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The Type Iα Inositol Polyphosphate 4-Phosphatase Generates and Terminates Phosphoinositide 3-Kinase Signals on Endosomes and the Plasma Membrane

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Endosomal trafficking is regulated by the recruitment of effector proteins to phosphatidylinositol 3-phosphate [PtdIns(3)P] on early endosomes. At the plasma membrane, phosphatidylinositol-(3,4)-bisphosphate [PtdIns(3,4)P2] binds the pleckstrin homology (PH) domain-containing proteins Akt and TAPP1. Type Iα inositol polyphosphate 4-phosphatase (4-phosphatase) dephosphorylates PtdIns(3,4)P2, forming PtdIns(3)P, but its subcellular localization is unknown. We report here in quiescent cells, the 4-phosphatase colocalized with early and recycling endosomes. On growth factor stimulation, 4-phosphatase endosomal localization persisted, but in addition the 4-phosphatase localized at the plasma membrane. Overexpression of the 4-phosphatase in serum-stimulated cells increased cellular PtdIns(3)P levels and prevented wortmannin-induced endosomal dilatation. Furthermore, mouse embryonic fibroblasts from homozygous Weeble mice, which have a mutation in the type I 4-phosphatase, exhibited dilated early endosomes. 4-Phosphatase translocation to the plasma membrane upon growth factor stimulation inhibited the recruitment of the TAPP1 PH domain.

INTRODUCTION

Phosphoinositides generated by phosphoinositide 3-kinases (PI 3-kinases) regulate cell survival, proliferation, migration, and vesicular trafficking (Cantley, 2002; Czech, 2003). In agonist-stimulated cells, phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3] is a transient plasma membrane (PM) signal, rapidly hydrolyzed to phosphatidylinositol-(3,4)-bisphosphate [PtdIns(3,4)P2] by the inositol polyphosphate 5-phosphatases (5-phosphatases) (Stephens et al., 1993; Mitchell et al., 2002). PtdIns(3,4)P2 is a more sustained signal lasting for up to 60 min, stimulated by B-cell activation, oxidative stress, or irreversible platelet aggregation (Banfic et al., 1998; Van der Kaay et al., 1999; Marshall et al., 2002).

PtdIns(3,4,5)P3 effectors include proteins with pleckstrin homology (PH) domains such as the serine/threonine kinases Akt and PDK1; the Tec family of tyrosine kinases; various adapter proteins; and exchange factors for small GTPases (Czech, 2003; Lemmon, 2003). PtdIns(3,4)P2 recruits and activates an overlapping and distinct subset of effector proteins relative to PtdIns(3,4,5)P3. For example, Akt, a key regulator of apoptotic and insulin signaling, contains a PH domain that in vitro binds both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 with comparable affinity (Lemmon, 2003). Both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are required for the full in vivo activation of Akt (Scheid et al., 2002). The PH domain of the dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP1) binds both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Ferguson et al., 2000), whereas the PX domain of p47phox binds both PtdIns(3,4)P2 and PtdIns(3,5)P3 (Konai et al., 2001). Specific PtdIns(3,4)P2 binding proteins, the tandem PH-domain-containing proteins TAPP1 and TAPP2, have been characterized recently (Dowler et al., 2000).

PtdIns(3,4)P2 is hydrolyzed to form PtdIns(3)P by the inositol polyphosphate 4-phosphatases, but the intracellular site of this reaction remains to be determined. Furthermore, it is unknown whether the PtdIns(3)P generated by the 4-phosphatase contributes to a functional PtdIns(3)P pool at the PM or other intracellular membranes. Two human 4-phosphatase isoforms (type I and II) have been identified, sharing 37% amino acid identity. Both isoforms contain a catalytic “CX5R” motif found in both protein tyrosine phosphatases and dual specificity protein/lipid phosphatases (Norris et al., 1995, 1997a). The type Iα 4-phosphatase regulates megakaryocyte cell growth, acting downstream of the transcription factor GATA-1. Overexpression of this 4-phosphatase Akt and PDK1; the Tec family of tyrosine kinases; various adapter proteins; and exchange factors for small GTPases (Czech, 2003; Czech, 2003). PtdIns(3,4)P2 recruits and activates an overlapping and distinct subset of effector proteins relative to PtdIns(3,4,5)P3. For example, Akt, a key regulator of apoptotic and insulin signaling, contains a PH domain that in vitro binds both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 with comparable affinity (Lemmon, 2003). Both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are required for the full in vivo activation of Akt (Scheid et al., 2002). The PH domain of the dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP1) binds both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Ferguson et al., 2000), whereas the PX domain of p47phox binds both PtdIns(3,4)P2 and PtdIns(3,5)P3 (Konai et al., 2001). Specific PtdIns(3,4)P2 binding proteins, the tandem PH-domain-containing proteins TAPP1 and TAPP2, have been characterized recently (Dowler et al., 2000).

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phatase in NIH 3T3 fibroblasts suppresses cellular proliferation, without affecting apoptosis (Vyas et al., 2000). A spontaneous mutant mouse (Wieble), which has a phenotype of neuronal loss in the cerebellum/hippocampus, severe ataxia, and neonatal death, has been identified and is associated with mutation in the type I 4-phosphatase gene (Nystuen et al., 2001).

In this study, we show that the type I 4-phosphatase localizes to endosomal membranes, generating PtdIns(3,4)P₂ via hydrolysis of PtdIns(3,4,5)P₃, thereby rescuing the phenotype of wortmannin-induced endosomal dilatation. After epidermal growth factor (EGF) stimulation, the 4-phosphatase translocates to the PM and inhibits the PM recruitment of the TAPPI PH domain. The 4-phosphatase contains an N-terminal tandem C2 domain that is essential for catalytic activity, endosomal localization, and regulation of the recruitment of effectors with PtdIns(3,4)P₂-specific PH domains to the PM. The 4-phosphatase hence functions in both generating PtdIns(3,4)P₂ on endosomal membranes and maintaining PtdIns(3,4)P₂ signals at the PM.

**MATERIALS AND METHODS**

**General Reagents and Vectors**

Restriction and DNA-modifying enzymes were from New England Biolabs (Beverly, MA), Fermentas (Hanover, MD), or Promega (Madison, WI). Big dye terminator cycle sequencing was from PerkinElmer Life and Analytical Sciences (Boston, MA), synthetic peptides were from Chiron Mimotopes (Clayton, Australia), [γ-³²P]ATP was from PerkinElmer Life and Analytical Sciences, phospholipids were from BIOMOL Research Laboratories (Plymouth Meeting, PA), oligonucleotides were from Monash University (Clayton, Australia), pTrcHis vector was from Invitrogen (Carlsbad, CA), pGEX-KG was from American Type Culture Collection (Manassas, MD), and pEGFP-C2 was from BD Biosciences Clontech (Palo Alto, CA). pEFBOS-FLAG vector was from American Type Culture Collection (Manassas, MD), and pEGFP-C2 was from BD Biosciences Clontech (Palo Alto, CA). pEFBOS-FLAG vector was from Dr. B. Hok-Toh, Monash University.

**Purification of Recombinant 4-Phosphatase**

Human 4-phosphatase type I sequence was PCR amplified and subcloned into the KpnI site of pTrcHisC (pTrcHisC-4ptase) or the HindIII site of pGEX-KG (pGEX-4ptase). Recombinant purified enzymes were used to generate PtdIns(3,4,5)P₃ (pGEX-4ptase) via hydrolysis of PtdIns(3,4,5)P₃, thereby rescuing the phenotype of wortmannin-induced endosomal dilatation. After epidermal growth factor (EGF) stimulation, the 4-phosphatase translocates to the PM and inhibits the PM recruitment of the TAPPI PH domain. The 4-phosphatase contains an N-terminal tandem C2 domain that is essential for catalytic activity, endosomal localization, and regulation of the recruitment of effectors with PtdIns(3,4)P₂-specific PH domains to the PM. The 4-phosphatase hence functions in both generating PtdIns(3,4)P₂ on endosomal membranes and maintaining PtdIns(3,4)P₂ signals at the PM.

**Antibodies**

Two New Zealand White rabbits were immunized with the 4-phosphatase type I peptides [MTEARESHPRGHGARARA]₃C and [EESKDQRRPVRTS-VDTVNVR]₃C conjugated via the terminal cysteine to diphtheria toxin. Serum was purified on an immunopeptide-coupled thiopropyl-Sepharose 6B resin. Other antibodies or stains were obtained as indicated: Xpress antibody (Sigma-Aldrich); hemagglutinin (HA) antibody (Sigma-Aldrich, Missouri); green fluorescent protein (GFP) antibody, phalloidin, and Alexa Fluor-conjugated secondary antibodies (Molecular Probes); FLAG antibody (Sigma-Aldrich); and anti-HA antibody (Zymed Laboratories, South San Francisco, CA), and 5 μl of sample buffer for 10 min at RT; and pelleted at 100,000 g.

**Lipid 4-Phosphatase Assays**

PtdIns[32P-3,4]P₂ was formed by incubating 56 μg of PtdIns(4)P with 20 μg of phosphatidylserine (PtdSer), 1 μg of recombinant PI 3-kinase (Layton et al., 1996), 50 μM ATP, 20 μl of [γ-³²P]ATP (0.2 mCi, 3000 Ci/mM; MP Biomedicals, Irvine, CA), and 5 μl of 20% kinase buffer (400 mM Tris pH 7.4, 5 mM MgCl₂, and 10 μM EGTA) in 100 μl. Lipid products were extracted and suspended in 20 mM Tris pH 7.4, 1 mM EDTA. Lipid 4-phosphatase assays (100 μl) were performed by adding PtdIns[32P-3,4]P₂ to the recombinant wild-type or mutant 4-phosphatase catalytic domain in 50 mM 3-(N-morpholino)propanesulfonic acid, 10 mM EDTA, and 200 mM NaCl at 37°C for 30 min. Extracted lipids were analyzed by thin-layer chromatography (TLC) in chloroform/methanol/acetic acid/water [4:5:3:8:5:7 (vol/vol)]. Phosphoinositide products were excised from the TLC and counted using liquid scintillation counting.

**Liposome Binding Assays**

Phosphatidylethanolamine with PtdSer, PtdIns3P, PtdIns4P, PtdIns(3,4)P₂, or PtdIns(4,5)P₂ at a 5% molar ratio mixture was dried under N₂ and resuspended in 20 mM HEPES, pH 7.2, 100 mM NaCl, and 500 μM EDTA; sonicated (5 min) to form liposomes; incubated with GST-fusion proteins in 50 μl of sample buffer for 10 min at RT; and pelleted at 100,000 × g for 30 min. The pellet was resuspended in 35 μl of SDS-PAGE reducing buffer, analyzed by 10% SDS-PAGE, and silver stained. Densitometry was performed using GelPro software (Media Cybernetics, Silver Spring, MD).
(Kisseleva et al., 2002), by using 293T-Rex cells (Invitrogen). Addition of 100 ng of tetracycline per milliliter to the growth medium induced the expression of the 4-phosphatase. Cells were labeled in 35-mm tissue culture dishes in 1 ml of medium containing 400 μCi of [32P]orthophosphate (PerkinElmer Life and Analytical Sciences) for 2–4 h at 37°C in a phosphate-free DMEM (Invitrogen) containing 5% FCS dialyzed against 0.85% NaCl. Cells were harvested, spun, and lysed by an addition of 1.8 ml of methanol/chloroform/8% HClO4 (20:10:1). Lipids were extracted by the addition of 500 μl of chloroform and 500 μl of 1% HClO4, and the lower organic phase was collected, washed twice with 1% HClO4, and evaporated. Deacylation was as described previously (Caldwell et al., 1991). Glycerophosphorylinositol derivatives were resolved on an anion-exchange column (PartiSphere Sax, 4.6 × 125 mm; Whatman, Maidstone, United Kingdom) by using a 0–1.25 M NaH2PO4, pH 4.5, gradient as described previously (Kisseleva et al., 2002). Radiolabeled deacylated phospholipids were detected by a β-RAM in-flow detection system (IN/US Systems, Tampa, FL). Data were normalized to the total [32P] counts in PtdIns(4)P, which were stable in all cell lines regardless of construct expressed, accounting for 10% of [32P]orthophosphate incorporated.

Isolation of Mouse Embryonic Fibroblasts (MEFs)

MEFs were isolated from 12.5-d-old embryos from a heterozygous Weeble mouse mating (The Jackson Laboratory, Bar Harbor, ME). These cells were expanded for two passages before analysis by immunofluorescent confocal microscopy, which was performed as described above. Lysates from MEFs were immunoblotted using a previously described C-terminal 4-phosphatase antibody (Norris et al., 1997b). Genotyping of MEFs was performed as described previously (Nystuen et al., 2001).

RESULTS

4-Phosphatase Localizes to Intracellular Vesicles in Quiescent Cells

To investigate the intracellular localization of the type I 4-phosphatase, we developed antipeptide antibodies to two 4-phosphatase peptide sequences (aa 1–16 and 174–193), which showed no identity to any sequences in the protein databases. The antibody immunoblotted FLAG-4-phosphatase and the N-terminal domain (aa 1–304) to which the antibody was raised (Figure 1A). Moreover, this peptide affinity-purified antibody detected a 105-kDa polypeptide in human embryonic kidney 293, CHO, HL60 (myeloid), U937 (promonocytic), MEG-01 (megakaryocytic), and faintly in PC12 (neuroendocrine) cells, consistent with the endogenous enzyme (Figure 1A).

The intracellular localization of endogenous 4-phosphatase was determined in serum-starved CHO cells by indirect immunofluorescence, by using anti-4-phosphatase antibodies, which labeled distinct punctate vesicles throughout the cytosol, reminiscent of endocytic organelles (Figure 1B, left). Preimmune serum was nonreactive (our unpublished data). GFP-tagged wild-type or catalytically inactive 4-phosphataseC856S (right) were visualized by confocal microscopy, or lysates were immunoblotted with GFP antibody. Bar, 5 μm.

Figure 1. 4-Phosphatase intracellular localization. (A) Triton-soluble fraction of cell lysates from the indicated cell lines or COS-1 cells expressing FLAG-tagged wild-type 4-phosphatase, or the N-terminal 304 aa, were immunoblotted with affinity-purified 4-phosphatase antibody. (B) Serum-starved CHO cells were stained with affinity-purified 4-phosphatase antibody (left). COS-1 cells transiently expressing GFP-tagged wild-type (middle) or inactive 4-phosphataseC856S (right) were visualized by confocal microscopy, or lysates were immunoblotted with GFP antibody. Bar, 5 μm.
lytic site resulting in complete loss of enzyme activity (Norris et al., 1997a), was expressed in COS-1 cells (Figure 1B, middle and right, respectively). GFP-tagged 4-phosphatase localized in a perinuclear distribution, with punctate vesicular and faint cytosolic staining. A similar distribution was noted when the 4-phosphatase was expressed with a FLAG tag (our unpublished data). Cytosolic staining was more prominent in cells overexpressing the 4-phosphatase at high levels. The catalytically inactive mutant, whether expressed with an N-terminal GFP, or FLAG-tag (our unpublished data), demonstrated vesicular and faint cytosolic staining, with less prominent vesicle clustering noted in the perinuclear region than for the wild-type enzyme (Figure 1B). In control studies, GFP- and FLAG-tagged (our unpublished data) wild-type and catalytically inactive 4-phosphatase polypeptides were expressed without significant proteolysis (Figure 1B). The structural integrity of Golgi cisternae, endosomal compartments, and lysosomes is maintained by tethering of these organelles to microtubules emanating from the microtubule-organizing center (MTOC) (Matteoni and Kreis, 1987). On nocodazole treatment, the MTOC collapsed and microtubules dispersed (evident on costaining with anti-β-tubulin), correlating with cytosolic redistribution of 4-phosphatase–decorated vesicles (our unpublished data). In cells treated with dimethyl sulfoxide vehicle alone, 4-phosphatase perinuclear staining persisted, overlapping with the MTOC (our unpublished data). The 4-phosphatase therefore localizes to microtubule-tethered vesicles and its overexpression may promote vesicle clustering in the perinuclear region.

4-Phosphatase Colocalizes with Early and Recycling Endosomes in Quiescent Cells

The subcellular localization of the 4-phosphatase was determined by colocalization of GFP- or FLAG-4-phosphatase with specific endocytic or secretory markers (Figure 2, A–C). A significant proportion of 4-phosphatase–positive vesicles colocalized with the EEA-1 (Figure 2A, a, arrows in merged image). FLAG-4-phosphatase also colocalized extensively with GFP-Rab5 (Figure 2A, b, arrows in merged image). Rab11 is a small GTPase that localizes to perinuclear recycling endosomes and the trans-Golgi network (Ren et al., 1998). GFP-4-phosphatase–decorated vesicles colocalized with both recombinant XPRESS-tagged and endogenous Rab11, most intensely in the perinuclear region (Figure 2A, c and d, arrow in merged images).

The colocalization of the 4-phosphatase with the hTfnR was performed at an ultrastructural level. The TfnR traverses the entire endocytic pathway from early/sorting to perinuclear recycling endosomes and back to PM (Maxfield and McGraw, 2004). CHO cells were transiently cotransfected with GFP-4-phosphatase and hTfnR, incubated with 25 μg/ml Tfn-HRP and Tfn-positive endosomes labeled with the electron-dense peroxidase DAB reaction product (Stoorvogel et al., 1996). Ultrathin cryosections were incubated with an anti-GFP antibody followed by 10-nm gold-conjugated anti-mouse IgG secondary antibody (Figure 2B). GFP-4-phosphatase–decorated clusters of tubulo-vesicular structures, ~100 nm in diameter, which were DAB and hence Tfn positive. In addition 4-phosphatase-labeled vesicles were detected as densely staining DAB-positive vesicle clusters in the peri-Golgi region (Figure 2B, a, box, which is magnified in b). The 4-phosphatase–stained vesicles were not surrounded by an outer membrane and were hence distinct from multivesicular bodies (Figure 2B, b). The striking colocalization of the 4-phosphatase with Tfn-positive structures suggests it localizes to early and recycling endosomes. In control studies, gold particles were not detected using isotype IgG1 antibodies to tyrosine hydroxylase (Figure 2B, c).

No colocalization of the 4-phosphatase was demonstrated with Rab7 (late endosome/lysosome), LAMP-2 (lysosome), or mannose 6-phosphate receptor (M6PR) (Golgi to lysosome) (Figure 2C), or with the markers of the constitutive secretory pathway as shown by coexpression with GFP-tagged temperature-sensitive mutant of vesicular stomatitis virus glycoprotein (our unpublished data). Furthermore, the 4-phosphatase did not colocalize with endogenous Rab8, a marker of secretory vesicles (Figure 2C). 4-Phosphatase–expressing cells also were stained with antibodies to the Golgi-specific markers β-COP (Figure 2C, bottom), γ-adapin and Rab6a (our unpublished data), but no colocalization was observed. Moreover, 4-phosphatase perinuclear localization remained unperturbed after brefeldin A-induced Golgi collapse (our unpublished data). Collectively, these studies demonstrate that in quiescent cells, the 4-phosphatase colocalizes most consistently with markers of early and recycling endosomal compartments.

4-Phosphatase Accumulates on Early and Recycling Endosomes upon Expression of Specific Rab GTPase Mutants

We examined 4-phosphatase localization upon coexpression with specific Rab GTPase mutants. Rab5 regulates clathrin-coated vesicle formation, endosome motility, and early endosome fusion (Smythe, 2002). Overexpression of the GTPase-deficient Rab5 mutant, Rab5Q79L, causes enlargement of early endosomes, resulting from enhanced homotypic and heterotypic fusion (Barbieri et al., 1996). Under these conditions, the 4-phosphatase colocalized significantly with the enlarged Rab5Q79L-positive vesicles (Figure 3). Rab7 facilitates the correct aggregation and fusion of late endocytic structures and the biogenesis of lysosomes. Overexpression of the active GFP-Rab7Q67L causes enlargement and increased aggregation of late endocytic vesicles in the perinuclear region (Bucci et al., 2000). No significant colocalization between Rab7-decorated vesicles and the 4-phosphatase was apparent (Figure 3). Overexpression of Rab11Q70L, a dominant-positive GTPase-deficient mutant, increases the rate of Tfn uptake and accumulation of TfnR in early endosomes, as well as enhancing perinuclear recycling and inhibiting endosome to TGN trafficking (Wilcke et al., 2000). The GDP-locked mutant, Rab11S25N, localizes to the cytosol as GTP hydrolysis is required for recruitment of Rab11 and its effectors to endosomal membranes (Wilcke et al., 2000). In cells overexpressing Rab11S25N, the 4-phosphatase showed a more prominent cytosolic distribution with loss of endosomal localization (Figure 3). When coexpressed with Rab11Q70L, the 4-phosphatase colocalized with Rab11Q70L-positive vesicles (Figure 3). Interestingly, under these conditions the 4-phosphatase also localized to the PM in quiescent cells (Figure 3, row 4, arrow), suggesting that this constitutively active Rab11 mutant may promote the recycling of 4-phosphatase–decorated vesicles to the PM. In control studies, no change in the localization of EEA-1 (early endosomes) or Sec15 (recycling endosomes) (Zhang et al., 2004) was observed upon expression of Rab11Q70L (Figure 3, bottom two rows). This demonstrates that the 4-phosphatase may associate with and/or dissociate from early/recycling endosomes upon overexpression of specific Rab GTPase mutants.
4-Phosphatase Translocates to the Plasma Membrane upon EGF Stimulation

EGF treatment stimulates PtdIns(3,4)P₂ synthesis at the PM (Cantley, 2002), and under these conditions, within 5 min, both the endogenous (our unpublished data) and the recombinant 4-phosphatase (Figure 4A, arrows) were detected at the PM persisting up to 30 min in the majority of cells (>300 cells scored from three independent transfections). In addition we noted 4-phosphatase endosomal localization persisted in EGF-stimulated cells. The 4-phosphatase colocalized with areas of intense actin staining at the leading edge of the EGF-stimulated cells (membrane ruffles), as shown by the PM.
Figure 3. 4-Phosphatase localization upon expression of Rab GTPase mutants. COS-1 cells were cotransfected with wild-type 4-phosphatase and Rab5Q79L, Rab7Q67L, dominant-negative Rab11 (Rab11S25N), or Rab11Q70L; serum-starved overnight; and visualized by confocal microscopy. Arrow shows 4-phosphatase PM localization upon coexpression with Rab11Q70L. Control studies in Rab11Q70L-expressing cells demonstrate unchanged EEA1 (early endosome) and Sec15 (recycling endosome) localization. Merged images are shown on the right in top three panels only. Bar, 5 μm.
phalloidin staining (Figure 4A). Minimal colocalization of the 4-phosphatase was demonstrated with actin stress fibers, either in quiescent or stimulated cells. Notably, in cells over-expressing the recombinant 4-phosphatase, after EGF stimulation, no decrease in the number of membrane ruffles formed was detected relative to empty vector-expressing

Figure 4. EGF stimulates 4-phosphatase plasma membrane translocation. COS-1 cells transiently expressing GFP-4-phosphatase (A) were serum-starved overnight, stimulated with 100 ng/ml EGF, fixed, counterstained with phalloidin, and visualized by confocal microscopy. Arrow indicates GFP-4-phosphatase plasma membrane localization. (B) COS-1 cells expressing GFP-4-phosphatase were serum-starved overnight and treated with 30 μM nocodazole for 45 min, washed, and stimulated with 100 ng/ml EGF for 10 min and counterstained with anti-β-tubulin monoclonal antibody. Merged images are shown on the right. Bar, 5 μm.
cells. Depolymerization of microtubules upon nocodozole treatment resulted in complete abrogation of 4-phosphatase translocation to the PM, indicating that 4-phosphatase PM translocation is microtubule dependent (Figure 4B).

4-Phosphatase Contains an N-Terminal Tandem C2 Domain

The human type I 4-phosphatase sequence (GENPEPT [NP_004018]) was used as a probe in PSI-BLAST searches (Altschul et al., 1997) and showed significant sequence similarity (expect score 3 × 10^{-27}) in the N terminus (residues 31–206) to the synaptotagmin tandem C2 domains (C2A/C2B) (pdb identifier 1DQV) (Bai and Chapman, 2004) and similarity to numerous other C2 domains (expect score 3 × 10^{-27}) (Hurley and Meyer, 2001). The 4-phosphatase central domain (residues 305–608) seems unique, sharing no sequence similarity to other protein families. The C-terminal catalytic domain has been defined previously (Norris et al., 1997b). Based on these data and the structural analysis of the synaptotagmin tandem C2 domains, we propose that the N-terminal region of the human type I 4-phosphatase (residues 1–151) contains a C2 domain (C2A), and we predict that a second, tandem C2 domain (C2B) is located between residues 156–304 (Figure 5). The extent of sequence similarity is low (<15%) within the second putative C2 domain, and accurate assignment of domain boundaries based upon informatics data alone is difficult. A variety of 4-phosphatase constructs were developed to functionally assign individual regions that regulate enzyme activity and/or subcellular localization (Figure 5). Structural studies of PTEN and tyrosine phosphatases reveal that the catalytic CxR motif forms a tight loop that binds the substrate’s phosphate moiety and contains the catalytic cysteine responsible for the nucleophilic attack (Lee et al., 1999; Das et al., 2003). In addition, a conserved aspartic acid (Asp) located on a mobile loop N-terminal to the catalytic cysteine acts as a general acid essential for catalytic activity. We have identified a conserved candidate Asp residue in all 4-phosphatases, including the archael SopB and SigD, as D693 (Figure 5), (Norris et al., 1998).

To characterize the role of the C2 domains and/or candidate catalytic Asp (D693) in PtdIns[3,4]P₂ hydrolysis, we generated wild-type and mutant 4-phosphatases that lacked the N-terminal tandem C2 domain (ΔC2AB) or had a mutation in D693 (D693N) (Figure 5). His-tagged wild-type 4-phosphatase, D693N-4-phosphatase, and a mutant lacking the C2AB domain (ΔC2AB) were expressed and purified (Figure 6A). Recombinant wild-type 4-phosphatase dephosphorylated PtdIns[32P-3,4]P₂ forming PtdIns[32P-3]P. However, mutation of the conserved Asp, D693N, resulted in complete loss of lipid phosphatase activity at all enzyme concentrations tested (Figure 6B). Despite the presence of this essential catalytic Asp and the CxR motif, characteristic of tyrosine phosphatases, the 4-phosphatase exhibited no tyrosine phosphatase activity (our unpublished data). Recombinant 4-phosphatase which lacked the tandem C2 domain (4-phosphataseΔC2AB) showed little PtdIns[3,4]P₂ 4-phosphatase activity, suggesting that this domain may significantly regulate either substrate binding and/or sub-

Figure 5. Type I 4-phosphatase domain organization. 4-Phosphatase domains and summary of constructs which are NH₂-terminally His₆, GST, FLAG, HA, or GFP tagged. Shown also are the residues critical for catalysis, including D693 and C856.
strate orientation with respect to the catalytic domain (Figure 6B).

**4-Phosphatase C2AB Domain Binds to Phosphoinositides In Vitro**

We investigated the potential role of the tandem C2 domain in PtdIns(3,4)P₂ binding, given that the 4-phosphatase C2 domain deletion mutants lose catalytic activity. Liposomal binding studies were undertaken to examine the interaction of the C2AB domains versus the catalytic domain with PtdIns(3,4)P₂ and the 4-phosphatase reaction product PtdIns(3)P. GST-C2AB bound PtdIns(3,4)P₂ and to a lesser extent phosphatidylserine, but failed to bind PtdIns(3)P, although it could bind other monophosphorylated phosphoinositides such as PtdIns(4)P (Figure 6C). A 4-phosphatase mutant that contains the catalytic domain, but not the C2AB domain (∆C2AB), bound both PtdIns(3,4)P₂ and PtdIns(3)P (Figure 6D). In control studies, GST did not bind any phospholipids (our unpublished data). Thus, both the C2AB domain and the catalytic domain facilitate PtdIns(3,4)P₂ binding, whereas the catalytic domain also binds the product of 4-phosphatase PtdIns(3,4)P₂ hydrolysis, PtdIns(3)P. C2AB domain binding to PtdIns(3,4)P₂ may contribute to 4-phosphatase substrate orientation with respect to residue D693 and thus facilitate PtdIns(3,4)P₂ D4 phosphate hydrolysis.

**4-Phosphatase C2AB Domain Mediates Endosomal Localization**

We investigated the subcellular targeting of the 4-phosphatase by specific domains. The individual C2A or C2B domains localized diffusely in the cytosol (Figures 7A and 5, for constructs). However, the C2AB domain showed distinct vesicular staining with little cytosolic distribution. These vesicles colocalized with Rab11 (our unpublished data). 4-phosphatase mutants lacking the C2A, or the C2AB domain (∆C2A and ∆C2AB, respectively) showed predominantly cytosolic staining. A 4-phosphatase mutant lacking the C-terminal half of the catalytic domain (∆1/2 catalytic) localized to endosomal vesicles and the cytosol like the intact wild-type 4-phosphatase but without the prominent perinuclear vesicle clustering. The catalytic domain localized diffusely in the cytosol and the perinuclear region, but endosomal vesicles were not as clearly delineated. Hence, the C2 domains play a significant role in directing the 4-phosphatase to endosomes. Immunoblot analysis showed intact expression of all mutants (Figure 7A).

We also investigated which 4-phosphatase domain directs PM localization. After EGF stimulation (10 min), the isolated C2AB domain was not detected at the PM; however, the addition of the central domain to the C2AB domain (C2AB + central) resulted in prominent PM localization, suggesting that this central domain contains critical motifs that facilitate PM targeting (Figure 7B, arrow). Mutant 4-phosphatase lacking either the C2A domain (∆C2A) or both the N-terminal C2 domains (∆C2AB) showed PM recruitment (Figure 7B, arrow). The isolated 4-phosphatase catalytic domain also localized to the PM on EGF stimulation (Figure 7B, arrow). Therefore, 4-phosphatase PM relocalization is facilitated by the catalytic and central but not the C2AB domains.

**Expression of the Catalytically Active 4-Phosphatase Rescues the Wortmannin-induced Dilatation of Early Endosomes**

The studies reported here have revealed a previously unrecognized localization of the 4-phosphatase to endosomes. PtdIns(3)P is synthesized on early endosomes by the wort-
mannin-sensitive PI3-kinase hVPS34, which is recruited to endosomal membranes by Rab5GTP (Christoforidis et al., 1999). Several studies have proposed that PtdIns(3)P regulates early endosomal fusion, because inhibition of PI3-kinase activity by using the inhibitor wortmannin is associated with early endosomal dilatation and inhibition of in vitro early endosomal fusion (Jones and Clague, 1995; Li et al., 1995; Jones et al., 1998). EEA-1 localizes to early endosomal membranes, by an interaction between its FYVE domain, PtdIns(3)P, and Rab5-GTP (Stenmark et al., 1996). Treatment of cells with 100 nM wortmannin blocks PtdIns(3)P synthesis on early endosomes, leading to EEA-1 dissociation (Patki et al., 1997), and the early endosomes become dilated, looking like large, hollow rings (Simonsen et al., 1998).

PtdIns(3,4)P$_2$ accumulates both at the plasma membrane and on intracellular membranes, including tubulo-vesicular structures, the endoplasmic reticulum, and multivesicular bodies upon agonist stimulation (Watt et al., 2004). We reasoned that overexpression of the 4-phosphatase may catalyze the hydrolysis of PtdIns(3,4)P$_2$, forming PtdIns(3)P on endosomal membranes. The effect of 4-phosphatase overexpression on wortmannin-induced endosomal dilatation was therefore examined. To this end, COS-1 cells, serum-starved or grown in serum, the latter being the conditions under which PtdIns(3,4)P$_2$ levels increase (Figure 8, A and B, respectively), transiently expressing the wild-type or inactive 4-phosphataseC856S were treated with 100 nM wortmannin (30 min) and counterstained with EEA-1 antibody.
starved, wortmannin-treated cells developed dilated early endosomes in vector and inactive 4-phosphatase–expressing cells (our unpublished data) and also upon overexpression of the wild-type 4-phosphatase, as shown by the appearance of dilated “EEA-1–stained rings” (Figure 8A, a–c, magnified in d). In f, upper box is magnified in h; in i, lower box magnified in j; and box in k is magnified in m. Arrows indicate endosomal enlargement. Bar, 5 μm. (D) Cells stably overexpressing the 4-phosphatase or empty vector were grown in serum and labeled for 2 h with [32P]orthophosphate. Deacylated lipids were analyzed by SAX HPLC. 32P counts (cpm) derived from PtdIns(3,4)P2 or PtdIns(3)P for vector cells showing mean and SE of three experiments (black bars); cells overexpressing type I 4-phosphatase showing mean and SE of four experiments (open bars). PtdIns(3,4)P2 to PtdIns-3P ratios in vector versus type I 4-phosphatase–overexpressing cells are shown in boxed area.

Figure 8. 4-Phosphatase rescues the wortmannin-induced dilatation of early endosomes. COS-1 cells transiently expressing GFP-wild type (A and B) or catalytically inactive (C856S) 4-phosphatase (C) were treated with 100 nM wortmannin for 30 min after serum starvation overnight (A) or growth in serum (B and C) and were fixed and counterstained with EEA-1 antibody. White box in b is magnified in d; in f, upper box is magnified in h; in i, lower box magnified in j; and box in k is magnified in m. Arrows indicate endosomal enlargement. Bar, 5 μm. (D) Cells stably overexpressing the 4-phosphatase or empty vector were grown in serum and labeled for 2 h with [32P]orthophosphate. Deacylated lipids were analyzed by SAX HPLC. 32P counts (cpm) derived from PtdIns(3,4)P2 or PtdIns(3)P for vector cells showing mean and SE of three experiments (black bars); cells overexpressing type I 4-phosphatase showing mean and SE of four experiments (open bars). PtdIns(3,4)P2 to PtdIns-3P ratios in vector versus type I 4-phosphatase–overexpressing cells are shown in boxed area.
inhibits hVPS34-mediated synthesis of PtdIns(3)P on early endosomes, the PtdIns(3,4)P2 pool hydrolyzed by the 4-phosphatase in wortmannin-treated cells may be synthesized by a wortmannin-resistant PI3-kinase, such as the class II C2/H9251 PI 3-kinase (Domin et al., 2000).

We investigated whether the 4-phosphatase regulates the total cellular levels of PtdIns(3)P by using stable cell lines overexpressing this enzyme, generated using the T-Rex tetracycline-regulated system (Kisseleva et al., 2002). These studies were performed on cells grown in serum, conditions under which we demonstrated overexpression of the 4-phosphatase rescued the wortmannin-induced dilatation of early endosomes. Deacylated 32P-labeled phosphoinositides were analyzed by anion-exchange high-performance liquid chromatography (HPLC). The levels of PtdIns(3,4)P2 and PtdIns(3)P are as shown in Figure 8D. In cells stably transfected with type I 4-phosphatase, PtdIns(3,4)P2 was reduced by 20% compared with the empty vector cells, accompanied by a 1.76-fold increase in PtdIns(3)P. In vector cells, the PtdIns(3)P/PtdIns(3,4)P2 ratio was 2.8 (Figure 8D), whereas in cells overexpressing the 4-phosphatase, this ratio increased to 6. In control studies, the levels of PtdIns(4)P and PtdIns(4,5)P2 were comparable in vector and 4-phosphatase-overexpressing cells (our unpublished data). Therefore, under conditions of serum stimulation, the total intracellular levels of PtdIns(3)P are increased upon 4-phosphatase overexpression.

To further reveal whether the 4-phosphatase generates a functional PtdIns(3)P pool, we examined the endosomes of MEFs derived from homozygous Weeble mice (Nystuen et al., 2001). The Weeble mutation was localized by genetic mapping to the 4-phosphatase type I gene. Deletion of a single nucleotide 744G in the 4-phosphatase sequence, results in a frame shift, creating a stop codon at aa 263. As anticipated, 4-phosphatase type I immunoblot analysis of cell lysates from wild-type (WT), heterozygous, and homozygous Weeble MEFs revealed the absence of the 105-kDa 4-phosphatase in Weeble/H11002/H11002 MEF lysates (Figure 9A). Wild-type MEFs grown in serum demonstrated punctate EEA-1 staining of endosomes (Figure 9B, a and ai). In contrast, +/− MEFs from heterozygotes (Figure 9B, b), and more notably homozygous Weeble MEFs showed significant enlargement of endosomes with the appearance of dilated EEA-1–decorated rings (Figure 9B, c and ci, arrows). Furthermore, Rab11-decorated endosomes also demonstrated dilatation in Weeble/H11002/H11002 MEFs (Figure 9B, g and gi, arrows) that was not detected in the wild-type MEFs (Figure 9B, e and ei). These studies strongly indicate that the 4-phosphatase generates a functional PtdIns(3)P pool on endosomes.

4-Phosphatase Regulates the PM Recruitment of the TAPP1 PH Domain

The 4-phosphatase regulation of the recruitment of PtdIns(3,4)P2-specific effectors to the PM has not been re-
Figure 10. 4-Phosphatase regulates the PM recruitment of a PtdIns(3,4)P₂-specific effector. COS-1 cells transiently expressing GFP-TAPP1 PH domain and FLAG empty vector (A) or FLAG-4-phosphatase (B) or HA-4-phosphatase catalytic domain (aa 608–938) (C). Cells were serum-starved overnight and stimulated with 100 ng/ml EGF and visualized by confocal microscopy. Cells were scored for TAPP1 PM localization. Data are expressed as the mean number of cells ± SEM (n = 3) showing no (white bars), moderate (gray bars), or extensive (black bars) GFP-PH-TAPP1 PM staining (20 cells scored for each of three independent transfections).
ported previously. To address this question, we examined the effect of overexpression of the intact 4-phosphatase versus the isolated catalytic domain on the PM recruitment of the PH-domain of TAPP1 (GFP-PH-TAPP1), upon EGF stimulation (Figure 10). Cotransfected cells from three independent transfections were scored for extensive, moderate or absent PM GFP-PH-TAPP1. In serum-starved cells expressing empty FLAG-vector and GFP-PH-TAPP1, GFP fluorescence was detected diffusely throughout the cytoplasm and the nucleus (Figure 10A). On EGF stimulation, GFP-PH-TAPP1 PM staining was evident within 5 min, persisting up to 45 min (Marshall et al., 2002) (Figure 10A, arrowheads). After 10 min EGF stimulation, the majority of cells showed extensive PM staining. By 45 min, the intensity of PM staining had decreased. Significantly, upon coexpression of GFP-PH-TAPP1 with wild-type FLAG-4-phosphatase, no PM GFP fluorescence was detected after 5- to 10-min EGF stimulation (Figure 10B). In some cells, faint PM fluorescence was evident by 20 and 45 min EGF stimulation, but this was significantly reduced relative to the empty vector-expressing cells. As described here, the 4-phosphatase mutants lacking C2 domains can bind PtdIns(3,4)P₂, but they do not possess PtdIns(3,4)P₂ 4-phosphatase activity (Figure 6). The isolated catalytic domain which lacks the C2 domains (HA-catalytic) was coexpressed with GFP-PH-TAPP1 (Figure 10C). After 10-min EGF stimulation, moderate-to-extensive GFP-PH-TAPP1 fluorescence was detected at the PM in the majority of cells (Figure 10C, arrows). Therefore, the C2AB domain is essential for 4-phosphatase catalytic activity and thereby regulates the PM recruitment of PtdIns(3,4)P₂-specific PH domains.

**DISCUSSION**

The results of this study have revealed that the type I 4-phosphatase localizes to early and recycling endosomes and generates an endosomal pool of PtdIns(3)P, via hydrolysis of PtdIns(3,4)P₂. After EGF-stimulated PI 3-kinase activation, the 4-phosphatase translocates to membrane ruffles, the site of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ synthesis, where the enzyme inhibits the PM recruitment of the PtdIns(3,4)P₂-specific TAPP1 PH domain. The 4-phosphatase C2 domains are critical for the enzyme’s endosomal targeting, PtdIns(3,4)P₂ 4-phosphatase catalytic activity, and in agonist-stimulated cells, 4-phosphatase-mediated inhibition of PM recruitment of TAPP1 PH domain. Collectively, these studies demonstrate a functional role for the 4-phosphatase in regulating PI 3-kinase signaling events, both in the endosomal pathway and at the PM.

The spatiotemporal dynamics of membrane trafficking events are governed by reversible recruitment of effector proteins to restricted intracellular membranes decorated with phosphoinositide signals such as PtdIns(3)P and PtdIns(3,4)P₂ (Cantley, 2002; Czech, 2003). Molecular mechanisms mediating the concentration of such lipid signaling molecules to specific microdomains of intracellular compartmental membranes are in turn regulated by the subcellular targeting of the specific kinases and/or phosphatases that respectively synthesize or degrade these phosphoinositides. Consistent with the contention that PtdIns(3)P and/or PtdIns(3,4)P₂ signals are turned over at high rates in restricted locations, we have shown here that the type I 4-phosphatase localizes to endosomal membranes, colocalizing with early and/or recycling endosomes. Although we cannot exclude the possibility that the C2AB domain mediates 4-phosphatase endosomal association via protein–protein rather than protein–phosphoinositide interaction, as has been shown for other C2 domains (Hurley and Meyer, 2001; Bai and Chapman, 2004). The C2AB domain also was critical for 4-phosphatase catalytic activity, as shown by in vitro enzyme assays. In addition, the wild-type 4-phosphatase, but not mutants lacking the C2AB domain, inhibited the PtdIns(3,4)P₂-dependent translocation of PH domain of TAPP1 to the PM, consistent with a significant functional role for the C2 domains in facilitating either PtdIns(3,4)P₂ binding and/or orientation with respect to the catalytic domain. Studies of the lipid phosphatase PTEN, which also uses PtdIns(3,4)P₂ as a substrate and contains an N-terminal C2 domain, have revealed that both its phospha-
A widely accepted concept in the field is that the lipid phosphatases act to terminate PI 3-kinase signals. However, with this study we have presented a paradigm shift, showing that the 4-phosphatase also can function in signal generation, forming PtdIns(3)P. In addition, in agonist-stimulated cells, the 4-phosphatase terminates PI 3-kinase signals at the PM via hydrolysis of PtdIns(3,4)P 2, and thereby abrogates the recruitment of its effector proteins to this site. Therefore, the activity and the subcellular targeting of the 4-phosphatase are critical to its function in both signal generation and termination at specific subcellular sites.

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Dyson, J. M., O’Malley, C. J., Becanovic, J., Munday, A. D., Berndt, M. C., Coghill, I. D., Pandolfi, P., and Pavletich, N. P. (1999). Crystal structure of the 6-phosphatase catalytic domain and the C2 domain mediating the enzyme’s association with the PM. PtdIns(3,4)P 2 functions as a signaling molecule in growth factor-stimulated pathways and also is generated independently of PtdIns(3,4,5)P 3 in response to oxidative stress (Van der Kaay et al., 1999). The major metabolic pathway for PtdIns(3,4)P 2 synthesis in growth-factor-stimulated cells is via 5-phosphatase hydrolysis of PtdIns(3,4,5)P 3. It is noteworthy that several PtdIns(3,4,5)P 3 5-phosphatases localize to membrane ruffles in stimulated cells (Dyson et al., 2001; Gurung et al., 2003). In addition, the TAPP1 PH domain specifically binds PtdIns(3,4)P 2 and also localizes to membrane ruffles, suggesting that this is the site of PtdIns(3,4)P 2 synthesis (Marshall et al., 2002). We have shown that upon EGF-stimulation, the 4-phosphatase translocates to membrane ruffles, where the enzyme regulates the PM recruitment of the PH domain of TAPP1. The targeting of 4-phosphatase to endosomes in quiescent cells, away from the PM, and its delayed recruitment to membrane ruffles upon agonist stimulation may represent a molecular mechanism mediating delayed and sustained PtdIns(3,4)P 2 signaling at the PM.

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4-Phosphatase Localizes to Endosomes


