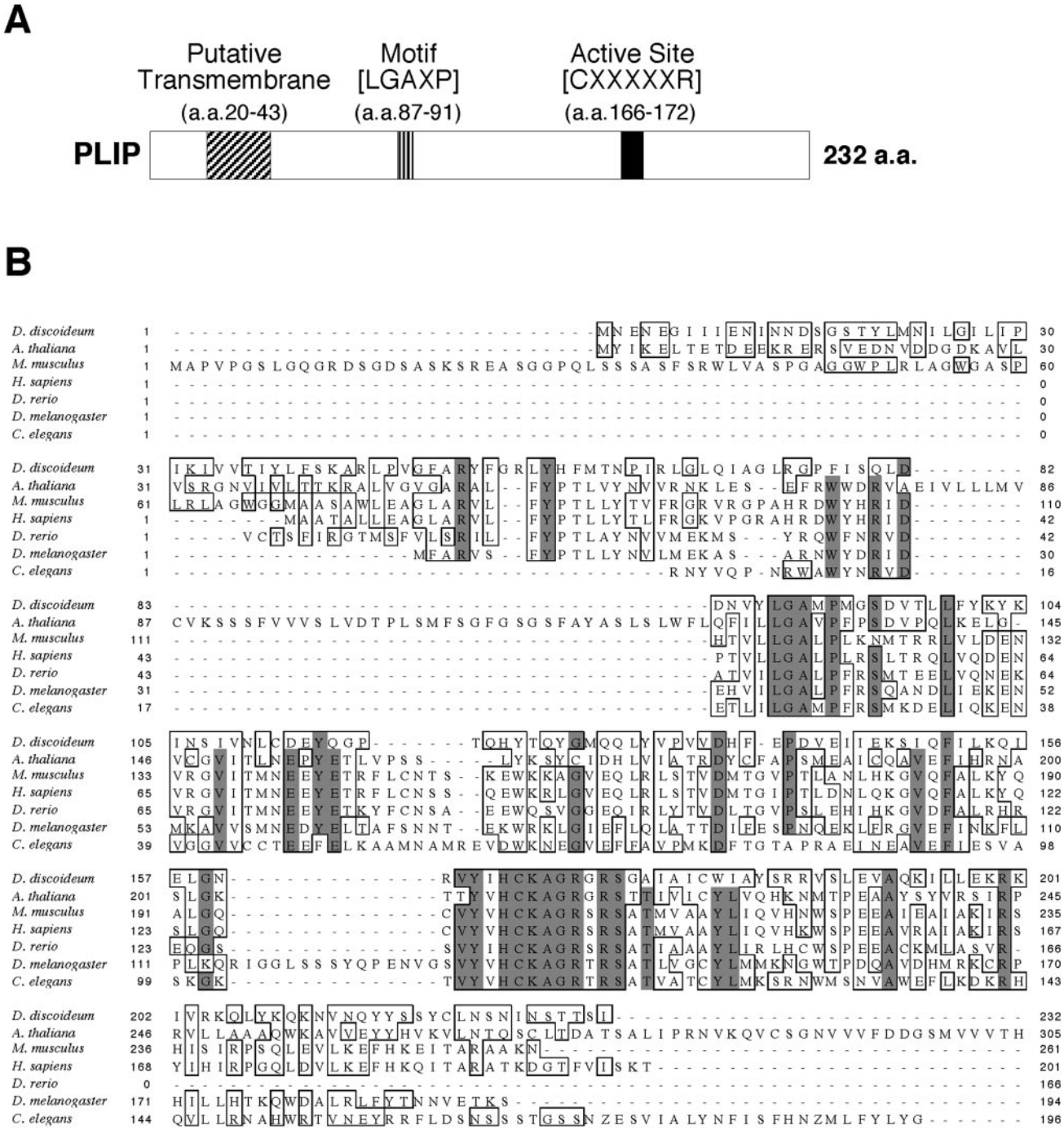

FIG. 2. **PLIP phosphatase family.** *A*, structure of the *Dictyostelium* PLIP protein. *a.a.*, amino acids. *B*, sequence alignment obtained by ClustalW of PLIP homologs from different organisms: *Arabidopsis thaliana* (GenBank™ accession number gi 25408407), *Mus musculus* (gi 20839608), *H. sapiens* (gi 18044254), *Danio rerio* (deduced from cDNA sequence gi 5759841), *Drosophila melanogaster* (gi 7301043), and *Caenorhabditis elegans* (deduced from cDNA sequence gi 14832687).

ment of this cell fraction with 500 mM NaCl and 2.5% Triton X-100 led to PLIP-HA solubilization. PLIP-GFP exhibited identical properties (data not shown), consistent with PLIP being an integral membrane protein.

To examine whether the PLIP catalytic domain is in the cytoplasmic or luminal compartment, we treated a crude cell lysate expressing PLIP-GFP with the non-membrane-permeant protease trypsin (Fig. 4*B*). In these experiments, we used golvesin fused to GFP at either the N or C terminus as an internal control (29). Golvesin is a Golgi protein that contains a short N-terminal domain followed by a transmembrane domain and a long C-terminal domain. PLIP-GFP is rapidly degraded by trypsin, producing a band corresponding to GFP, which is resistant to trypsin digestion (44). GFP-golvesin is also rapidly digested by trypsin, producing a GFP-sized fragment. In contrast, the treatment of golvesin-GFP with trypsin resulted in the formation of two slightly shorter peptides recognized by anti-GFP antibody, consistent with only the short N-terminal domain of golvesin being accessible to the protease. These results support a spatial model in which the C terminus of PLIP containing the phosphatase domain faces the cytoplasmic compartment, whereas the C terminus of golvesin is luminal (Fig. 4*C*).

PLIP Has Phosphatidylinositol Phosphatase Activity—Based

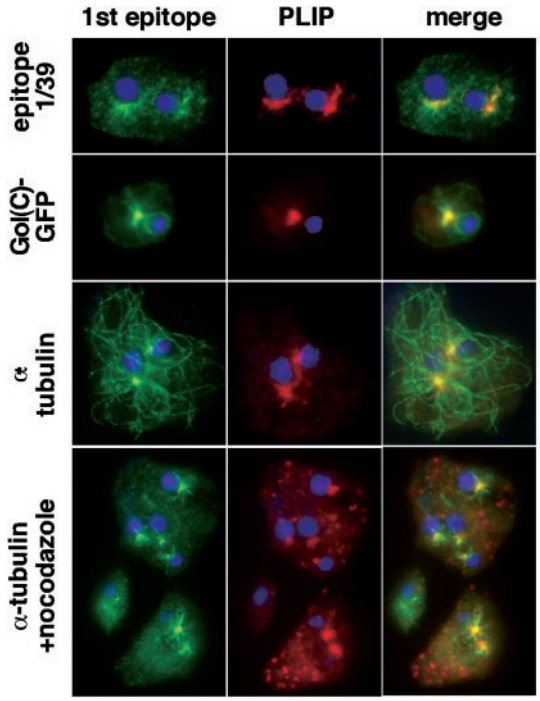

FIG. 3. **Immunolocalization of PLIP.** PLIP-HA was expressed in KAx-3 cells and golvesin-GFP-expressing cells. *Left panels*, cells were labeled for the specified protein using a secondary antibody coupled to fluorescein isothiocyanate (*green*). Golvesin-GFP (*Gol(C)-GFP*) was revealed by GFP fluorescence. *Middle panels*, PLIP was detected using anti-HA antibody and the secondary antibody coupled to TRITC (*red*). *Right panels*, shown are images of superimposed pictures from the *left* and *middle panels*. Overlaps between PLIP-HA and the second protein appear in *yellow*. In all panels, nuclei stained with Hoechst appear in *blue*. In the *lower panels*, cells were treated for 2 h with 10 μM nocodazole before fixation.

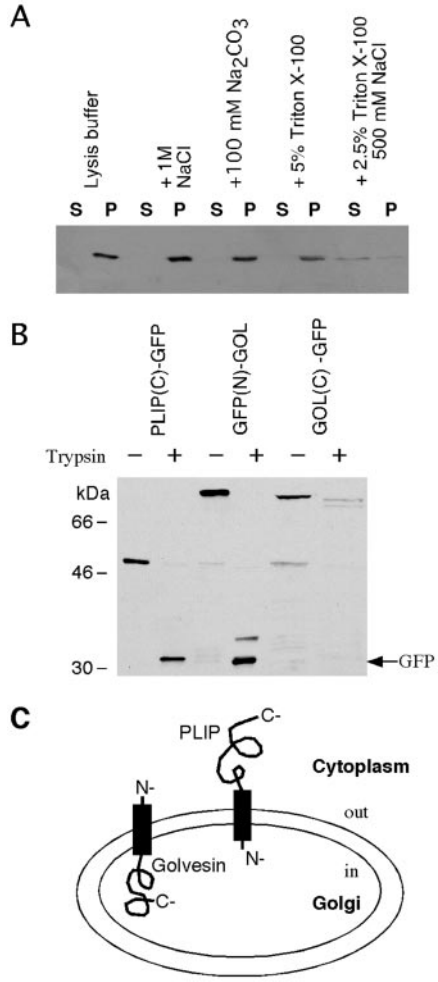

FIG. 4. **Analysis of PLIP topology.** *A*, lysates of cells expressing PLIP-GFP were fractionated as soluble (*S*) and insoluble pellet (*P*) fractions by centrifugation at 100,000 × *g* in different salt and detergent combinations. Proteins were resolved by SDS-PAGE and labeled with anti-GFP antibody. *B*, cell lysates from PLIP-GFP-, GFP-golvesin (*GOL*)-, and golvesin-GFP-expressing cells were incubated with (+) or without (−) trypsin. Proteins were resolved by SDS-PAGE and labeled with anti-GFP antibody. *C*, shown is a model for the topology of PLIP and golvesin in the Golgi apparatus.

on primary sequence similarity between PLIP and known phosphoinositide phosphatases related to PTEN in the putative P-loop region, we predicted that PLIP would exhibit phosphoinositide phosphatase activity. To test this, full-length glutathione *S*-transferase-PLIP was expressed in *E. coli*, purified to near homogeneity, and tested for phosphoinositide phosphatase activity *in vitro* against a panel of di-C_8-phosphoinositide substrates (see "Experimental Procedures"). Although the overall specific activity of PLIP was significantly lower than that of other known phosphoinositide phosphatases, PLIP was able to dephosphorylate each phosphoinositide substrate, including 3-phosphoinositides (Fig. 5*A*). Surprisingly, PLIP exhibited a preference for PI(5)P. Furthermore, incubation of PLIP with 50 mM *N*-ethylmaleimide, a thiol-modifying reagent known to react with the active-site cysteine nucleophile of protein tyrosine phosphatases (20), reduced the activity of PLIP for PI(5)P by ~10-fold. Although we have not analyzed many potential proteinaceous substrates, the ability of an enzyme to act as a protein phosphatase *in vivo* is often accurately reflected by its ability to utilize *p*-nitrophenyl phosphate as a substrate (30). Fig. 5*B* shows that the *in vitro* *p*-nitrophenyl phosphate activity of PLIP is 4 orders of magnitude lower than that of laforin, a known protein phosphatase, supporting the hypothesis that PLIP acts as a phosphoinositide phosphatase rather than a protein phosphatase *in vivo*.

Phenotype of PLIP Null Cells—To access the role of PLIP *in vivo*, two independent PLIP knockout mutants were created by homologous recombination and confirmed by Southern blot analysis (see "Experimental Procedures") (data not shown). RNA blot analysis revealed that PLIP mRNA, which is expressed in vegetative cells and throughout development, was not detectable in either PLIP null strain (Fig. 6*A*).

When PLIP null cells were plated for multicellular development on non-nutrient phosphate-buffered agar at a standard cell density (8×10^5 cells/cm^2), we observed no difference between the null strain and the parental wild-type control strain (Fig. 6*B*). However, at a lower cell density (2×10^5 cells/cm^2), aggregation was significantly delayed in PLIP null cells. Thus, although PLIP is not essential for *Dictyostelium* development, PLIP is required for proper aggregation under conditions in which cells must signal over longer distances. Aggregation is mediated by the chemotaxis of cells toward the chemoattractant cAMP, which is regulated, in part, by PI(3,4,5)P_3 signaling at the plasma membrane (6, 11, 33, 34). However, analysis of chemotaxis of PLIP null cells to a micropipette emitting cAMP and the kinetics and level of cAMP-stimulated Akt/protein kinase B activation did not reveal any difference between PLIP null cells and the wild-type control (data not shown). These results suggest that PLIP is not directly involved in the dephosphorylation of PI(3,4,5)P_3 at the plasma membrane, consistent with the Golgi localization of PLIP, and that the deletion (mutation) of the PLIP gene does not significantly affect PI(3,4,5)P_3 metabolism at the plasma membrane.

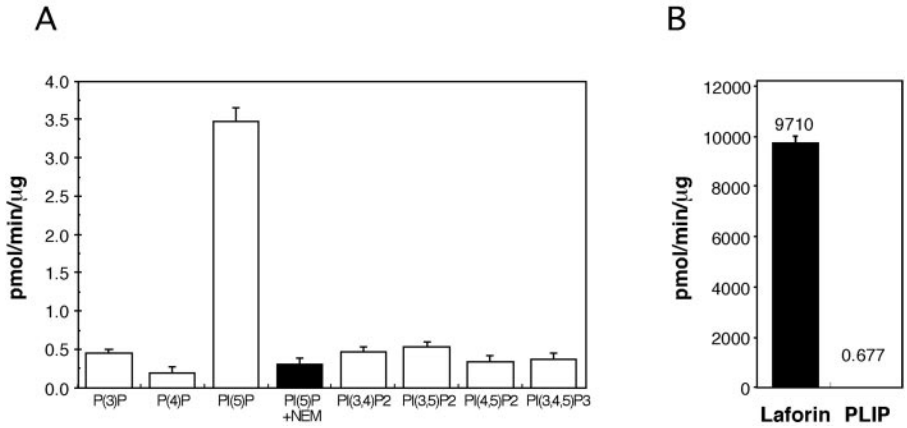

FIG. 5. **PLIP phosphatase activity.** *A*, PLIP phosphatase activity against various phosphoinositide substrates as outlined under "Experimental Procedures." The *black bar* represents the activity of PLIP against PI(5)P after incubation with 50 mM *N*-ethylmaleimide (*NEM*) on ice for 5 min. *B*, phosphatase activity against *p*-nitrophenyl phosphate of PLIP and laforin, a known protein phosphatase.

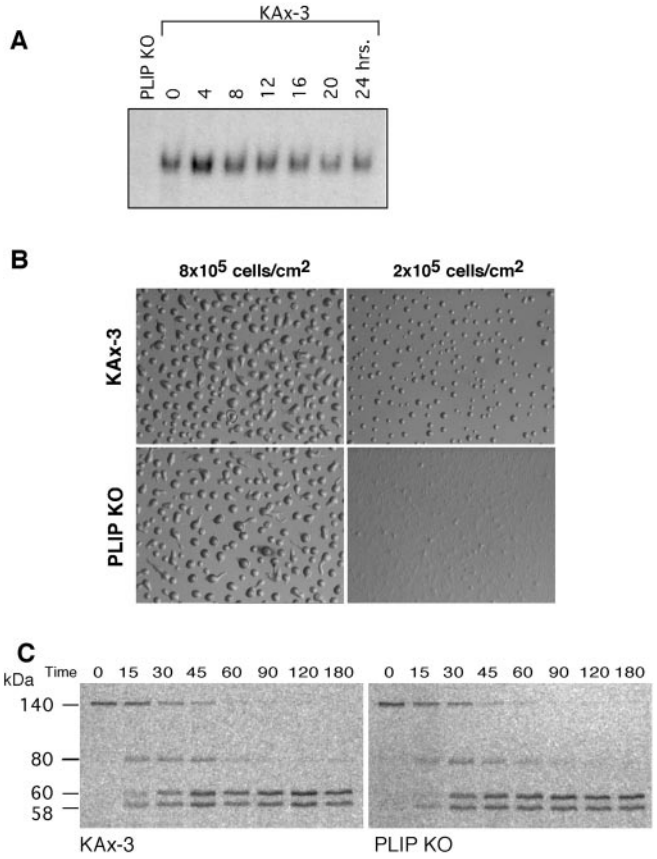

FIG. 6. **Analysis of the PLIP knockout mutant.** *A*, Northern blot analysis of PLIP mRNA expression in vegetative parental wild-type strain KAx-3 and PLIP knockout (*KO*) cell lines and during KAx-3 development after 0, 4, 8, 12, 16, 20, and 24 h of starvation. *B*, developmental analysis of KAx-3 and PLIP knockout cells. Cells were plated on sodium/potassium-agar medium and starved for 8 h. Cells were plated at a high density (8×10^5 cells/cm^2; *left panels*) and at a lower density (2×10^5 cells/cm^2; *right panels*). *C*, kinetics of α-mannosidase maturation in KAx-3 and PLIP knockout cells. Cells were labeled with [^{35}S]Met; and at different time points after the beginning of the chase, cells were lysed, and α-mannosidase was immunoprecipitated. Precipitated proteins were resolved by SDS-PAGE and detected with a radioactivity-sensitive screen.

This finding may not be unexpected considering the preference of PLIP for PI(5)P *in vitro*. We then investigated whether PLIP null cells exhibit a defect in Golgi structure or function. PLIP null and wild-type cells were transformed with PLIP-HA and PLIP(D136A)-HA, a putative phosphatase-inactive PLIP mutant in which a conserved Asp residue in the catalytic domain that is essential for lipid and protein tyrosine phosphatase activity in other members of this protein family has been mutated to Ala (23, 45). Immunofluorescence studies indicated that the Golgi structures with PLIP(D136A)-HA and PLIP-HA in wild-type and PLIP null cells were indistinguishable, indicating that disruption of PLIP does not visibly affect the Golgi structure as assayed by light microscopy.

Phosphoinositides have been implicated in membrane trafficking (2, 46). We therefore wondered whether PLIP is involved in the regulation of protein processing in the Golgi or transport from the Golgi to the lysosome. To examine this, we analyzed the maturation of α-mannosidase and β-glucosidase in PLIP null cells (47, 48). α-Mannosidase is synthesized as a 140-kDa precursor and subsequently processed to an 80-kDa intermediate form and a mature 58-kDa subunit in the late Golgi or early endosome. The 80-kDa intermediate is then further processed in the lysosome to form the mature 60-kDa α-mannosidase. To examine the processing of α-mannosidase, we performed pulse-chase experiments and examined the time course of α-mannosidase processing (32). As shown in Fig. 6*C*, no difference was seen in α-mannosidase maturation between PLIP null and wild-type cells. β-Glucosidase is synthesized as a 105-kDa precursor and then processed to a 100-kDa protein in the lysosome. We did not observe any defect in β-glucosidase maturation in PLIP null cells (data not shown). These results suggest that PLIP is not required for the maturation of this class of proteins in the Golgi system or their transport to the lysosome compartments.

DISCUSSION

In this study, we identified a new protein in *Dictyostelium* called PLIP, which has an unexpected phosphoinositide phosphatase activity. Although PLIP was identified based on its similarity to PTEN in the CX_5R phosphatase signature motif, PLIP activity is clearly distinct from the previously identified PTEN and other members of the family of phosphoinositide 3-phosphatases. *In vitro*, PLIP was able to dephosphorylate a broad panel of phosphoinositides, including 3-phosphoinositides. Surprisingly and in contrast to PTEN and the related protein PTEN2 (36), PLIP preferred PI(5)P as substrate. To our knowledge, this is the first example of a phosphatase with a preference for this phosphoinositide. However, as PI(5)P was discovered only recently, it was not used to characterize the activity of other phosphoinositide phosphatases such as TPIP (35) and 5-phosphoinositide phosphatases (22). It is therefore possible that other previously identified phos-

phoinositide phosphatases may also exhibit activity against PI(5)P.

Further studies will be needed to identify the physiological substrate(s) of PLIP *in vivo*. Our results suggest that it might be PI(5)P. PI(5)P was discovered only recently as a naturally occurring phosphoinositide present at low concentration in both animals and plants, but not in yeast (17, 18, 49). PI(5)P concentration increases in response to osmotic shock (49) or in platelet cells after thrombin treatment (50), suggesting that PI(5)P may play a role as a signaling molecule. *In vivo*, PI(5)P may be produced by phosphorylation of phosphatidylinositol by PIKfyve kinase (17, 51) and, as recently shown, by dephosphorylation of PI(3,5)P_2 by the 3′-phosphatase myotubularin (16). Type II phosphatidylinositol phosphate kinase phosphorylates PI(5)P to produce PI(4,5)P_2. Although this pathway is considered minor compared with the pathway involving PI(4)P and type I phosphatidylinositol-phosphate kinase, it may be of biological significance in certain cells, cell compartments, or physiological conditions (18). As with other phosphoinositides, PI(5)P may interact with protein domains to regulate their localization or activity. A PI(5)P-mediated regulation is involved in the activation of the myotubularin MTM1, MTMR3, and MTMR6 proteins (16). However, so far, the localization of PI(5)P and its biological role in the cell context remain unknown.

Analysis of plant and metazoan data bases showed that PLIP is the founding member of a potentially new class of phosphatidylinositol phosphatases. This family is related by homologies within and just N-terminal to the catalytic core domain conserved between the broader members of the PTEN superfamily phosphatidylinositol 3-phosphatases. Our analysis indicated that PLIP is an integral protein localized in the Golgi. The subcellular localization of other members of this family is not known. The N-terminal transmembrane domain identified in *Dictyostelium* PLIP is not conserved in other organisms. Although a putative transmembrane domain as predicted by TMHMM Version 2.0 is found in the PLIP sequences from *Arabidopsis* and zebrafish, there is no experimental evidence demonstrating that these proteins have a transmembrane domain or that they are localized to the Golgi. Therefore, until the subcellular localization of other members of this protein family is determined, we do not know whether the conservation is only in the sequence motifs N-terminal to and within the catalytic core domain or whether subcellular localization of the proteins is also conserved.

We created a PLIP gene knockout by homologous recombination and examined the phenotypes of these cells. Analysis of the mutant strain indicated that PLIP is required for aggregation of cells to form a multicellular organism under conditions of low cell density. PLIP localized in the Golgi, with its phosphatase domain facing the cytoplasmic compartment. PLIP is thus spatially poised to potentially regulate membrane trafficking by dephosphorylating phosphatidylinositides. However, we were unable to correlate the aggregation phenotype with a defect in the Golgi structure or function.

It is possible that PLIP functions in a pathway not assayed in this study. The phenotype of the mutant cells may result from complex but small changes in phospholipid concentration and localization. PLIP was able to dephosphorylate *in vitro* a broad panel of phosphoinositides. Furthermore, if PI(5)P is the physiological substrate of PLIP, mutation of this phosphatase may increase, at least locally, the synthesis of PI(4,5)P_2 and therefore PI(3,4,5)P_3 through phosphorylation by the type II phosphatidylinositol-phosphate kinase (18) and may increase the dephosphorylation of PI(3)P and PI(3,5)P_2 through the stimulation of myotubularin (16).

Finally, the function of PLIP may also be carried out by other lipid phosphatases, resulting in a weak cellular phenotype exhibited by PLIP null cells. Although we did not identify other PLIP homologs or proteins related to PTEN2, which is in the Golgi (36), we identified, in the *Dictyostelium* genome, several members of the phosphoinositide 3-phosphatase myotubularin family. It was recently reported that *Dictyostelium* has at least four potential phosphoinositide 5-phosphatases (52), but their localization and their activity for PI(5)P are not known. The analysis of PLIP homologs in other organisms will help to unravel the physiological function of this new class of phosphatases and contribute to a better understanding of the role of phosphatidylinositides, in particular PI(5)P, in membrane signaling.

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