The identification and characterization of two phosphatidylinositol-4,5-bisphosphate 4-phosphatases

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Numerous inositol polyphosphate 5-phosphatases catalyze the degradation of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) to phosphatidylinositol-4-phosphate (PtdIns-4-P). An alternative pathway to degrade PtdIns-4,5-P_2 is the hydrolysis of PtdIns-4,5-P_2 by a 4-phosphatase, leading to the production of PtdIns-5-P. Whereas the bacterial IpgD enzyme is known to catalyze this reaction, no such mammalian enzyme has been found. We have identified and characterized two previously undescribed human enzymes, PtdIns-4,5-P_2 4-phosphatase type I and type II, which catalyze the hydrolysis of PtdIns-4,5-P_2 to phosphatidylinositol-5-phosphate (PtdIns-5-P). Both enzymes are ubiquitously expressed and localize to late endosomal/lysosomal membranes in epithelial cells. Overexpression of either enzyme in HeLa cells increases EGF-receptor degradation upon EGF stimulation.

cell signaling | lysosome | phosphatidylinositol 5-phosphate

Inositol-signaling molecules regulate many cellular processes, including actin cytoskeletal dynamics, apoptosis, cell division, and intracellular-membrane trafficking (1–4). Identification of the enzymes that regulate the levels of inositol second messengers is, therefore, important to understand how major cellular events are spatially and temporally coordinated.

A large number of kinases and phosphatases have been described that regulate the levels of phosphatidylinositols in human cells (2, 5). The production of PtdIns-5-P and its signaling functions are among the least well understood (6). Recent work implied that a significant amount of cellular PtdIns-5-P might be regulated by lipid phosphatases rather than kinases, because overexpression of the relevant phosphatidylinositol kinases did not significantly affect PtdIns-5-P levels in HeLa or Cos7 cells (7). Candidate human phosphatases include some of the myotubularins that convert phosphatidylinositol-3,5-bisphosphate to PtdIns-5-P (8, 9).

Some bacterial pathogens, such as *Salmonella dublin*, secrete virulence factors (SopB) that are inositol polyphosphate phosphatases into host cells to modulate the inositol-signaling pathways and improve their survival and propagation (10). The *Shigella flexneri* virulence factor IpgD is an inositol polyphosphate 4-phosphatase that converts PtdIns-4,5-P_2 to PtdIns-5-P, leading to an accumulation of PtdIns-5-P in cells infected with the pathogen. Epithelial cells infected with *Shigella flexneri* exhibit membrane blebbing and actin cytoskeletal changes, indicating the importance of this reaction (11). Whether such a reaction normally occurs in mammalian cells and whether there are any endogenous enzymes that can catalyze this reaction have not been established.

We now report the identification of two human PtdIns-4,5-P_2 4-phosphatases, PtdIns-4,5-P_2 4-phosphatase type I (PtdIns-4,5-P_2 4-Ptase I) and PtdIns-4,5-P_2 4-phosphatase type II (PtdIns-4,5-P_2 4-Ptase II). Both enzymes are ubiquitously expressed, localize to late endosomal/lysosomal membranes, and appear to affect the lysosomal degradation of internalized plasma membrane receptors. We conclude that two different pathways exist in mammalian cells to degrade PtdIns-4,5-P_2. One is catalyzed by inositol polyphosphate 5-phosphatases, leading to the production of PtdIns-4-P. The other pathway involves the type I and II PtdIns-4,5-P_2 4-Ptases and produces PtdIns-5-P.¶

Materials and Methods

Reagents and Chemicals. All chemicals were purchased from Sigma , unless stated otherwise. BSA for immunofluorescence was obtained from Jackson ImmunoResearch. Paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA). [^{3}H]inositol was from American Radiolabeled Chemicals (St. Louis), and all other [^{3}H]inositol polyphosphates were from PerkinElmer. [^{32}P]dCTP was from Amersham Pharmacia.

Antibodies and Fluorescent Probes. Rabbit polyclonal antibodies were raised against a peptide comprised of the N-terminal 109-aa residues of PtdIns-4,5-P_2 4-Ptase I. The following commercial polyclonal antibodies were used: anti-giantin, Covance (Princeton, NJ); anti-EGF receptor (EGFR), Santa Cruz Biotechnology; and anti-GFP, Invitrogen. Sources of monoclonal antibodies were: anti-GFP, Covance; anti-early endosomal antigen 1 (EEA1), BD Biosciences; anti-Lamp-1, Linton Traub (University of Pittsburgh School of Medicine, Pittsburgh); and anti-β-Tubulin, Sigma. Lysotracker, EGF-Alexa Fluor 555, and all fluorescently labeled secondary antibodies were from Molecular Probes. Horseradish-peroxidase-linked secondary antibodies and enhanced chemiluminescence reagents for Western blots were from Pierce.

Cloning. Full-length type I and type II PtdIns-4,5-P_2 4-Ptases in pTrcHis2 plasmids were cloned by PCR using the human image cDNA clones, 3940519 (BC002867, type I) and 4820065 (BC33892, type II) (Open Biosystems, Huntsville, AL) as templates. EcoR1 and KpnI restriction sites were added to the 5′ and 3′ ends, respectively, of both type I and type II PtdIns-4,5-P_2 4-Ptases by using a PCR strategy that also deleted the first methionine codon in the ORF. FLAG–peptide fusion constructs were prepared by subcloning PCR products into pBakPak9 HF plasmids (BD Biosciences). The full-length inserts corresponding to type I and type II PtdIns-4,5-P_2 4-Ptase cDNAs were digested with BamHI and XhoI and subcloned into pcDNA4/TO (Invitrogen) and pEGFP (C1) (Clontech).

Small Interfering (si)RNA Sequence. The PtdIns-4,5-P_2 4-Ptase I siRNA duplex was obtained from Ambion (Austin, TX); sense,

Conflict of interest statement: No conflicts declared.

Abbreviations: EEA1, early endosomal antigen 1; EGFR, EGF receptor; PtdIns-4,5-P_2, phosphatidylinositol-4,5-bisphosphate; PtdIns-5-P, phosphatidylinositol-5-phosphate; PtdIns-3,5-P_2, phosphatidylinositol-3,5-bisphosphate; PtdIns-4,5-P_2 4-Ptase I, PtdIns-4,5-P_2 4-phosphatase type I; PtdIns-4,5-P_2 4-Ptase II, PtdIns-4,5-P_2 4-phosphatase type II; siRNA, small interfering RNA.

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GCAUCAGCAUGUAGUCAAUUG; antisense, UUUGACUACAUGCUGAUGCAU.

Transient Transfections. HeLa or Cos7 cells were transfected by using Lipofectamine 2000 according to the manufacturer's instructions. In the EGFR-degradation assay, HeLa cells were nucleofected according to the manufacturer's protocol (Amaxa, Cologne, Germany). All experiments were performed 1–3 days after transfection.

PtdIns-4,5-P_2 4-Phosphatase Assays. [^{3}H] PtdIns-4,5-P_2 (10,000 cpm per assay), unlabeled PtdIns-4,5-P_2, and phosphatidylserine were dried under nitrogen and resuspended in buffer containing 10 mM Tris·HCl, pH 7.4, 130 mM KCl, and 20 mM NaCl, followed by brief sonication. Enzyme assays were performed in a reaction volume of 25 μl containing 25 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 100 μg/ml BSA, and 20 μM PtdIns-4,5-P_2. Reactions were started by the addition of the enzyme and carried out for 30 min at 37°C. Reactions were terminated by the addition of 50 μl of 1 N HCl. Lipids were extracted with 500 μl of chloroform/methanol/12 N HCl (20:10:1) and separated by thin-layer chromatography. The plates (Silica gel 60, EMD Chemicals, Darmstadt, Germany) were developed with chloroform/methanol/H_2O/15 N NH_4OH (90:90:20:7). To analyze reaction products derived from [^{3}H] PtdIns-4,5-P_2 hydrolysis, the extracted lipids were deacylated (12) and identified by anion-exchange HPLC (PartiSphere SAX, 4.6 × 125 mm, Whatman) by comparison with known [^{32}P]-labeled standards obtained from metabolically labeled HEK293 cells, as described in ref. 13.

Analysis of Phosphatidylinositol Levels in Cells. HEK293 cells stably transfected with either pcDNA4/TO empty vector or PtdIns-4,5-P_2 4-Ptase I were labeled with 10 μCi/ml (1 Ci = 37 GBq) [^{3}H] *myo*-inositol for four days in 35-mm dishes. Expression of PtdIns-4,5-P_2 4-Ptase I was induced with 200 ng/ml tetracycline 48 h before cells were harvested. Cells were lysed by the addition of 1.8 ml of methanol/chloroform/8% $HClO_4$ (20:10:1). After the addition of 500 μl of chloroform and 500 μl of 1% $HClO_4$, the lower organic phase was collected, washed twice with 1% $HClO_4$, and evaporated. Samples were deacylated and analyzed by HPLC, as described above.

Northern Blot Analysis. Full-length human type I and type II PtdIns-4,5-P_2 4-Ptases in pTrcHis2 plasmids (Invitrogen) were digested with BstnI (PtdIns-4,5-P_2 4-Ptase I) or MboII (PtdIns-4,5-P_2 4-Ptase II). Bands corresponding to a 281-bp fragment of PtdIns-4,5-P_2 4-Ptase I and a 163-bp fragment of PtdIns-4,5-P_2 4-Ptase II were gel purified (Qiagen, Valencia, CA) and labeled by using the Rediprime II DNA-labeling system (Amersham Pharmacia). Labeled probe was hybridized to human multiple tissue expression arrays (Clontech) by using ExpressHyb hybridization solution (Clontech).

Protein Purification. Full-length FLAG-tagged type I and type II PtdIns-4,5-P_2 4-Ptases were expressed in Sf9 cells grown in serum-free medium (Invitrogen) by using the BakPAK baculoviral expression system (Clontech). Infected cell pellets were lysed in 20 ml of homogenization buffer (20 mM Hepes, pH 7.6, 140 mM NaCl, 10% glycerol, 0.2% Nonidet P-40, 10 mM benzamidine, 40 μM iodoacetamide, 1 μM pepstatin A, 40 μM leupeptin, 10 μM bestatin, 50 μg/ml chymostatin, 2 μM calpain inhibitor, 10 μg/ml antipain, 1 μM microcystin, and 1 mM

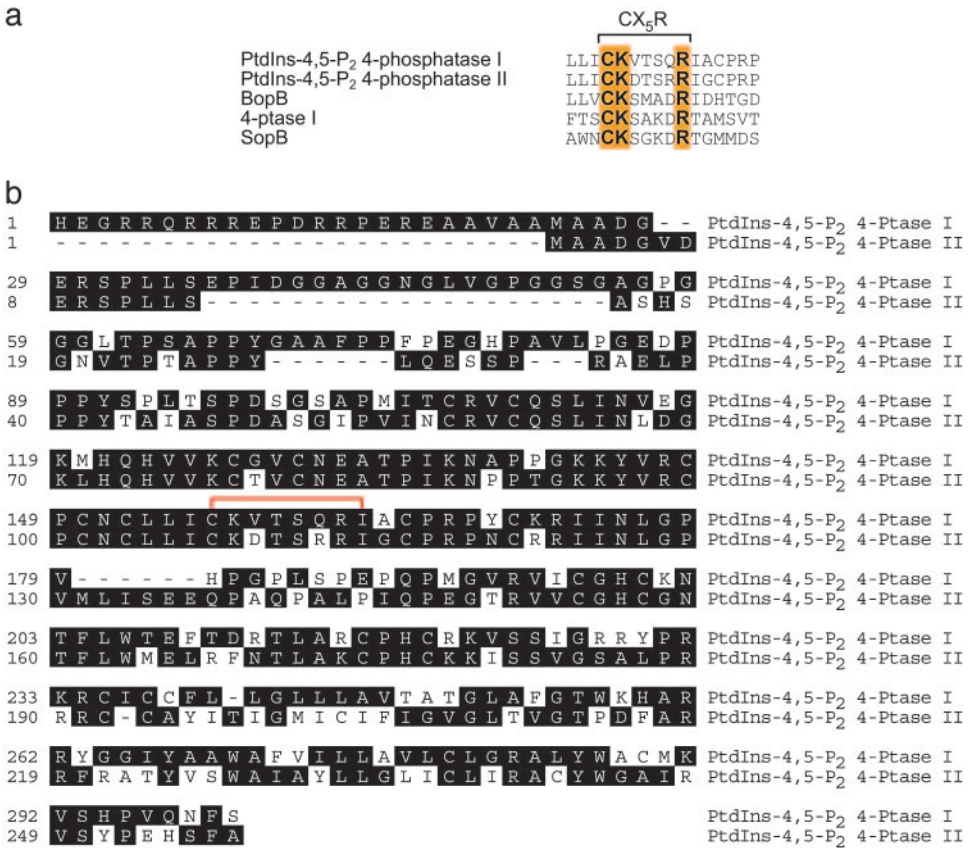

Fig. 1. Identification of two putative human phosphatases containing a CX_5R motif. (*a*) Alignment of the CX_5R motif of SopB, BopB, type I 4-phosphatase, and the two previously uncharacterized human phosphatases PtdIns-4,5-P_2 4-Ptase type I and type II. (*b*) The amino acid sequences of PtdIns-4,5-P_2 4-Ptase type I and type II are 51% identical. The location of the active site is indicated in red.

PMSF). Lysates were allowed to bind to M2 anti-FLAG agarose beads (Sigma) and were washed with Tris-buffered saline, and proteins were eluted in 500-μl fractions with 0.1 mg/ml FLAG peptide (Sigma) in Tris-buffered saline. Samples containing the majority of protein, as measured by absorbance at 280 nm, were pooled and dialyzed against Tris-buffered saline before their use in *in vitro* phosphatase assays.

Immunofluorescence Microscopy. Cells grown on coverslips were fixed for 5 min with IF fixation buffer (10 mM Pipes pH 6.5, 127 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 1 mM EGTA, and 4% paraformaldehyde) then washed with Tris-buffered saline and solubilized with 0.5% Triton X-100 in PBS for 10 min. Primary and secondary antibodies were diluted in PBS/0.1%Triton X-100/5% BSA. Cells were incubated for 1 h with primary and for 30 min with secondary antibodies at 37°C. Washes after antibody incubations were performed by dipping coverslips into PBS 30 times. Cells were mounted in Prolong mounting media (Molecular Probes). Lysotracker and EGF-Alexa Fluor 555 were used according to the manufacturer's instructions. Images were taken by using a Nikon Eclipse TE2000-S inverted microscope.

EGFR-Degradation Assay. EGFR degradation was assayed exactly as described by Bache *et al.* (14). HeLa cells were either mock electroporated, or electroporated with GFP-PtdIns-4,5-P_2 4-Ptase I, GFP-PtdIns-4,5-P_2 4-Ptase II, or both. At 24 h after electroporation, the cells were plated in 35-mm dishes. At 48 h after transfection, the medium (DMEM/10% FBS) was replaced with the same medium containing 10 μg/ml cycloheximide and 200 ng/ml EGF-Alexa Fluor 555. Cells were washed once with PBS and harvested after 0, 180, and 240 min by adding 50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 1% Triton X-100, and protease inhibitors (Roche Applied Science, Indianapolis). The amount of EGFR was analyzed by Western blotting for EGFR. β-Tubulin blots were done to control for loading.

Results

Two Previously Uncharacterized Putative Human Phosphatases Containing a CX_5R Motif. We searched the human genome for proteins homologous to the *Burkholderia pseudomallei* virulence factor BopB, a putative phosphatase that contains a CX_5R phosphatase motif (Fig. 1*a*) (15). Two previously uncharacterized proteins with about 12% identity to BopB were identified (Fig. 1*b*). The two BopB-like proteins are encoded on chromosomes 14q11.2 (type I) and 8q21.3 (type II). Interestingly, both proteins contain the conserved CX_5R motif, indicating that they may be phosphatases as well. We noticed that the arginine was followed by an isoleucine residue as opposed to a threonine found in type I 4-phosphatase and the *Salmonella dublin* SopB phosphatase. Mutations of this Thr to Ala in SopB decreases activity to 10% of wild-type levels (Norris, F. A. and P.W.M., unpublished data). Because there is precedent for bacteria manipulating the host's inositol-signaling cascade by SopB, we sought to determine whether BopB can act as an inositol polyphosphate phosphatase. Unfortunately, we have been unable to purify recombinant bacterial BopB protein. Considering the conserved CX_5R motif, we cloned and expressed the two human BopB-like proteins to assay them for inositol phosphate phosphatase activity.

Identification of Two Human PtdIns-4,5-P_2 4-Phosphatases. The cDNA for each putative phosphatase was cloned into baculoviral expression vectors, and recombinant FLAG-tagged protein was purified from infected Sf9 cells. Initially, we assayed for phosphatase activity toward inositol phosphates, including Ins-1,2,3,4,5,6-P_6, Ins-1,3,4,5,6-P_5, Ins-1,4,5,6-P_4, Ins-1,3,4,6-P_4, Ins-1,3,4,5-P_4, Ins-1,4,5-P_3, Ins-1,4-P_2, and Ins-4-P. Neither putative human phosphatase dephosphorylated any of these inositol phosphate substrates in *in vitro* assays (data not shown).

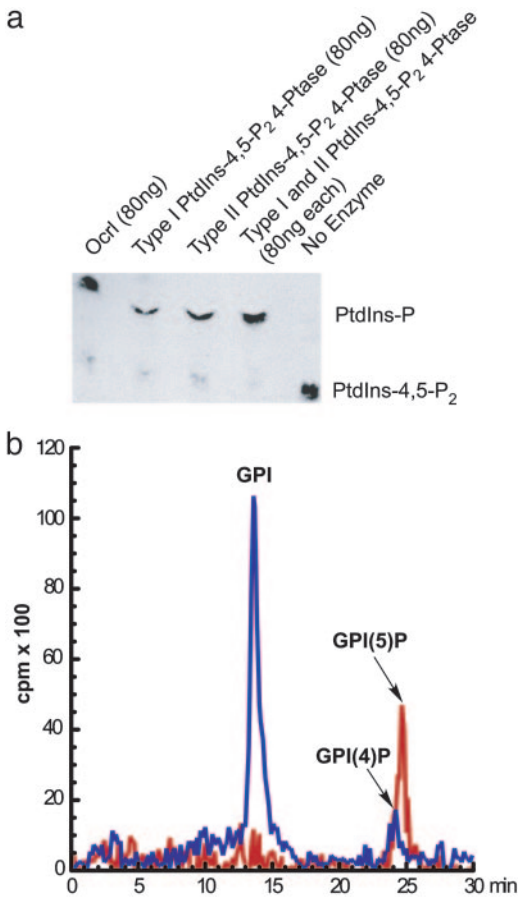

Fig. 2. Enzymatic activity of PtdIns-4,5-P_2 4-phosphatases. (*a*) Thin-layer chromatography separation of the PtdIns-4,5-P_2 hydrolysis products of *in vitro* reactions containing PtdIns-4,5-P_2 4-Ptase I, PtdIns-4,5-P_2 4-Ptase II, PtdIns-4,5-P_2 4-Ptase I and II combined, and OcrI (a type II inositol polyphosphate 5-phosphatase). (*b*) HPLC chromatogram of PtdIns-4,5-P_2 4-Ptase I hydrolysis product. Both type I and type II PtdIns-4,5-P_2 4-Ptases hydrolyze the 4-phosphate of PtdIns-4,5-P_2 to produce PtdIns-5-P. Glycerophosphorylinositol (GPI) (5)P elutes after GPI (4)P.

Next, we tested phosphatidylinositols as substrates, including PtdIns-3,4,5-P_3, PtdIns-4,5-P_2, PtdIns-3,4-P_2, PtdIns-3,5-P_2, PtdIns-5-P, PtdIns-4-P, and PtdIns-3-P. The two recombinant proteins both specifically hydrolyzed PtdIns-4,5-P_2 but none of the other substrates (Fig. 2*a* and data not shown). HPLC analysis of the reaction products showed that these two enzymes catalyze the hydrolysis of the 4-position phosphate on PtdIns-4,5-P_2 (Fig. 2*b*). No such activity has been described in mammals, and we designated the two enzymes PtdIns-4,5-P_2 4-phosphatase type I and PtdIns-4,5-P_2 4-phosphatase type II. When the type I and type II PtdIns-4,5-P_2 4-phosphatases were incubated in equal amounts, the catalytic activity was exactly twice that of each enzyme alone, indicating that the two enzymes do not work synergistically under the reaction conditions tested (Fig. 2*a*).

Both PtdIns-4,5-P_2 4-Phosphatases Are Expressed Ubiquitously and Localize to Late Endosomal/Lysosomal Membranes. Because the PtdIns-4,5-P_2 4-Ptases I and II have the same catalytic properties, we reasoned that they may have different expression patterns. Northern blot analysis of human tissues showed that both phosphatases are ubiquitously expressed. We did notice higher levels of PtdIns-4,5-P_2 4-Ptase II compared with PtdIns-

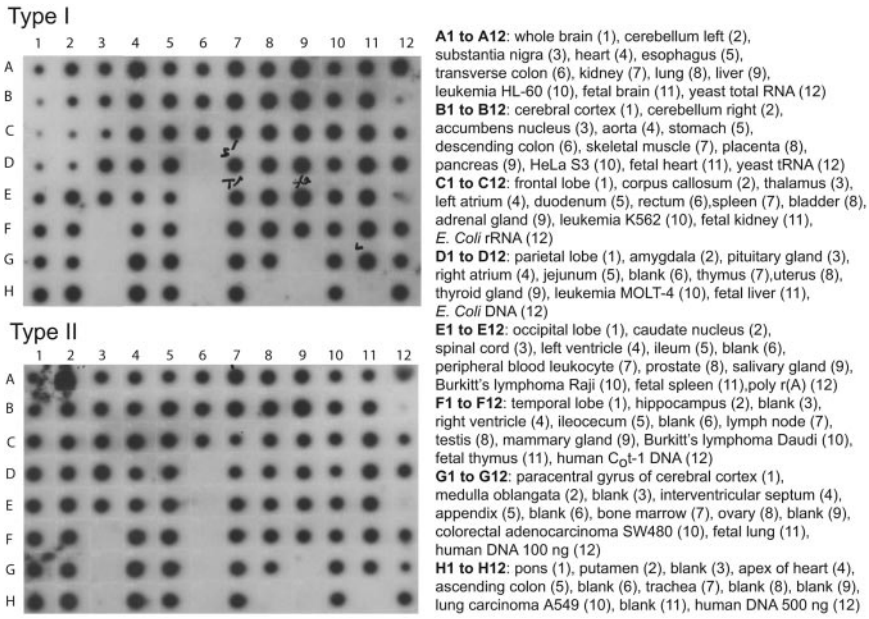

Fig. 3. Northern blot analysis of type I and type II PtdIns-4,5-P_2 4-Ptase expression in human tissues and cells.

4,5-P_2 4-Ptase I mRNA in several areas of the brain. However, we found relatively greater PtdIns-4,5-P_2 4-Ptase I expression in the liver, spleen, thymus, adrenal, thyroid, and salivary glands (Fig. 3).

Next, we studied the intracellular localization of the two enzymes. We created GFP fusion constructs and transiently expressed the phosphatases in Cos7 cells. Both GFP fusion proteins exhibited a late endosomal/lysosomal membrane distribution, as was indicated by colocalization with lamp-1 and lysotracker staining (Fig. 4*c* and data not shown). To determine whether endogenous PtdIns-4,5-P_2 4-Ptases are also associated with lysosomal membranes, polyclonal antisera were raised in rabbits. The PtdIns-4,5-P_2 4-Ptase I antibody staining clearly shows the endogenous protein on lamp-1-positive, lysotracker-

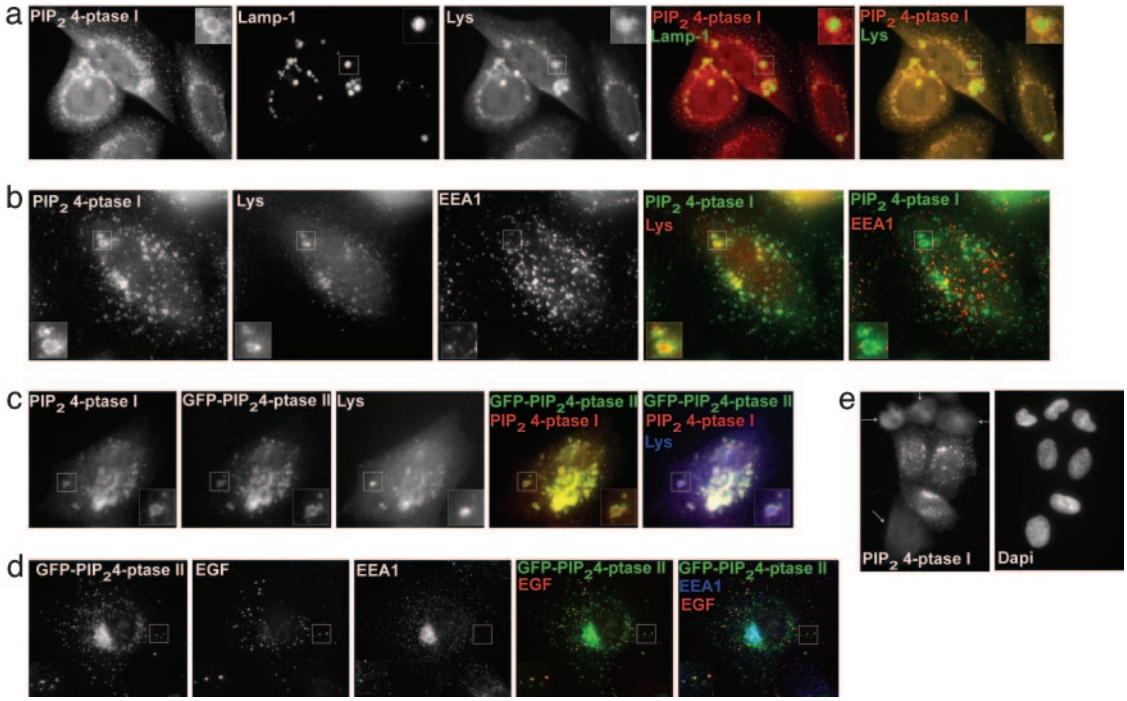

Fig. 4. PtdIns-4,5-P_2 4-Ptase I and II localize to lamp-1, lysotracker-positive late endosomal/lysosomal membranes. (*a*) PtdIns-4,5-P_2 4-Ptase I (Alexa Fluor 488), lamp-1 (Alexa Fluor 647), and lysotracker (DND-99) are colocalized in HeLa cells. (*b*) PtdIns-4,5-P_2 4-Ptase I (Alexa Fluor 488), lysotracker (DND-99), and EEA1 (Alexa Fluor 647) staining in HeLa cells. (*c*) PtdIns-4,5-P_2 4-Ptase I (Alexa Fluor 647), GFP-PtdIns-4,5-P_2 4-Ptase II (GFP), and lysotracker (DND-99) staining in HeLa cells. (*d*) GFP-PtdIns-4,5-P_2 4-Ptase II (Alexa Fluor 488), EGF-Alexa Fluor 555, and EEA1 (Alexa Fluor 647) staining in Cos7 cells. Cos7 cells were incubated with 200 ng/ml EGF-Alexa Fluor 555 for 30 min. (*e*) PtdIns-4,5-P_2 4-Ptase I staining in HeLa cells transfected with Type I PtdIns-4,5-P_2 4-Ptase siRNAs indicates specificity of staining (arrows indicate transfected cells). The boxed regions of interest are shown at higher magnification (*Insets*).

containing membranes in HeLa cells (Fig. 4*a*). To ensure the specificity of staining, HeLa cells transfected with PtdIns-4,5-P_2 4-Ptase I siRNAs were stained, and no staining was apparent in those cells (Fig. 4*e*). PtdIns-4,5-P_2 4-Ptase I antiserum also does not recognize PtdIns-4,5-P_2 4-Ptase II on Western blots, so we conclude that the antibodies are specific to PtdIns-4,5-P_2 4-Ptase I. The type II PtdIns-4,5-P_2 4-phosphatase antibodies are not suitable for immunofluorescence. Neither enzyme colocalizes significantly with the early endosomal marker EEA1 (Fig. 4 *b* and *d*). After a 30-min uptake of fluorescently labeled EGF, which is sorted with EGFRs to lysosomes through early endosomes, some EGF punctae colocalize with PtdIns-4,5-P_2 4-Ptase-positive lysosomal structures, whereas others colocalize with EEA1-positive endosomes (Fig. 4*d*). Both PtdIns-4,5-P_2 4-Ptases contain two putative transmembrane domains near the C terminus, suggesting that these enzymes may be lysosomal transmembrane proteins.

Overexpression of PtdIns-4,5-P_2 4-Ptase I Decreases Cellular PtdIns-4,5-P_2 Levels. To address how much of the cellular PtdIns-4,5-P_2 and PtdIns-5-P levels in cells are controlled by the PtdIns-4,5-P_2 4-phosphatases, we created HEK293 cells stably expressing PtdIns-4,5-P_2 4-Ptase I under a tetracycline-inducible promoter. These cells were labeled with [^{3}H] *myo*-inositol for 4 days and induced with tetracycline for 48 h. Deacylated phosphatidylinositol derivatives were analyzed by HPLC. PtdIns-4,5-P_2 levels normalized to PtdIns-4-P levels were significantly lower in cells overexpressing PtdIns-4,5-P_2 4-Ptase I compared with vector control cells (0.59 ± 0.05 (PtdIns-4,5-P_2 4-Ptase I) versus 0.79 ± 0.03 (vector), unpaired *t* test, $P < 0.03$) (Fig. 5*a*). This result shows that PtdIns-4,5-P_2 4-Ptase I can significantly change total PtdIns-4,5-P_2 levels *in vivo*. A concomitant increase in PtdIns-5-P levels would be predicted but has yet to be shown experimentally.

PtdIns-4,5-P_2 4-Ptases I and II Affect EGFR Degradation upon EGF Stimulation. Considering the localization of the PtdIns-4,5-P_2 4-Ptases, we postulated that they might affect lysosomal functions. An effect on lysosomal function could be achieved by either the formation of PtdIns-5-P or the depletion of PtdIns-4,5-P_2.

To test whether overexpression of the enzymes might indicate a regulatory function at lysosomes, HeLa cells were transiently transfected with GFP-PtdIns-4,5-P_2 4-Ptase I, GFP-PtdIns-4,5-P_2 4-Ptase II, or both enzymes. EGFR degradation was measured after stimulation with 200 ng/ml EGF in the presence of 10 μg/ml cycloheximide. The degradation of EGFRs was enhanced in the PtdIns-4,5-P_2 4-Ptase-expressing cells compared with the control cells (Fig. 5*b*). Thus, PtdIns-4,5-P_2 might have an inhibitory effect on lysosomal degradation of internalized receptors, or PtdIns-5-P may stimulate this process. Transfection of HeLa cells with PtdIns-4,5-P_2 4-Ptase I and II siRNAs did not affect PtdIns-4,5-P_2 levels or EGFR degradation, indicating that PtdIns-5-P likely is responsible for the effects on EGFR degradation (data not shown).

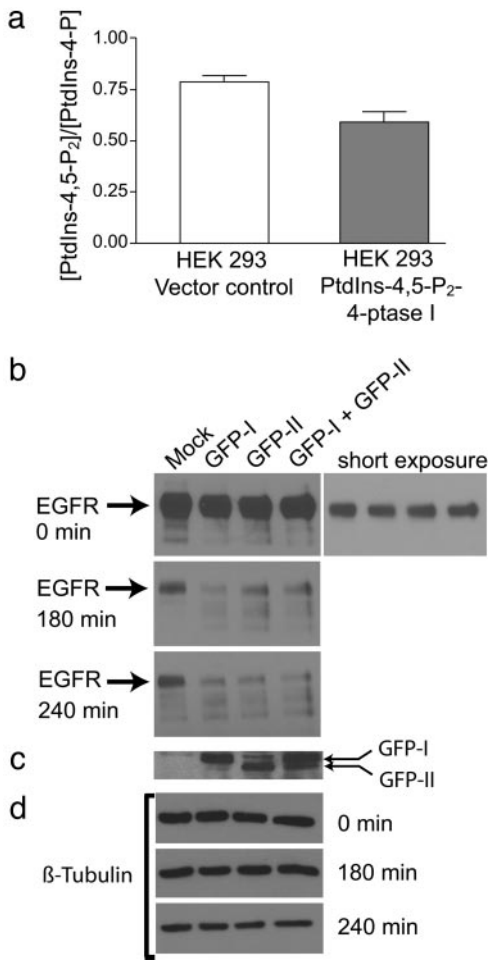

Fig. 5. Effects of overexpression of Type I and/or Type II PtdIns-4,5-P_2 4-Ptases. (*a*) Ratio of [PtdIns-4,5-P_2]/[PtdIns-4-P] in HEK 293 cells overexpressing PtdIns-4,5-P_2 4-Ptase I or empty vector [0.59 ± 0.05 (PtdIns-4,5-P_2 4-Ptase I) vs. 0.79 ± 0.03 (vector), unpaired *t* test $P < 0.03$]. (*b*) Analysis of EGFR degradation in cells transiently transfected with no DNA (mock), GFP-PtdIns-4,5-P_2 4-Ptase I (GFP-I), GFP-PtdIns-4,5-P_2 Ptase II (GFP-II), and both GFP-I and GFP-II (GFP-I + GFP-II). EGFR levels were analyzed by Western blotting after 0, 180, and 240 min of 200-ng/ml EGF stimulation in the presence of 10 μg/ml cycloheximide. (*c*) The expression of the PtdIns-4,5-P_2 4-Ptases I and II was confirmed by anti-GFP Western blotting. (*d*) Western blotting for β-tubulin was done to assess loading.

Discussion

We have characterized a class of inositol polyphosphate phosphatases, the PtdIns-4,5-P_2 4-Ptases I and II. These enzymes hydrolyze the 4-position phosphate of PtdIns-4,5-P_2 to produce PtdIns-5-P, providing a pathway for how PtdIns-5-P can be produced in cells. Signaling through PtdIns-4,5-P_2 has been studied extensively, and this lipid affects many cellular processes, including actin cytoskeletal dynamics and intracellular-membrane trafficking (2, 16). PtdIns-5-P was shown to exist in cells relatively recently, and its cellular-signaling function is poorly understood (6). It is known that PtdIns-5-P levels increase in the nucleus during the G_1 phase of the cell cycle (17), increase in platelets in response to thrombin stimulation (18), and also increase after insulin stimulation of adipocytes (19). Hypoosmotic shock and histamine decrease PtdIns-5-P levels (7, 20), and hyperosmotic shock increases PtdIns-5-P levels in L6 myotubes overexpressing the myotubularin protein MTM1 (9). Possible mechanisms of action of PtdIns-5-P signaling include the regulation of inositol lipid phosphatases, such as the myotubularins, and the regulation of the interaction between the putative tumor-suppressor protein ING2 and p53 (21–23).

The similarity between the PtdIns-4,5-P_2 4-Ptases and *B. pseudomallei* BopB is of interest and indicates that BopB may also be an inositol polyphosphate 4-phosphase. A possible clue to the normal function of the PtdIns-4,5-P_2 4-Ptases may result from the study of how BopB aids *B. pseudomallei* infection of human cells.

Our results suggest that PtdIns-4,5-P_2 and PtdIns-5-P play an important role on the lysosomal membrane, because overexpression of type I and/or type II PtdIns-4,5-P_2 4-Ptase increases the amount of EGFR degradation that occurs within the first 4 h of

EGF stimulation. One possibility is that PtdIns-4,5-P_2 plays an inhibitory role in EGFR degradation on lysosomal membranes, and, thus, depletion of this lipid leads to improved receptor clearance. PtdIns-4,5-P_2 plays a key role in AP-2-mediated clathrin-coated vesicle formation at the plasma membrane (2). AP-2 containing clathrin-coated buds have been shown to form on lysosomes *in vitro*, raising the possibility that PtdIns-4,5-P_2 might allow the recycling of some EGFRs from late endosomes before degradation in a PtdIns-4,5-P_2-dependent manner (24). Alternatively, PtdIns-5-P might promote the degradation of EGFRs, either directly or indirectly, as by affecting the concentration of PtdIns-3,5-P_2. Both PtdIns-3-P and PtdIns-3,5-P_2 are important in the sorting of proteins into multivesicular bodies (25–27). Endosomal PtdIns-3-P can be produced by two pathways, one via the phosphorylation of PtdIns to PtdIns-3-P by hVps34 and the other via the production and subsequent degradation of PtdIns-3,4,5-P_3 by type I PI3 kinase (D3 phosphorylation of PtdIns-4,5-P_2), type II inositol polyphosphate 5-phosphatase, and type Iα inositol polyphosphate 4-phosphatase (28). Similarly, there may be multiple ways to produce PtdIns-3,5-P_2 on multivesicular bodies, either by phosphorylation of PtdIns-3-P by PikFYVE or by the degradation of PtdIns-4,5-P_2 to PtdIns-5-P by the PtdIns-4,5-P_2 4-Ptases and subsequent 3-phosphorylation by type I PI3 kinase, a reaction that has been shown to occur *in vitro* (6, 26).

Overexpression of PtdIns-4,5-P_2 4-Ptase I resulted in a significant decrease in total cellular PtdIns-4,5-P_2 levels. Given the lysosomal localization of PtdIns-4,5-P_2 4-Ptase I, the phosphatidylinositol composition of late endosomal and lysosomal membranes is likely to have undergone even greater changes. The decrease in total PtdIns-4,5-P_2 also indicates that a significant portion of cellular PtdIns-4,5-P_2 is accessible to the PtdIns-4,5-P_2 4-Ptase enzymes on late endosomes/lysosomes.

In this article, we describe two inositol polyphosphate 4-phosphatases that hydrolyze PtdIns-4,5-P_2 to PtdIns-5-P. PtdIns-4,5-P_2 4-Ptase I and II localize to lysosomal membranes and, when overexpressed, increase EGF-stimulated EGFR degradation. Future studies are needed to define the functions of both PtdIns-4,5-P_2 and PtdIns-5-P on late endosomal and lysosomal membranes.

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