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The Type I α 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 I α 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. The 4-phosphatase contains C2 domains, which bind PtdIns(3,4)P₂, and C2-domain-deletion mutants lost PtdIns(3,4)P₂ 4-phosphatase activity, did not localize to endosomes or inhibit TAPP1 PH domain membrane recruitment. The 4-phosphatase therefore both generates and terminates phosphoinositide 3-kinase signals at distinct subcellular locations.

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 ki-

nases 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 p47^{phox} binds both PtdIns(3,4)P₂ and PtdIns(3)P (Kanai *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 I α 4-phosphatase regulates megakaryocyte cell growth, acting downstream of the transcription factor GATA-1. Overexpression of this 4-phos-

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Abbreviations used: EEA, early endosomal antigen; 4-phosphatase, inositol polyphosphate 4-phosphatase; 5-phosphatase, inositol polyphosphate 5-phosphatase; MTOC, microtubule organizing center; PtdIns(3,4)P₂, phosphatidylinositol (3,4)-bisphosphate; PtdIns3-P, phosphatidylinositol 3-phosphate; PI 3-kinase, phosphoinositide 3-kinase; PM, plasma membrane; TAPP1, tandem PH domain-containing protein-1.

phatase in NIH 3T3 fibroblasts suppresses cellular proliferation, without affecting apoptosis (Vyas *et al.*, 2000). A spontaneous mutant mouse (*Weeble*), 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 (Nys-tuen *et al.*, 2001).

In this study, we show that the type I 4-phosphatase localizes to endosomal membranes, generating PtdIns(3)P via hydrolysis of PtdIns(3,4)P₂, 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 TAPP1 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)P on endosomal membranes and terminating 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), pTrcHisC 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 Dr. Willson (Walter and Eliza Hall Institute, Melbourne, Australia). Rab5 and Rab5Q79L cDNAs were kindly provided by Dr. R. Parton (University of Queensland, Brisbane, Australia). The PH domain of TAPP1 (aa 95–404) cDNA was from Dr. S. Dowler (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, United Kingdom). Other reagents were from Sigma-Aldrich (St. Louis, MO).

Antibodies

Two New Zealand White rabbits were immunized with the 4-phosphatase type I peptides (¹MTAREHSPRHGARARA¹⁶C and ¹⁷⁴EEEKSDQRPVTRSDVTVNGR¹⁹³C) conjugated via the terminal cysteine to diphtheria toxoid. Serum was purified on an immunopeptide-coupled thiopropyl-Sepharose 6B resin. Other antibodies or stains were obtained as indicated: Xpress antibody (Invitrogen); FLAG antibody (Sigma-Aldrich); hemagglutinin (HA) antibody (Silenus, Melbourne, Australia); green fluorescent protein (GFP) antibody, phalloidin, and Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR); glutathione S-transferase (GST) antibody (Promega); β -tubulin, γ -adaptin, and β -COP, Rab11 antibody (Zymed Laboratories, South San Francisco, CA); Rab8 (BD Biosciences, San Jose, CA), LAMP-2 antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); and human early endosomal antigen (EEA)-1 antibody (gift 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-KG-4ptase), adding N-terminal hexa-His and GST tags in frame. Asp693 was PCR mutated to asparagine and ligated into the *KpnI* site of pTrcHisC (pTrcHisC-D693N). N-terminal GST-tandem C2 domains were PCR amplified and subcloned into the pGEX-KG *HindIII* site. 4-Phosphatase (aa 305–939) was PCR amplified and subcloned into the *KpnI* site of pTrcHisC or into the *HindIII* site of pGEX-KG (pTrcHisC-4ptase Δ C2AB and pGEX-KG-4ptase Δ C2AB). Constructs were transformed into BL21 *Escherichia coli*, incubated to an OD₆₀₀ = 0.6, and protein was induced with isopropyl β -D-thiogalactopyranoside (1 mM final). Pelleted bacteria were resuspended in 50 mM Tris, pH 8, 1 M NaCl, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) lysed by 3 \times sonication 30 s on ice, pelleted 19,000 \times g (15 min) and purified by Ni²⁺ affinity chromatography. Ni-NTA resin (2 ml) (BD Biosciences, Palo Alto, CA), preequilibrated with lysis buffer, was added to the cleared bacterial lysate and rocked at 4°C 1 h, pelleted at 700 \times g for 5 min, washed, proteins eluted with 10 ml of 75 mM imidazole, 50 mM Tris, pH 8, 1 M NaCl, and concentrated using a Vivaspinn 10,000 molecular weight cut-off

(Vivascience, Stonehouse, United Kingdom). Two milliliters of glutathione Sepharose resin, preequilibrated with phosphate-buffered saline (PBS), was added to the cleared bacterial lysate, rocked at 4°C for 1 h, pelleted at 500 \times g for 5 min, washed with ice-cold PBS, and eluted with 10 mM glutathione, 50 mM Tris, pH 8.

Mammalian Expression Plasmids

Wild-type 4-phosphatase cDNA was subcloned into pEGFP-C2 (*KpnI* site) and pEFBOS (*MluI* site) encoding GFP- and FLAG-tagged 4-phosphatase, respectively. The 4-phosphatase catalytic mutant C856S was PCR generated and cloned into pEGFP-C2 (*KpnI* site) and pEFBOS (*MluI* site). Various 4-phosphatase mutants or domains were PCR amplified and subcloned into the *KpnI* sites of pEGFP-C2, or pCGN and/or the *MluI* site of pEFBOS. All PCR product sequences were confirmed by Big-Dye Terminator DNA sequencing.

Cell Culture, Transient Transfections, and Microscopy

COS-1 and Chinese hamster ovary (CHO) cells were cultured in DMEM and Ham's-F-12, respectively, with 10% fetal calf serum (FCS) (CSL Biosciences, Parkville, Australia), 100 U/ml penicillin/streptomycin, and 2 mM glutamine. COS-1 cell transfections were performed by electroporation, using the Gene Pulser II System (Bio-Rad, Hercules, CA). For confocal microscopy, transfected cells were grown on coverslips and allowed to recover for 20–40 h. Nontransfected or transfected cells (24 h posttransfection) were serum-starved for 20 h and stimulated with 100 ng/ml EGF or grown in DMEM 10% FCS, or Ham's-F-12, or serum-starved for 1–2 h and incubated with nocodazole (30 mM for 45 min), or 100 nM wortmannin (for 30 min or 60 min), or brefeldin A, and fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. Primary antibodies were incubated for 45 min at room temperature (RT) in the following dilutions: anti-EEA-1 (1:10,000); anti-FLAG and anti-HA (1:2000); anti-Xpress (1:500); anti- β -tubulin, anti- γ -adaptin, and anti- β -COP (1:50); anti-Rab11, anti-Rab8, and anti-LAMP2 (1:20); and anti-4-phosphatase (1:5). Antibodies were detected using Alexa-conjugated goat anti-mouse or mouse anti-rabbit secondary antibodies (1:800). Coverslips were washed three times with PBS and mounted onto glass slides by using SlowFade. Single plane or stacked images were obtained using a Leica TCS-NT Krypton or Fluoview confocal microscope.

For ultrastructural analysis of 4-phosphatase localization by using electron microscopy, CHO cells were transiently cotransfected with GFP-4-phosphatase and human transferrin receptor (hTfRn), incubated with 25 μ g/ml Tf-horseradish peroxidase (HRP), and Tf-positive endosomes were labeled with the electron-dense peroxidase 3,3'-diaminobenzidine (DAB) reaction product. Ultrathin cryosections were incubated with GFP antibody followed by 10-nm gold-conjugated anti-mouse IgG secondary antibody.

Preparation of Cell Lysates

Cell lines grown to confluence were washed with PBS, lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM benzamide, 2 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, and 2 μ g/ml aprotinin for 2 h at 4°C, and pelleted at 15,400 \times g (10 min) to isolate the Triton-soluble fraction.

Lipid 4-Phosphatase Assays

PtdIns[³²P-3,4]P₂ was formed by incubating 56 μ g of PtdIns(4)P with 20 μ g of phosphatidylserine (PtdSer), 1 μ g of recombinant PI 3-kinase (Layton *et al.*, 1998), 50 μ M ATP, 20 μ l of [γ -³²P]ATP (0.2 mCi, 3000 Ci/mM; MP Biomedicals, Irvine, CA), and 5 μ l of 20 \times kinase buffer (400 mM Tris, pH 7.4, 100 mM MgCl₂, and 10 mM EGTA) in 100 μ l. Lipid products were extracted and suspended in 20 mM Tris, pH 7.4, 1 mM EGTA. Lipid 4-phosphatase assays (100 μ l) were performed by adding PtdIns[³²P-3,4]P₂ to the recombinant wild-type or mutant 4-phosphatase 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 [43:38:5:7 (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 μ M EDTA; sonicated (5 min) to form liposomes; incubated with GST-fusion proteins in 50 μ l of sample buffer for 10 min at RT; and pelleted at 100,000 \times g for 30 min. The pellet was resuspended in 35 μ l of SDS-PAGE reducing buffer, analyzed by 10% SDS-PAGE, and silver stained. Densitometry was performed using GelPro software (Media Cybernetics, Silver Spring, MD).

Determination of Phosphoinositide Levels in Cells Overexpressing 4-Phosphatase I

A T-Rex tetracycline-regulated system (Invitrogen) was used to create cell lines stably overexpressing 4-phosphatase-1 as described previously

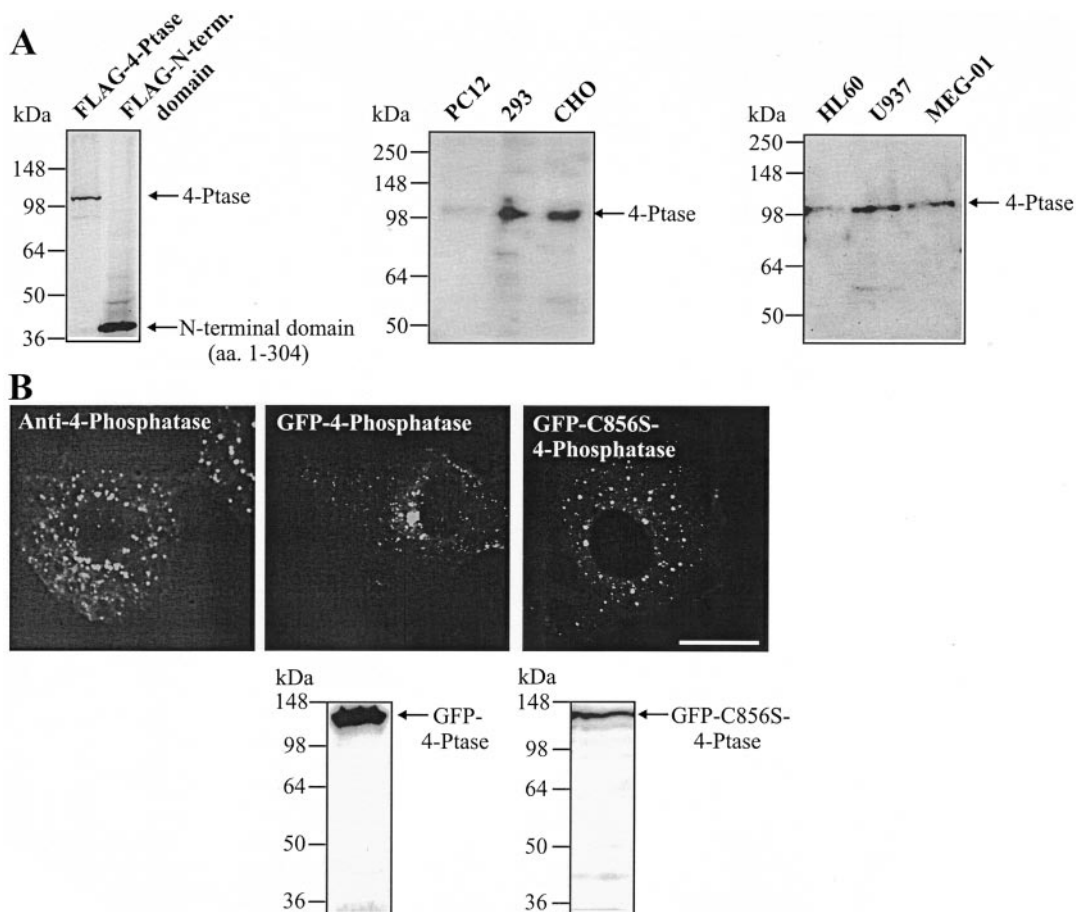


Figure 1. 4-Phosphatase intracellular localization. (A) Triton-soluble fraction of cell lysates from the indicated cell lines or COS-1 cells expressing FLAG-tagged wild-type 4-phosphatase, or the N-terminal 304 aa, were immunoblotted with affinity-purified 4-phosphatase antibody. (B) Serum-starved CHO cells were stained with affinity-purified 4-phosphatase antibody (left). COS-1 cells transiently expressing GFP-tagged wild-type (middle) or inactive 4-phosphatase^{C856S} (right) were visualized by confocal microscopy, or lysates were immunoblotted with GFP antibody. Bar, 5 μ m.

(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 μ Ci 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 μ l of chloroform and 500 μ l 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 \times 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 anti-peptide 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-phosphatase^{C856S}, which contains a C-S mutation in the CX₅R cata-

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 μ g/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, \sim 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 endo-

somes. 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 -adap-tin 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 homo- 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.

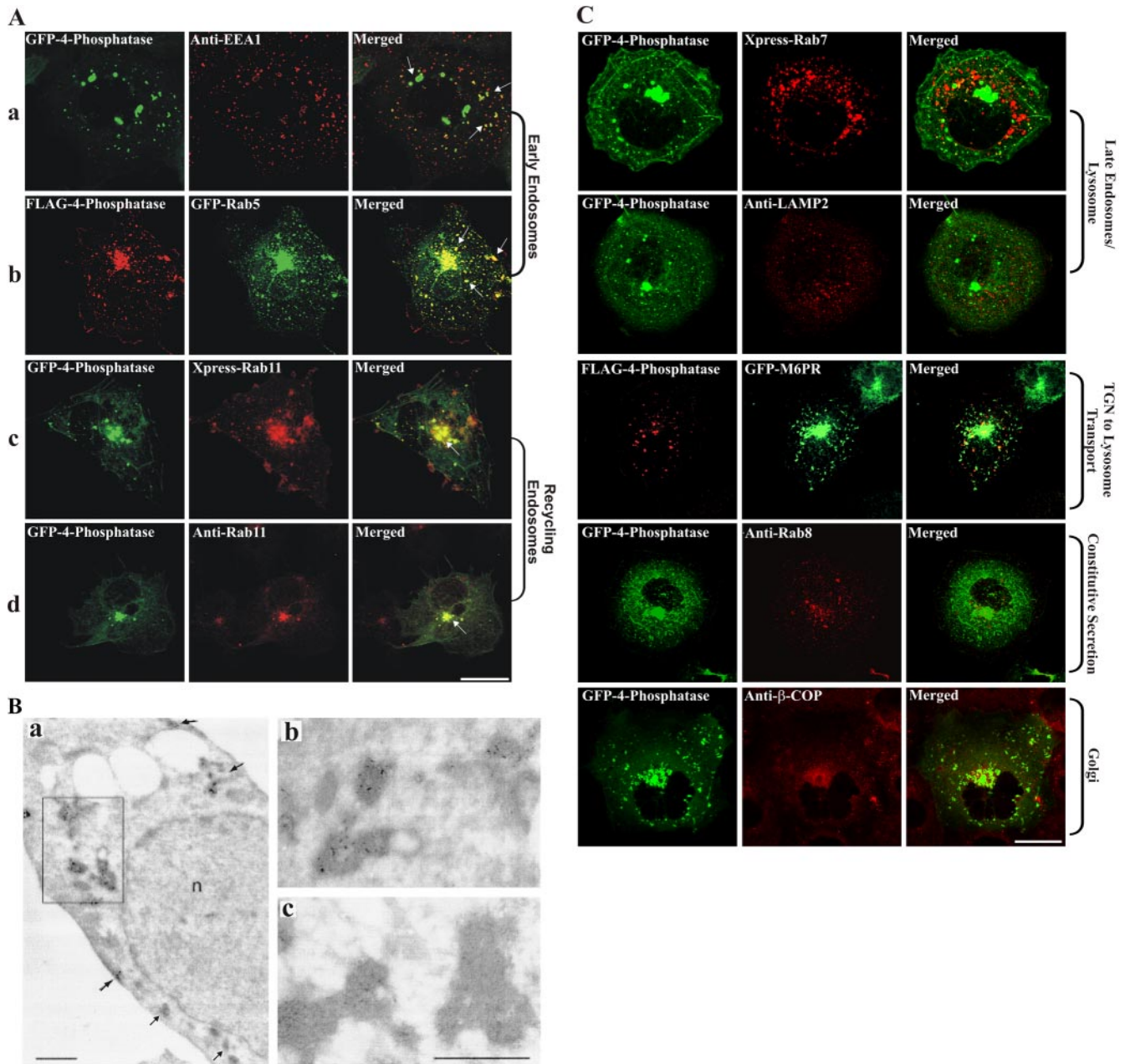


Figure 2. 4-Phosphatase colocalizes with endosomes. (A) COS-1 cells expressing GFP-4-phosphatase (green) or FLAG-4-phosphatase (row 2, red) were serum-starved overnight and colocalized with EEA-1 (a, red) or Rab11 (d, red) by using specific antibodies, or recombinant Xpress-Rab11 (c, red) or GFP-Rab5 (b, green), and visualized by confocal microscopy. Merged images are shown on the right. Arrows indicate colocalization. Cells were serum-starved overnight before fixation. Bar, 5 μm . (B) CHO cells expressing GFP-4-phosphatase and human TfnR were incubated with 25 $\mu\text{g}/\text{ml}$ Tfn-HRP for 30 min at 37°C and subjected to DAB cytochemistry. Ultrathin cryosections were incubated with GFP antibody and gold particle-conjugated anti-mouse IgG secondary antibody. (a) Low-power electron micrograph with DAB-positive/gold-labeled tubulo-vesicular structures (see arrows and boxed region). (b) High-power electron micrograph of boxed region in a. (c) High-power electron micrograph of vesicle clusters by using an anti-tyrosine hydroxylase IgG1 isotype control, showing DAB-positive regions with no immunogold labeling. n, nucleus. Bar, 1 μm . (C) COS-1 cells were either cotransfected with wild-type 4-phosphatase and Xpress-Rab7, or GFP-M6PR and serum-starved overnight. 4-Phosphatase-expressing cells were costained for endogenous lysosome (anti-Lamp-2), Golgi (anti- β -COP), or secretory vesicle (anti-Rab8) markers. All cells were serum-starved overnight before fixation. Bar, 5 μm .

4-Phosphatase Translocates to the Plasma Membrane upon EGF Stimulation

EGF treatment stimulates $\text{PtdIns}(3,4)\text{P}_2$ 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

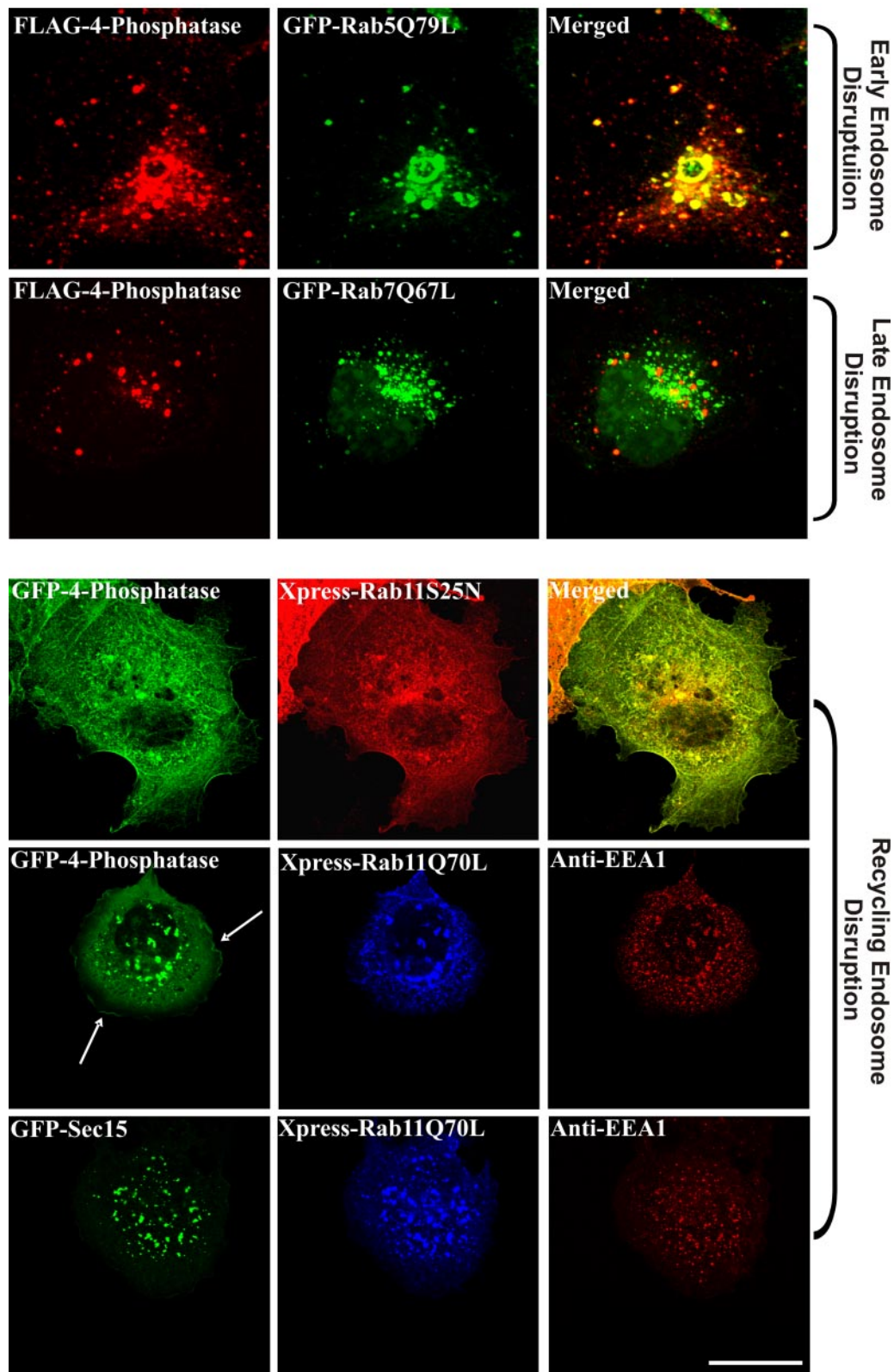


Figure 3. 4-Phosphatase localization upon expression of Rab GTPase mutants. COS-1 cells were cotransfected with wild-type 4-phosphatase and Rab5Q79L, Rab7Q67L, dominant-negative Rab11 (Rab11S25N), or Rab11Q70L; serum-starved overnight; and visualized by confocal microscopy. Arrow shows 4-phosphatase 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 μ m.

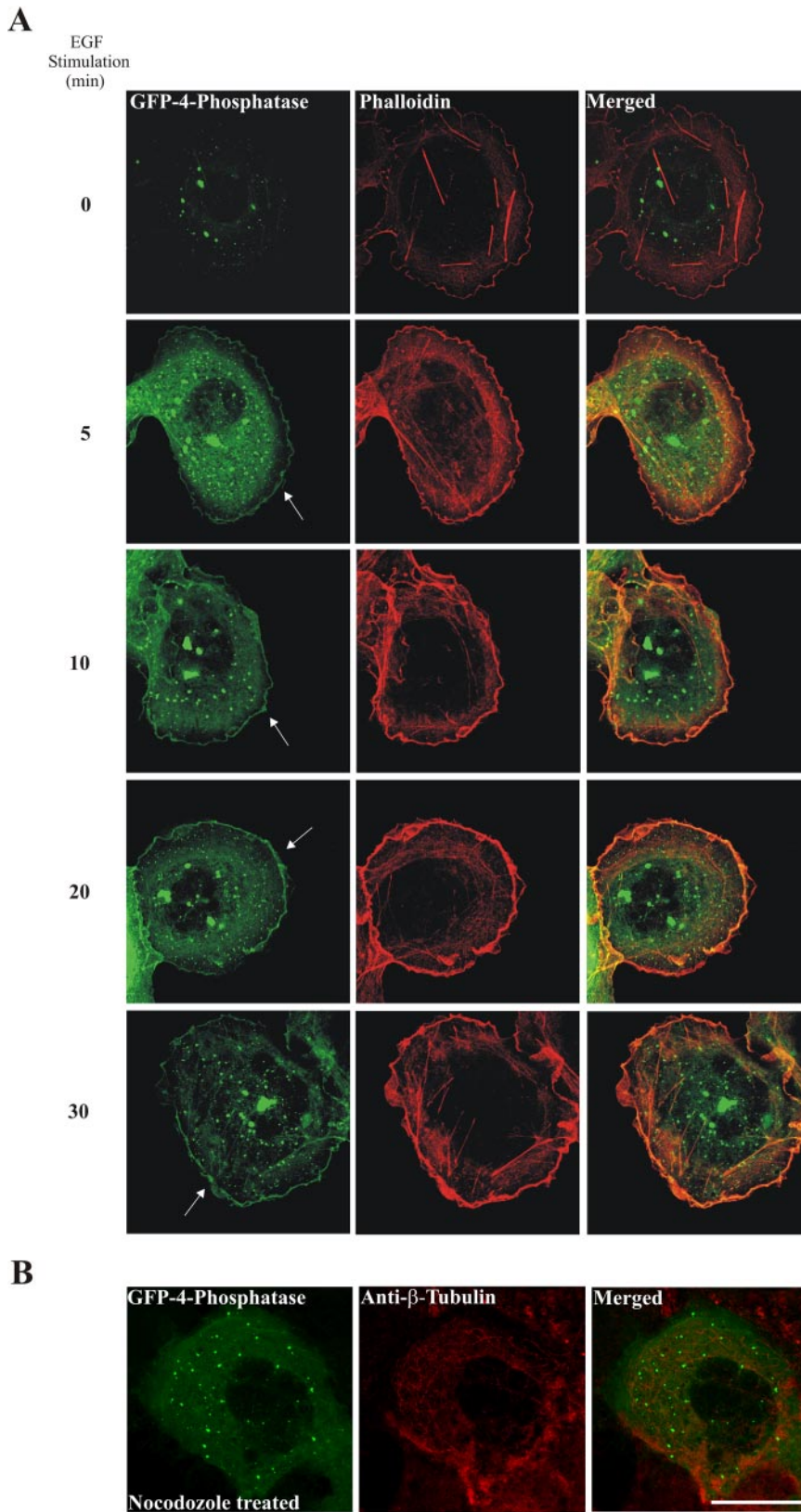


Figure 4. EGF stimulates 4-phosphatase plasma membrane translocation. COS-1 cells transiently expressing GFP-4-phosphatase (A) were serum-starved overnight, stimulated with 100 ng/ml EGF, fixed, counterstained with phalloidin, and visualized by confocal microscopy. Arrow indicates GFP-4-phosphatase plasma membrane localization. (B) COS-1 cells expressing GFP-4-phosphatase were serum-starved overnight and treated with 30 μ M 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 μ m.

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

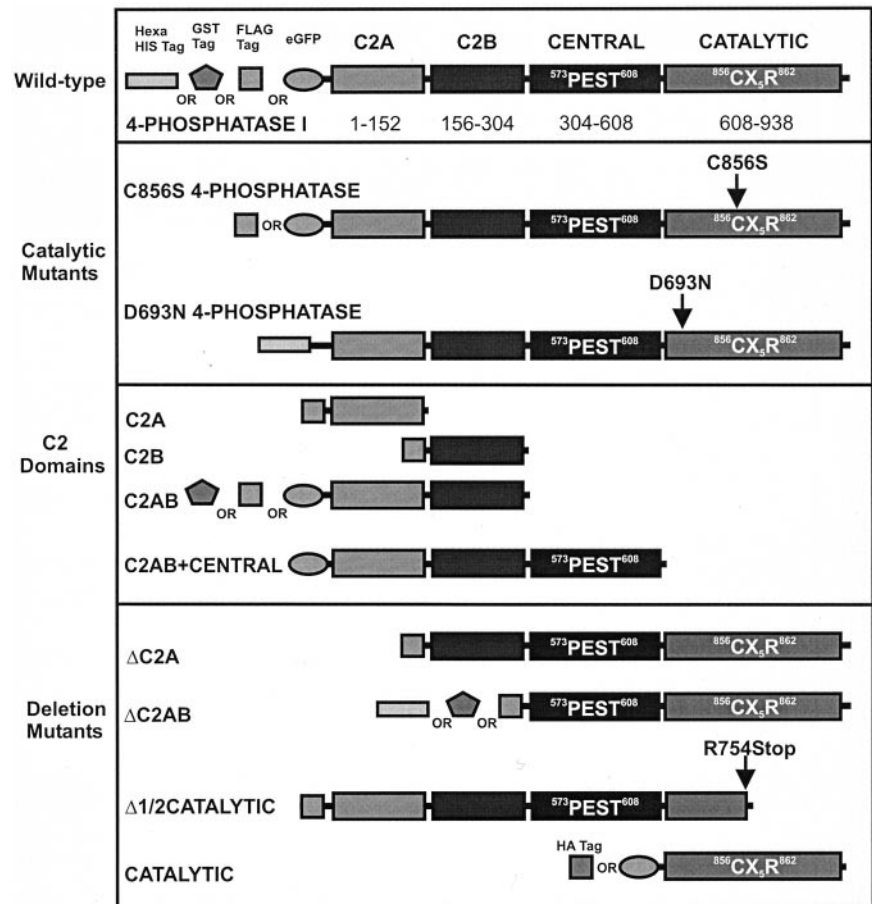


Figure 5. Type I 4-phosphatase domain organization. 4-Phosphatase domains and summary of constructs which are NH₂-terminally His₆, GST, FLAG, HA, or GFP tagged. Shown also are the residues critical for catalysis, including D693 and C856.

cells. Depolymerization of microtubules upon nocodazole 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 ty-

rosine phosphatases reveal that the catalytic CX₅R 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 archeal 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 CX₅R 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-

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 (Δ 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 (Δ C2A and Δ C2AB, respectively) showed predominantly cytosolic staining. A 4-phosphatase mutant lacking the C-terminal half of the catalytic domain (Δ 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 (Δ C2A) or both the N-terminal C2 domains (Δ 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-

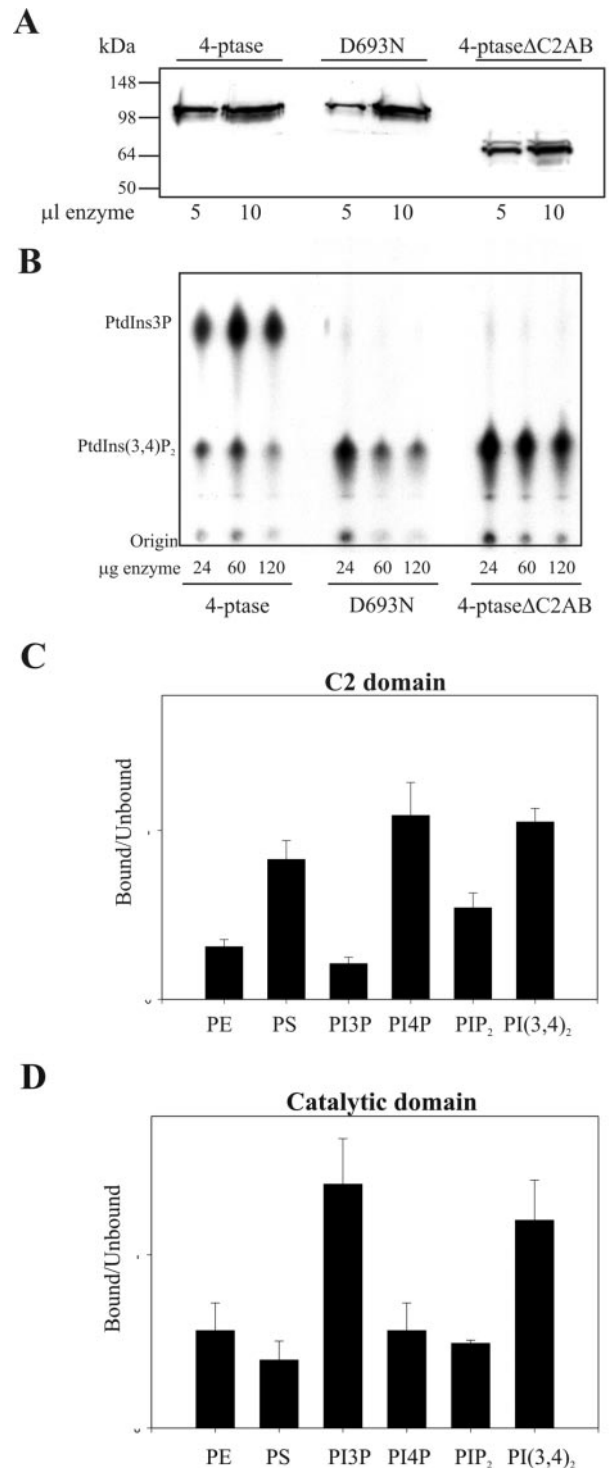


Figure 6. Phosphoinositide binding of 4-phosphatase domains. (A) Purified His₆-tagged wild-type, catalytically inactive 4-phosphatase (D493N), or 4-phosphatase lacking the C2AB domain (4-phosphatase Δ C2AB) were immunoblotted with anti-His₆ antibodies. (B) Hydrolysis of PtdIns[³²P-3,4]P₂ by the recombinant wild-type 4-phosphatase (24–120 μ g) or the indicated mutants. Extracted lipids were analyzed by TLC and autoradiography and compared with the migration of known standards. (C and D) Liposomes containing phosphatidylethanolamine/PtdSer and PtdIns3P, PtdIns4P, PtdIns(3,4)P₂, or PtdIns(4,5)P₂ (500 μ M) were mixed with GST-C2 domains (C) or GST-catalytic domain (D), and binding studies were performed as described under *Materials and Methods*. Data are the mean and SE of four assays.

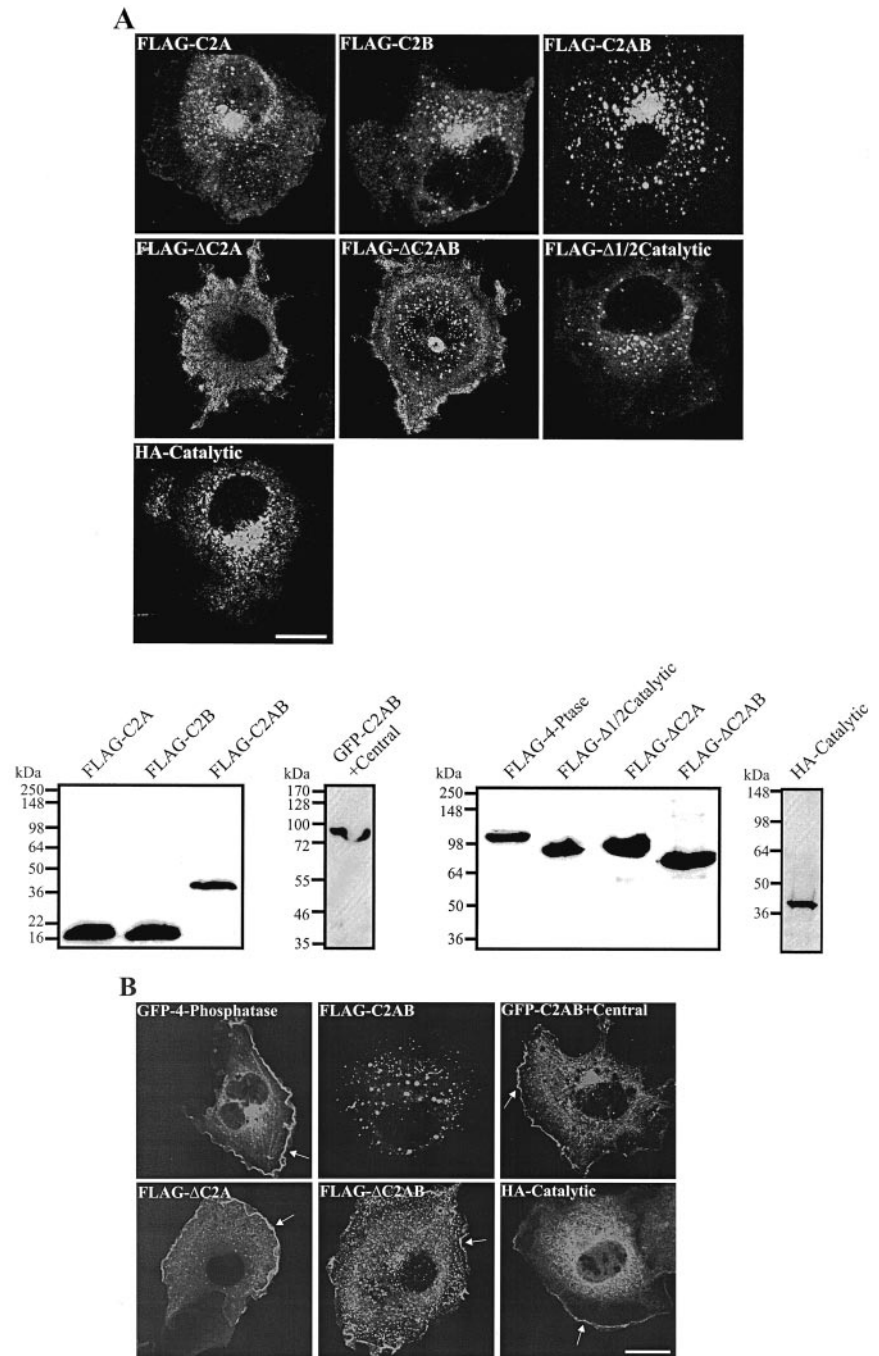


Figure 7. 4-Phosphatase endosomal targeting by the C2 domain. COS-1 cells expressing the indicated constructs were serum-starved overnight (A) and stimulated with 100 ng/ml EGF for 10 min (B), fixed, and stained with HA or FLAG antibodies, and visualized by confocal microscopy. Arrows in B indicate PM localization. Triton-soluble extracts of transfected cell lysates were immunoblotted with either FLAG, HA, or GFP antibodies (A). Molecular weight markers are shown on the left.

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)P₂ 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)P₂, 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)P₂ levels increase (Figure 8, A and B, respectively), transiently expressing the wild-type or inactive 4-phosphatase C856S were treated with 100 nM wortmannin (30 min) and counterstained with EEA-1 antibody. Serum-

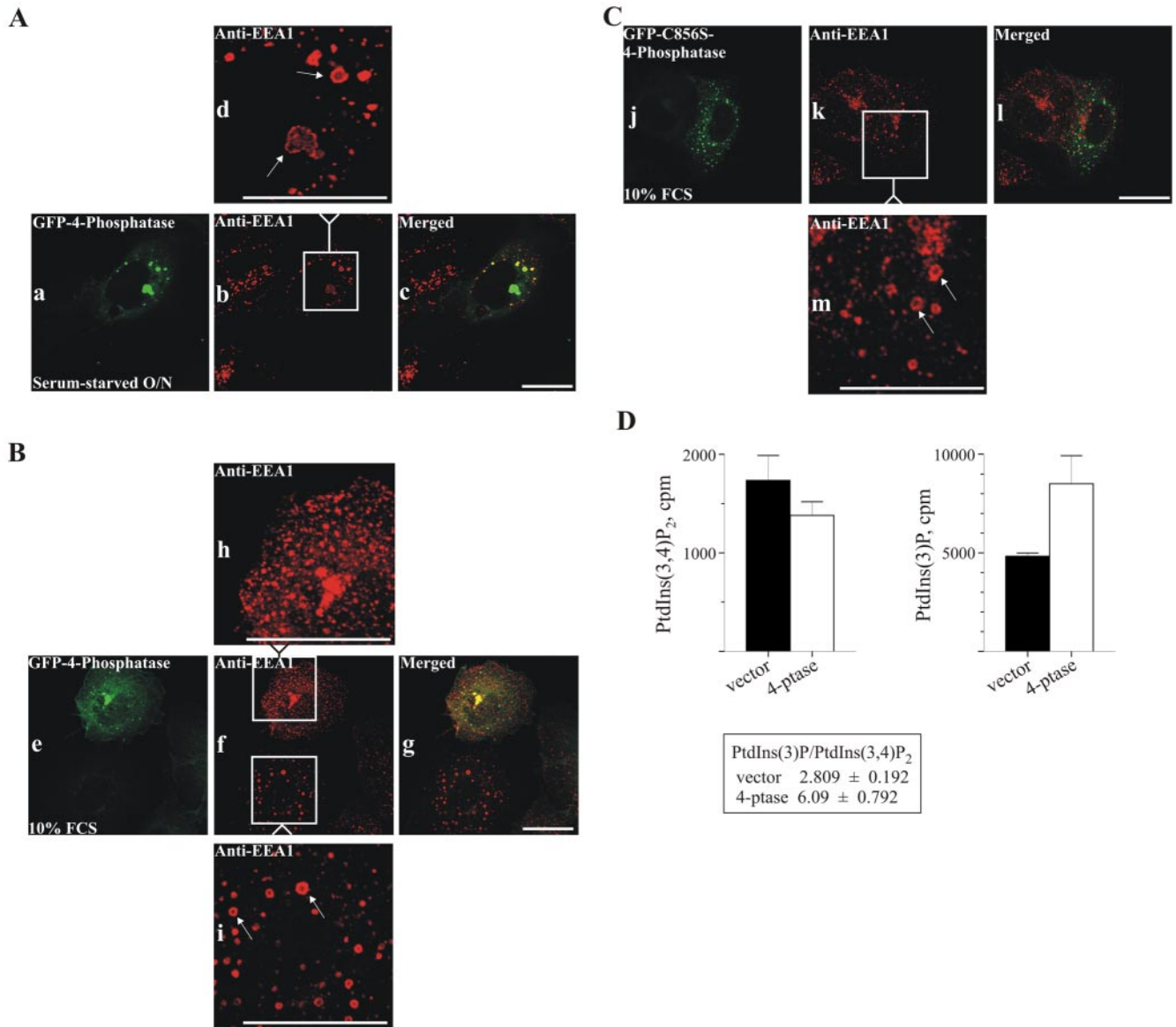


Figure 8. 4-Phosphatase rescues the wortmannin-induced dilatation of early endosomes. COS-1 cells transiently expressing GFP-wild type (A and B) or catalytically inactive (C856S) 4-phosphatase (C) were treated with 100 nM wortmannin for 30 min after serum starvation overnight (A) or growth in serum (B 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 f, lower box magnified in i; and box in k is magnified in m. Arrows indicate endosomal enlargement. Bar, 5 μ m. (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)P/PtdIns(3,4)P₂ to PtdIns-3P ratios in vector versus type I 4-phosphatase–overexpressing cells are shown in boxed area.

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). Although EEA-1 dissociates from endosomal membranes upon wortmannin treatment, we found endosomal EEA-1 staining was detected on dilated endosomes, consistent with reports that wortmannin-induced EEA-1 dissociation is incomplete in some cell lines (Chen and Wang, 2001; Tuma *et al.*, 2001b). Notably, when grown in serum, wortmannin-treated cells transiently overexpressing GFP-4-phosphatase demonstrated normal-sized EEA-1–decorated

endosomes (Figure 8B, magnified in h), whereas adjacent nontransfected cells showed endosomal dilatation. (Figure 8B, magnified in i). Significantly, in cells grown in serum, expression of the catalytically inactive 4-phosphatase C856S did not rescue the wortmannin-induced dilatation of endosomes, and EEA-1–decorated endosomes looked dilated, similar to nontransfected cells (Figure 8C, magnified in m). No colocalization of the inactive 4-phosphatase C856S with dilated endosomes was evident. Therefore, under serum-stimulated conditions resulting in increased PtdIns(3,4)P₂ synthesis, the 4-phosphatase may generate a PtdIns(3)P pool on early endosomes, via the hydrolysis of its cognate substrate PtdIns(3,4)P₂. Furthermore, given that wortmannin

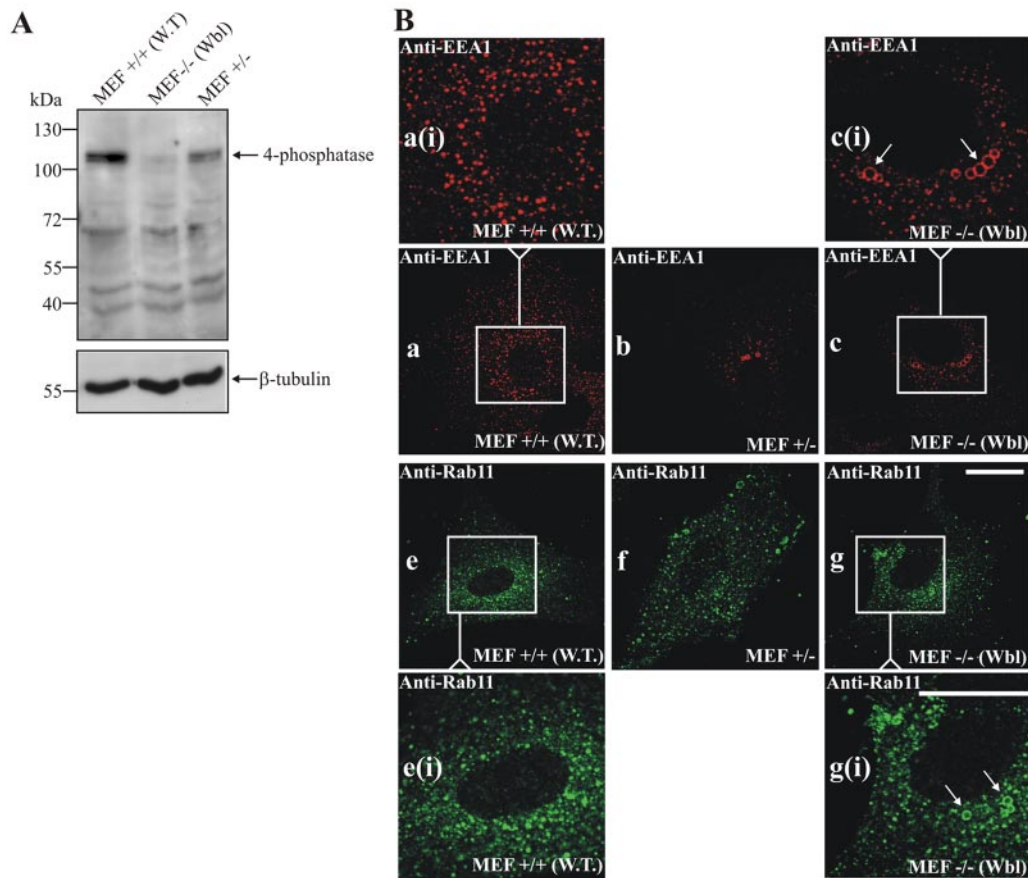


Figure 9. Type I 4-phosphatase $-/-$ MEFs show endosomal dilatation. (A) Detergent-soluble fractions of MEFs were immunoblotted using 4-phosphatase (C-terminal) anti-peptide antibodies or β -tubulin antibodies as a loading control. (B) MEFs, $+/+$ wild-type (WT), $+/-$ heterozygotes, or homozygous $-/-$ *Weeble* (*Wbl*) were stained with EEA-1 or Rab11 antibodies. The EEA-1-stained boxed area in a is shown enlarged in ai, the boxed area in c is shown enlarged in ci. Rab11-stained boxed area in e is shown enlarged in ei; boxed area in g is shown enlarged in gi. Arrows show endosomal dilatation. Bar, 5 μ m.

inhibits hVPS34-mediated synthesis of PtdIns(3)P on early endosomes, the PtdIns(3,4) P_2 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 α 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 32 P-labeled phosphoinositides were analyzed by anion-exchange high-performance liquid chromatography (HPLC). The levels of PtdIns(3,4) P_2 and PtdIns(3)P are as shown in Figure 8D. In cells stably transfected with type I 4-phosphatase, PtdIns(3,4) P_2 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) P_2 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) P_2 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 -/-* 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 -/-* 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) P_2 -specific effectors to the PM has not been re-

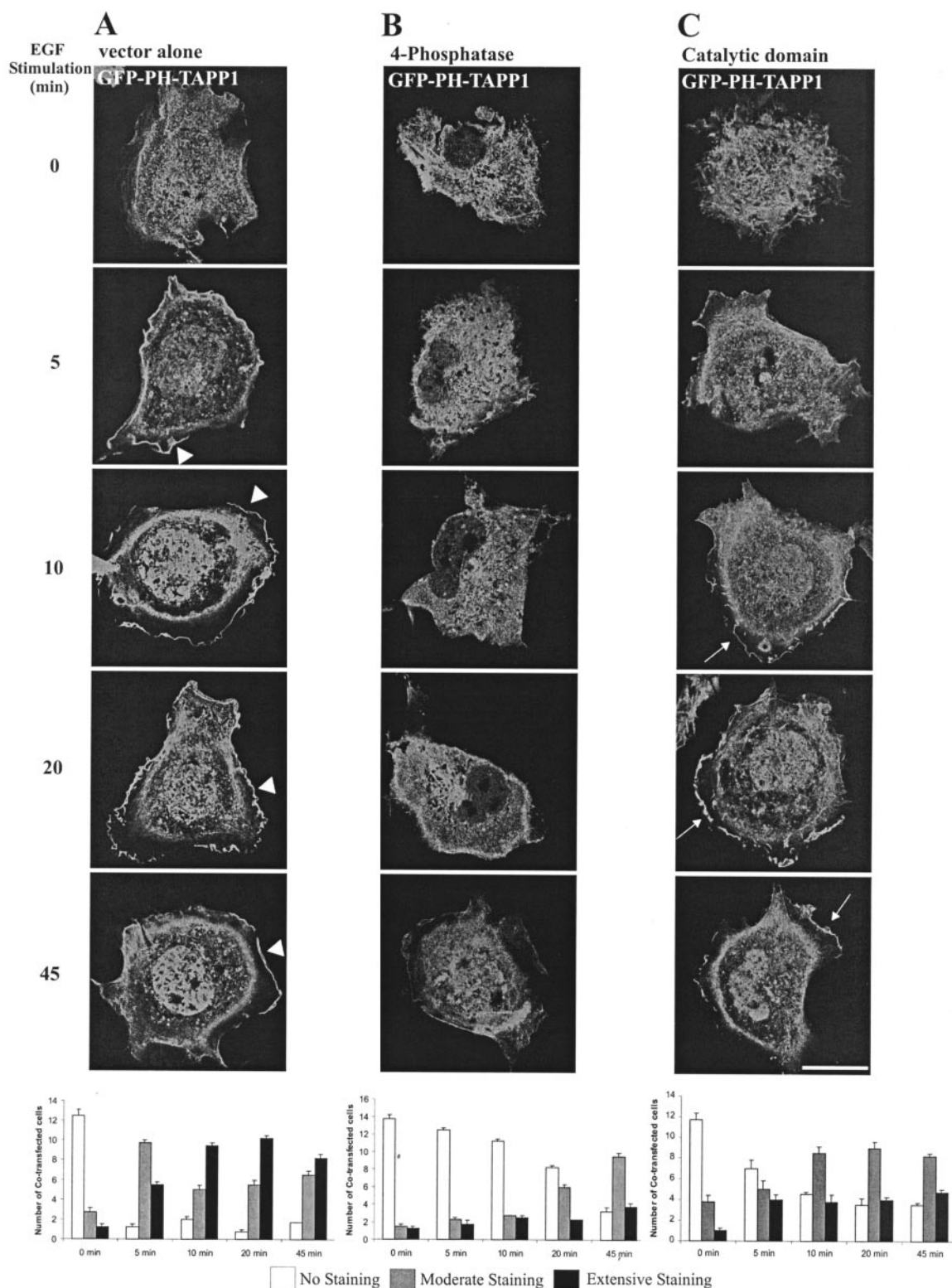


Figure 10. 4-Phosphatase regulates the PM recruitment of a PtdIns(3,4)P₂-specific effector. COS-1 cells transiently expressing GFP-TAPP1 PH domain and FLAG empty vector (A) or FLAG-4-phosphatase (B) or HA-4-phosphatase catalytic domain (aa 608–938) (C). Cells were serum-starved overnight and stimulated 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 the 4-phosphatase failed to demonstrate complete colocalization with early endosomal markers such as EEA-1 or the recycling endosomal marker Rab11, extensive colocalization with these markers was shown upon coexpression with

Rab5Q79L or Rab11Q70L. Furthermore, the lack of complete colocalization between 4-phosphatase and the endosomal marker EEA-1, is consistent with recent studies showing that PtdIns(3)P exists on endosomal membrane microdomains, associated with specific binding proteins. For example, Hrs, which binds PtdIns(3)P, does not show complete colocalization with EEA-1 (Gillooly *et al.*, 2003). Although cells overexpressing the 4-phosphatase showed some perinuclear staining, little evidence was demonstrated that this enzyme localizes to the Golgi, because the enzyme did not consistently colocalize with Golgi markers.

PtdIns(3)P promotes the association of EEA-1 with early endosomes (Stenmark *et al.*, 1996). The PI 3-kinase inhibitor wortmannin inhibits the synthesis of PtdIns(3)P, resulting in enlarged endosomes. (Simonsen *et al.*, 2001). Because wortmannin inhibits both class I and III PI 3-kinases, which synthesize PtdIns(3)P, PtdIns(3,4)P₂, PtdIns(3,4,5)P₃, and PtdIns(3,5)P₂, dissection of the trafficking pathways regulated by each specific phosphoinositide has not been directly evaluated. We have provided evidence here that 4-phosphatase hydrolysis of PtdIns(3,4)P₂ generates a functional pool of PtdIns(3)P. In agonist-stimulated cells, conditions resulting in PtdIns(3,4)P₂ synthesis, cells overexpressing the 4-phosphatase showed increased total cellular levels of PtdIns(3)P. Also, serum-stimulated but not serum-starved cells overexpressing the 4-phosphatase were resistant to wortmannin-induced endosomal dilatation. Finally, MEFs from 4-phosphatase-deficient mice exhibited endosomal enlargement, reminiscent of that seen in wortmannin-treated cells.

Recent studies using antibodies to class I and III PI 3-kinases demonstrated that the wortmannin-sensitive PI 3-kinases cannot account for all the endosomal PtdIns(3)P, suggesting that the wortmannin-insensitive class II PI 3-kinases may synthesize a PtdIns(3)P pool that maintains membrane transport (Tuma *et al.*, 2001a). The studies reported here support this hypothesis and furthermore indicate that a functional PtdIns(3)P pool can be generated via 4-phosphatase hydrolysis of PtdIns(3,4)P₂. In addition, it is an intriguing possibility that hVPS34 and the 4-phosphatase may generate distinct endosomal PtdIns(3)P pools. The functional analysis of endosomal trafficking in *Weeble* ^{-/-} MEFs may provide further evidence of the specific roles PtdIns(3,4)P₂ and the 4-phosphatase-generated PtdIns(3)P play in membrane transport and is the subject of ongoing laboratory investigation. The intracellular localization or function of the type II 4-phosphatase has not been reported, but given we have shown defects in endosomal morphology in *Weeble* MEFs, these two 4-phosphatases are likely to play nonredundant roles in distinct subcellular locations.

We have shown here that the 4-phosphatase tandem C2 domain binds PtdIns(3,4)P₂ and plays a significant role in directing 4-phosphatase to endosomes in quiescent cells. However, 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 phosphatase

tase domain and C2 domain are involved in electrostatic membrane binding (Das *et al.*, 2003). Like PTEN, we have shown that the 4-phosphatase catalytic domain and the C2 domains both facilitate membrane binding, the C2 domain being necessary and sufficient for endosomal membrane association and the catalytic domain mediating the enzyme's association with the PM.

PtdIns(3,4)P₂ functions as a signaling molecule in growth factor-stimulated pathways and also is generated independently of PtdIns(3,4,5)P₃ in response to oxidative stress (Van der Kaay *et al.*, 1999). The major metabolic pathway for PtdIns(3,4)P₂ synthesis in growth factor-stimulated cells is via 5-phosphatase hydrolysis of PtdIns(3,4,5)P₃. It is noteworthy that several PtdIns(3,4,5)P₃ 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)P₂ and also localizes to membrane ruffles, suggesting that this is the site of PtdIns(3,4)P₂ 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)P₂ signaling at the PM.

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)P₂ 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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