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Sec14p homology domains are found in a large number of proteins from plants, yeast, invertebrates, and higher eukaryotes. We report that the N-terminal Sec14p homology domain of the human protein tyrosine phosphatase PTP-MEG2 binds phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) in vitro and colocalizes with this lipid on secretory vesicle membranes in intact cells. Point mutations that prevented PtdIns(3,4,5)P_3 binding abrogated the capacity of PTP-MEG2 to induce homotypic secretory vesicle fusion in cells. Inhibition of cellular PtdIns(3,4,5)P_3 synthesis also rapidly reversed the effect of PTP-MEG2 on secretory vesicles. Finally, we show that several different phosphoinositide kinases colocalize with PTP-MEG2, thus allowing for local synthesis of PtdIns(3,4,5)P_3 in secretory vesicle membranes. We suggest that PTP-MEG2 through its Sec14p homology domain couples inositol phosphorylation to tyrosine dephosphorylation and the regulation of intracellular traffic of the secretory pathway in T cells. The Journal of Immunology, 2003, 171: 6661–6671.

Proteins destined for secretion are synthesized on membrane-bound ribosomes and are transported through the endoplasmic reticulum and Golgi to the trans-Golgi network, where they are sorted into small transport vesicles of either the constitutive or the regulated secretory pathways. Homotypic fusion of such transport vesicles results in condensing vacuoles that subsequently mature into secretory vesicles through an osmotic concentration process. Morphologically, most mature secretory vesicles have a dense core of amorphous material. In neuronal cells, they are also larger than the clear synaptic vesicles that contain small-molecule neurotransmitters.

Hemopoietic cells use the regulated secretory pathway for a variety of functions (1, 2). Platelets contain numerous vesicles with blood clotting factors, vasoactive agents, and growth factors. Mast cells are specialized to degranulate large secretory vesicles that contain histamine, vasoactive molecules, and leukotrienes, upon stimulation by Ag-IgE complexes. CTLs contain specialized granules with granzyme, perforin, and other lytic enzymes, which are released in a receptor-triggered and directional manner to kill target cells. In many cases, it appears that secretory vesicles have much in common with lysosomes and could perhaps be viewed as specialized lysosomes (2).

A hallmark of the regulated secretory pathway is its response to extracellular stimuli. In both endocrine and hemopoietic cells, the use of this pathway can be up-regulated severalfold, and in some instances, the contents of the vesicles are acutely expelled in response to receptor triggering. The main function of Th cells in the initiation of an immune response is to actively secrete a number of cytokines, which recruit and activate other cell types involved in inflammation and the ensuing innate and adaptive immune response (3). Depending on the subtype of Th cell, these polypeptides include numerous interleukins (e.g., IL-2, -4, -5, and -12), IFNs, and growth factors (e.g., for granulocytes and monocytes). Despite their importance in the immune system, secretory vesicles have been relatively little studied in lymphoid cells.

We have reported (4) that the tyrosine-specific protein phosphatase PTP-MEG2 is involved in regulation of vesicle traffic along the regulated secretory pathway in T cells and mast cells. This phosphatase contains a unique 250-aa, putative phospholipid-binding domain in its N terminus (5). This region has 28% identity with cellular retinaldehyde-binding protein and 24% identity with Sec14p, a yeast protein with phosphatidylinositol (PtdIns) transfer activity. A similar Sec14p homology domain is also found in two protein tyrosine phosphatases from Xenopus laevis, PTPX1 and PTPX10 (6), and numerous other proteins (7, 8), including many regulators of Rho, Rac, and Ras proteins (9).

We have studied the Sec14p homology domain of PTP-MEG2 and found that it binds a phospholipid with high affinity. This binding is not involved in targeting of PTP-MEG2 to the secretory vesicle compartment, but appears to be important for the function of PTP-MEG2 on these vesicles in intact cells. This represents a novel mechanism for cross talk between tyrosine phosphorylation and signaling by phosphoinositides.

Materials and Methods

Computer modeling

A three-dimensional model of aa 8–250 of PTP-MEG2 was built by comparative modeling, using the crystal structure of Sec14p (Brookhaven Protein Data Bank code 1aua) as a template. Several different alignments were

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generated using various alignment methods, including PSI-BLAST (10), FFAS (11), and SAM-99 (12). Despite the relatively low sequence identity between the Sec14p homology domain and Sec14p, the statistical significance of the alignment was high, ranging from an e value of $2 \times 10^{-4}$ for PSI-BLAST alignment to a Z-score of 65 for the FFAS alignment. These values suggested that the two proteins are certainly homologous, and that the alignments were very reliable. Each alignment was used to build a three-dimensional model using a MODELLER automated modeling program (13), and the resulting models were analyzed for quality using programs ProCheck (14), MatchMaker (Tripos, St. Louis, MO), and Profile3D (15). The resulting models had very high quality with a well-packed interior and a consistent pattern of interactions, again suggesting that the models were reliable and could be used for functional analysis of the Sec14p homology domain. The only differences between models obtained with different alignments concerned the exact length of the alignment, with the PSI-BLAST alignment resulting in a shorter model, with several residues clipped from the N and C termini of the PTP-MEG2 sequence.

**Abs and reagents**

The anti-influenza hemagglutinin (HA) tag epitope mAb 12CA5 conjugated to rhodamine isothiocyanate (TRITC) were from Roche Molecular Biochemicals (Indianapolis, IN). The 16B12 anti-HA from Berkeley Antibody (Richmond, CA) was used for immunoblotting. Anti-carboxypeptidase E was from Transduction Laboratories (Lexington, KY). Goat antirabbit IgG (F(ab)2), Alexa 488 was from Molecular Probes (Eugene, OR). Anti-GST mAb was from Santa Cruz Biotechnology (Santa Cruz, CA). All phosphoinositides and the anti-PtdIns-3,4,5-trisphosphate (PtdIns(3,4,5)P3) mAb were from Echelon (Salt Lake City, UT). Anti-PtdIns-3-kinase p85 Abs were from Upstate Biotechnology (Lake Placid, NY).

**Plasmid construction**

The cDNA for PTP-MEG2 (5) was kindly provided by P. Majerus (St. Louis, MO). It was subcloned into the pEF/HA vector (16), which adds an HA epitope to the N terminus of the cloned insert. The catalytically inactive mutant of PTP-MEG2, PTP-MEG2-C515S, the K55M, and K184M mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutations were verified by nucleotide sequencing. The nucleotides encoding the Sec14p homology domain, aa 1–261, were amplified by PCR from the PTP-MEG2 plasmid cDNA. The amplification product was subcloned into the prokaryotic expression vector pGEX-4T-1. The fusion protein was expressed in *Escherichia coli* strain DH5α and purified using glutathione-agarose as described previously (17).

**Dot blot assay for phospholipid binding (fat Westerns)**

The phospholipid binding specificity of the Sec14p homology domain of PTP-MEG2 was assayed as described (18). Nitrocellulose filters spotted with 100 pmol of phospholipids (PIP strips; Echelon) were blocked in 3% fatty acid-free BSA in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20 for 1 h and incubated with 0.1–1 µg/ml GST-fusion protein overnight at 4°C. The membrane was washed for 1 h and then incubated for 1 h with a 1/10000-diluted anti-GST mAb. The membrane was washed as before, and incubated for 1 h with 1/3000-diluted anti-mouse-HRP conjugate. Finally, the membrane was washed, and the GST fusion protein bound to the membrane by virtue of its interaction with phospholipid was detected by ECL (ECL kit; Amersham, Arlington Heights, IL).

**In vitro transcription and translation of full-length PTP-MEG2 for filter binding assay**

The cDNA for PTP-MEG2 was cloned into the pET30c vector (Novagen, Madison, WI), and the full-length S-tagged protein was generated using the TNT in vitro coupled transcription-translation system (Promega, Madison, WI). The reaction was first optimized using cold methionine, and the produced PTP-MEG2 protein was detected and quantified using an S-protein-HRP conjugate (Novagen) after SDS-PAGE and transfer of the reaction mixture to nitrocellulose filters. For the lipid overlay assays, PIP strips were blocked for 1 h as described above and then incubated for 1 h with 25 µl of the 35S-labeled in vitro translation reaction mixture diluted in 5 ml of blocking buffer. After washing extensively with blocking buffer, the strips were exposed to film.

**Cells and transient transfections**

Jurkat T leukemia cells were kept at logarithmic growth in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1-glutamine, and antibiotics. Transient transfections were conducted by electroporation as described before (19–23). Electroporation conditions typically contained 20 × 106 cells and a total of 5–20 µg of plasmid DNA, and in each transfection, the DNA amount was kept constant by the addition of empty vector. Cells were used for experiments 48 h after transfection.

**Immunoblot, immunoprecipitation, cell fractionation, and PtdIns-3-kinase assays**

Immunoprecipitation was performed as before (19–23). All immunoblotts were developed by the ECL technique (ECL kit; Amersham) according to the manufacturer’s instructions. Cell fractionation was done as before (24), and PtdIns-3-kinase assays were done as before (16, 21).

**Confocal microscopy**

Double immunofluorescence staining was done as before (4, 24). Briefly, cells were washed in PBS and fixed in freshly made 3.7% formaldehyde. Fixed cells were permeabilized with 0.1% saponin in PBS, then blocked in 2.5% normal goat serum in 0.1% saponin in PBS for 30 min at room temperature, and then incubated with primary and secondary Ab diluted in the same buffer for 1 h each at room temperature. After three washes with PBS, the cells were mounted onto glass slides and viewed under a confocal laser scanning microscope (MRC-1024; Bio-Rad, Hercules, CA). A differential interference contrast image was also taken of most cells.

**Results**

**Modeling of the Sec14p homology domain of PTP-MEG2**

As a rational approach to elucidating the function of the N-terminal Sec14p homology domain of PTP-MEG2, we built a three-dimensional model of aa 8–250 of this protein (see Materials and Methods). A comparison of this model with the crystal structure of Sec14p (25) shows that the two display a number of interesting differences despite being quite similar overall (Fig. 1, A–D). Notably, the phospholipid-binding pocket of Sec14p is well conserved in PTP-MEG2, but is shorter and has a cluster of basic amino acid residues in one end (Fig. 1E). This cluster contains Arg and Lys residues from the conserved Ala-Ara-Lys-Phe-Asp motif (aa 53–57 in PTP-MEG2), as well as Lys184, Lys209, Arg211, and Arg217, which are found in PTP-MEG2, but not in Sec14p (Fig. 1F). This model suggested that the PTP-MEG2 Sec14p homology domain may bind phospholipids that have a more acidic head group than the unphosphorylated inositol (Ins) of PtdIns bound by Sec14p.

The Sec14p homology domain of PTP-MEG2 binds PtdIns(3,4,5)P3.

To directly test the computer model prediction, we generated a GST-fusion protein containing the Sec14p homology domain of PTP-MEG2 and measured its capacity to bind phospholipids using a filter binding assay (18), in which the phospholipids are immobilized on nitrocellulose, while the protein is in solution and is subsequently detected by anti-GST or anti-PTP-MEG2 Abs. In this assay, 3–10 nM Sec14p homology domain of PTP-MEG2 bound best to PtdIns(3,4,5)P3 and, at a weaker, to PtdIns(3,5)P2 (Fig. 2). At 10 and 30 nM, some binding was also seen to PtdIns(3,4)P2, PtdIns(4,5)P2, PtdIns(4)P, and PtdIns(5)P. Other phospholipids did not bind at all to the GST-MEG2 protein. Control GST (Fig. 2F) or GST-hemopoietic protein tyrosine phosphatase (Fig. 4F) did not bind any phospholipids.

To verify that full-length PTP-MEG2 also binds the same phospholipids as the GST-Sec14p homology domain, we generated the 68-kDa PTP-MEG2 by in vitro transcription and translation of a bacterial expression plasmid encoding an N-terminally S-tagged PTP-MEG2 in the presence of [35S]methionine. The phospholipid filters were then incubated with the translation products, washed extensively, and exposed to film. In these experiments, full-length PTP-MEG2 bound the same phospholipids as the isolated Sec14p homology domain, namely PtdIns(3,4,5)P3 and PtdIns(3,5)P2 and less to other phosphoinositides (Fig. 3A). A control transcription/translation reaction without the DNA, but with [35S]methionine...
FIGURE 1. Computer model of the Sec14p homology domain of PTP-MEG2. A–D, Comparison of the crystal structure of Sec14p with the computer model of the Sec14p homology domain of PTP-MEG2, shown as a ribbon graphs (A and B) and as GRASP representation (C and D) with the surface topology of acidic (red) and basic (blue) charges. The two octylglucoside molecules found in the crystal of Sec14p are included as space-fill models. E, A close-up view of the putative binding pocket for an acidic head group of a ligand phospholipid in the Sec14p homology domain of PTP-MEG2. F, Amino acid sequence alignment of the Sec14p homology domain of PTP-MEG2 and Sec14p from *Candida albicans* (albi) and *Saccharomyces cerevisiae* (cerev). The basic residues pointed out in E are highlighted in pale blue.
and all other reagents, did not show any binding of phospholipids (Fig. 3B). The presence of full-length S-tagged PTP-MEG2 was also confirmed by anti-S immunoblotting (Fig. 3C).

Because yeast Sec14p binds more than just the polar head group of PtdIns (25, 26), we decided to test how well the polar head group of PtdIns(3,4,5)P₃, Ins(1,3,4,5)P₄, was able to compete with PtdIns(3,4,5)P₃ for binding to the Sec14p homology domain of PTP-MEG2. Although addition of Ins(1,3,4,5)P₄ to the binding assay had a dose-dependent inhibitory effect, this effect was relatively minor even at 1/₉₂₆ Ins(1,3,4,5)P₄ (Fig. 4). These data suggest (but do not prove) that PTP-MEG2 requires more than just the polar head group of PtdIns(3,4,5)P₃ for high-affinity binding.

Colocalization of PTP-MEG2 with PtdIns(3,4,5)P₃ in intact cells

PTP-MEG2 is normally found on the membrane of secretory vesicles in mast and T cells (4). Expression of catalytically active PTP-MEG2 results in a homotypic fusion of these normally 300-nm vesicles into giant vesicles with diameters of 1–2 μm. Catalytically inactive PTP-MEG2-C515S does not cause vesicle fusion (4). When cells expressing PTP-MEG2 were stained with a mAb specific for PtdIns(3,4,5)P₃, the resulting fluorescence was found to colocalize with PTP-MEG2 on the enlarged vesicles (Fig. 5, B and C). A weak plasma membrane staining was also seen in both untransfected and transfected Jurkat cells (Fig. 5A). As a control, we treated the cells with wortmannin, a fungal inhibitor of PtdIns 3-kinases, and as expected, PtdIns(3,4,5)P₃ was not detected in these cells (Fig. 5, B–F) (see below for effects of wortmannin in PTP-MEG2).

To verify these results with another technique, we used a fusion protein between green fluorescent protein (GFP) and the Btk kinase pleckstrin homology (PH) domain, which is highly specific for PtdIns(3,4,5)P₃ (27). In control Jurkat cells expressing the


FIGURE 4. Polar head group competition assay. A–D, Filter binding assays for phospholipid selection using 5 nM GST-Sec14p homology domain of PTP-MEG2 and the indicated concentrations of the GST-Sec14p homology domain of PTP-MEG2. E, Graph showing the densitometric quantitation of PtdIns(3,4,5)P₃ binding in A–D. F, A filter incubated with control GST. All filters were exposed to film for the exact same time.
FIGURE 5. Colocalization of PTP-MEG2 and PtdIns(3,4,5)P$_3$ in fused secretory vesicles in intact cells. A–F, Confocal microscopy of Jurkat cells double stained for PtdIns(3,4,5)P$_3$ with the specific mAb plus FITC-anti-mouse Ig (green), and then for PTP-MEG2 with the TRITC-conjugated anti-HA mAb (red). The cells were transfected with empty vector (A) or wild-type PTP-MEG2 (B–F). The cells in D–F were treated with 300 nM wortmannin for 30 min. Note that no PtdIns(3,4,5)P$_3$ can be seen in these cells. Differential interference contrast images of the same cells are shown in the right panels. G–N, Confocal microscopy of Jurkat cells transfected with the indicated GFP constructs plus empty vector (G–J) or PTP-MEG2 (K–N) and stained for PTP-MEG2 with the TRITC-conjugated anti-HA mAb (red). The cells in I were treated with 300 nM wortmannin for 30 min. Note that GFP-Btk-PH colocalizes with PTP-MEG2 in K and L, whereas there is no colocalization of GFP or GFP-EEA1–(FYVE)$_2$ with PTP-MEG2. O, Confocal microscopy of untransfected Jurkat cells stained for endogenous PTP-MEG2 (green).
GFP-Btk-PH construct, fluorescence was diffusely cytoplasmic with a clear enrichment under the plasma membrane (Fig. 5, G and H), which disappeared upon treatment of the cells with wortmannin (I). GFP alone was also diffusely distributed (J). In cells co-expressing PTP-MEG2, the GFP-Btk-PH protein accumulated in an intracellular vesicle structure that also stained for PTP-MEG2 (Fig. 5, K and L). In contrast, another fusion protein, consisting of GFP plus two tandem FYVE domains (from early endosomal Agl (EEA1); GFP-EEA1-(FYVE)2), which is specific for PtdIns(3)P (28–30), did not colocalize with PTP-MEG2 (Fig. 5M), as was the case also for GFP alone (N). Taken together, these experiments show that PTP-MEG2 colocalizes with PtdIns(3,4,5)P3 in intact cells.

**Binding of **PtdIns(3,4,5)P3** is required for PTP-MEG2 function**

To further test whether binding of PtdIns(3,4,5)P3 to the Sec14p homology domain of PTP-MEG2 is important for the function of PTP-MEG2, we created three mutants with reduced ability to bind this phospholipid. Aided by the computer model prediction that a number of basic amino acid residues in the Sec14p homology domain may be involved in binding to the acidic head group of PtdIns(3,4,5)P3 (Fig. 1), we chose two residues, Lys55 and Lys184, and mutated them individually or in combination to methionine residues. As assessed by dot blots (Fig. 6), the K55M- and K184M-mutated Sec14p homology domains both had a reduced ability to bind PtdIns(3,4,5)P3, whereas the double mutant did not bind at all. When expressed in cells, PTP-MEG2-K55M still caused fusion of secretory vesicles, although they tended to remain smaller, more numerous, and surrounded by small vesicles (Fig. 6, E–G), PTP-MEG2-K184M also caused incomplete fusion with numerous small vesicles (Fig. 6, I–K), whereas the double mutant, PTP-MEG2-K55M/K184M, was unable to cause any fusion (M–P). Instead, the fluorescence was mostly seen as small granules in the region of the Golgi apparatus and trans-Golgi network. Because this pattern is also seen with catalytically inactive PTP-MEG2-C515S (4), these results imply that the Sec14p homology domain of PTP-MEG2 needs to bind PtdIns(3,4,5)P3 for the enzyme to cause secretory vesicle fusion in intact cells.

**PTP-MEG2 mutants do not colocalize with PtdIns(3,4,5)P3**

To further verify that the K55M, K184M, and K55M/K184M mutants do not bind PtdIns(3,4,5)P3 in the intact cells, we used the GFP fusion proteins and costained the cells for PTP-MEG2. These experiments showed that only PTP-MEG2 with an intact Sec14p homology domain colocalized with the GFP-Btk-PH domain on the enlarged vesicles (Fig. 7A). In contrast, the three mutants did not colocalize with either GFP-Btk-PH or GFP-EEA1-(FYVE)2 (Fig. 7, B–H). Control blots showed that the mutant PTP-MEG2

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**FIGURE 6.** Sec14p homology domain mutants of PTP-MEG2 with reduced ligand binding. **A, D, H, and L,** Filter binding assays for phospholipid selection using 5 nM GST-Sec14p homology domain of PTP-MEG2 (A), or its K55M (D), K184M (H), or double K55M/K184M (L) point mutants. **B and C,** Confocal microscopy of cells transfected with PTP-MEG2 and stained for PTP-MEG2 with the anti-HA mAb. E–G, Confocal microscopy of cells transfected with PTP-MEG2-K55M and stained with the anti-HA mAb. I–K, Confocal microscopy of cells transfected with PTP-MEG2-K184M and stained with the anti-HA mAb. M–P, Confocal microscopy of cells transfected with PTP-MEG2-K55M/K184M and stained with the anti-HA mAb. The shown cells are representative of the majority of stained cells.
proteins were expressed at levels similar to intact PTP-MEG2 (Fig. 7I), indicating that they were stable. Control blots also showed that the two GFP fusion proteins and GFP alone were expressed as the expected-size proteins (Fig. 7J). A close-up of H shows that the location of PTP-MEG2-K55M/K184M remains granular and vesicular, although the size of these vesicles are much smaller.

**Inhibition of PtdIns(3,4,5)P₃ synthesis blocks PTP-MEG2 function**

The source of PtdIns(3,4,5)P₃ in the secretory vesicle compartment is not clear. In addition to the classical route of synthesis by phosphorylation of PtdIns(4,5)P₂ by type I PtdIns 3-kinases (31), there is a Golgi- and post-Golgi-localized synthetic (secretory/endo-cytic) pathway that is initiated by type III PtdIns 3-kinase (31, 32), which produces PtdIns(3)P. This lipid acts as a substrate for the D5-specific kinase PIKfyve (33, 34), which phosphorylates PtdIns(3)P to PtdIns(3,5)P₂. Both type III PtdIns 3-kinase and PIKfyve, as well as their products, are known to play regulatory roles in the secretory pathway in yeast (35). Thus, we favor this synthetic pathway over the classical route as the source of PtdIns(3,4,5)P₃ in the secretory vesicles. In support of this possibility, the Golgi apparatus and other intracellular membranes also contain D4-specific kinases (31), which could phosphorylate PtdIns(3,5)P₂ to PtdIns(3,4,5)P₃. However, it is interesting to note that PTP-MEG2 also bound PtdIns(3,5)P₂.

Regardless of the route by which PtdIns(3,4,5)P₃ is produced for PTP-MEG2 regulation, we reasoned that inhibition of D3-specific (and some D4-specific) kinases by treatment of cells with wortmannin should prevent its synthesis in the PTP-MEG2-containing vesicles. Indeed, as already shown in Fig. 5, wortmannin caused PtdIns(3,4,5)P₃ to disappear from the cells as assessed with the specific mAb (D–F) or the GFP-Btk-PH construct (I). Furthermore, when PTP-MEG2-containing cells were treated with 300 nM wortmannin (sufficient to also inhibit type III kinases (31)), the fused secretory vesicle compartment began to fragment and shrink in a time-dependent manner (Fig. 8, A–K). Within 15–30 min after addition of wortmannin, only a multitude of small PTP-MEG2-containing vesicles were seen (Fig. 8, E–H). A quantitation of vesicles >1 μm in diameter (Fig. 8K) also demonstrates the fast kinetics of the response. A lower dose of wortmannin (100 nM) was partly effective, whereas 50 nM had a minimal effect (not shown). Similarly, another inhibitor of PtdIns 3-kinases,
LY294002, also caused vesicle shrinking and fission at 50 μM, but was only partially effective at 25 μM (Fig. 8, L–N). These results support the notion that PtdIns(3,4,5)P₃ synthesis is required for the function of PTP-MEG2 in intact cells. The relatively high doses of inhibitors required for the effect may indicate that type III PtdIns 3-kinases, rather than type I, are involved. However, this issue remains to be addressed.

To distinguish between a real change in the morphology of the secretory vesicle compartment and a possible dissociation of PTP-MEG2 from the vesicle membrane, we also costained the cells for the secretory vesicle marker carboxypeptidase E, which recapitulated the result seen with PTP-MEG2 staining (Fig. 8, A–J). We also performed a subcellular fractionation experiment (Fig. 9), which showed that >90% of PTP-MEG2 was sedimented by ultracentrifugation at 100,000 × g of a postnuclear supernatant of sonicated cells, and the remaining fraction was pelleted at 300,000 × g. Following treatment of the cells with wortmannin, there was a small increase in the latter fraction, but no PTP-MEG2 appeared in the supernatant (Fig. 9, lane 3). Thus, PTP-MEG2 is membrane bound and remains so upon treatment of the cells with wortmannin. This notion is also supported by the close-up shown in Fig. 7K.

Intracellular location of D3-, D4-, and D5-specific phosphoinositide kinases in T cells

To further support the notion that PtdIns(3,4,5)P₃ is present on secretory vesicle membranes, where it can regulate PTP-MEG2, we stained control cells or PTP-MEG2-expressing cells with Abs against type I PtdIns 3-kinase p85 (Fig. 10, A–D), the human (h) type III PtdIns 3-kinase hVPS34 (E–H), PI4K (I and J), or the 68-kDa PIP5Kα (K–N). These experiments showed that all four 3-kinases, rather than type I, are involved. However, this issue remains to be addressed.

FIGURE 8. Reversal of PTP-MEG2-induced secretory vesicle fusion by PtdIns 3-kinase inhibitors. A–J. Time course of fragmentation of the fused secretory vesicles after addition of wortmannin. The cells were stained with anti-carboxypeptidase E mAb plus FITC-anti-mouse Ig (green) and then for PTP-MEG2 with the TRITC-conjugated anti-HA mAb (red). The shown cells were untreated (A and B), or incubated at 37°C for 5 min (C and D), 15 min (E and F), 30 min (G and H), or 60 min (I and J) with 300 nM wortmannin. The shown cells are representative of the majority of stained cells. K. Histogram showing the response as the fraction of cells with vesicles >1 μm in diameter. L–N. Confocal microscopy of cells transfected with PTP-MEG2 and treated with medium alone (L), or 25 μM (M) or 50 μM (N) LY294002 for 30 min.

FIGURE 9. Subcellular fractionation of cells expressing PTP-MEG2 or PTP-MEG2 mutants. Immunoblot with anti-HA mAbs of material pelleted by ultracentrifugation at 100,000 × g (top panel), 300,000 × g (middle panel), or remaining in the supernatant after the last centrifugation (bottom panel). The cells were transfected with the indicated construct, and the cells in lane 3 were treated with 300 nM wortmannin for 15 min before lysis.
kinases are well expressed in Jurkat T cells and reside in the cytosol and on many intracellular membranes. Expression of PTP-MEG2 had little effect on the localization of the two D3-specific kinases, but it was clear that some hVPS34 was enriched at the PTP-MEG2-containing vesicle membrane. However, there was also some PtdIns 3-kinase p85 in the vicinity (although it was not enriched), making it impossible to exclude type I PtdIns 3-kinase as a source of the D3 phosphorylation of secretory vesicle PtdIns(3,4,5)P3. Interestingly, both PI4Kβ and PIP5Kα were strongly enriched in the PTP-MEG2-containing vesicle membrane (Fig. 10, J, M, and N). Unfortunately, Abs against PIKfyve are not available. Taken together, these results indicate that the secretory vesicle membrane where PTP-MEG2 resides also contains phosphoinositide kinases that together can synthesize the PtdIns(3,4,5)P3 that we detect in this location and that can bind and regulate PTP-MEG2. However, the data do not allow us to determine which synthetic pathway is used for PtdIns(3,4,5)P3 production, and it may well be that both the classical and secretory/endocytic routes can be used in parallel or at different times.

FIGURE 10. Subcellular localization of inositide kinases in Jurkat T cells. A–D, Confocal microscopy of Jurkat cells transfected with empty vector (A and B) or PTP-MEG2 (C and D) double stained for type I PtdIns 3-kinase p85 plus goat anti-rabbit-Alexa 488 (green) and PTP-MEG2 with the TRITC-conjugated anti-HA mAb (red). E–H, Similar staining with an anti-type III PtdIns 3-kinase hVPS34 mAb plus anti-mouse-Alexa 488 (green) and PTP-MEG2 with the TRITC-conjugated anti-HA mAb (red). I and J, Similar staining for PI4Kβ with a polyclonal Ab plus goat anti-rabbit-Alexa 488 (green) and PTP-MEG2 with the TRITC-conjugated anti-HA mAb (red).
Discussion

The high-affinity binding of PtdIns(3,4,5)P$_3$ to the Sec14p homology domain of PTP-MEG2 represents a novel finding that introduces a new domain into the growing list of phosphoinositide-binding protein modules. Phosphoinositides are important second messengers in transmembrane signal transduction and intracellular vesicle traffic (36–38). Successful phosphorylation of the D3, D4, and D5 positions of the inositol head group by specific kinases (31) results in up to seven different phosphoinositides, many of which function as docking molecules for protein modules that have evolved to specifically interact with them (37). Such protein modules include the PH domains (27, 39), FYVE domains (28–30, 35), and C2 domains (40), and are found in protein and lipid kinases, phospholipases, adapter molecules, regulators of small GTP-binging proteins, cytoskeletal proteins, and components of endocytic pathways (28, 35, 37, 40, 41).

The yeast Sec14p protein binds PtdIns and phosphatidylcholine and functions to transport these lipids between cellular membranes. Phospholipid binding occurs through a deep elongated pocket with a lid-like structure that closes over the bound phospholipid (25), allowing the protein to extract the lipid from a bilayer and transport it to another location. In higher eukaryotes, this transport function has been taken over by a distinct class of PtdIns transport proteins, which play important roles in phosphoinositide-mediated signaling (26). Instead, Sec14p-like proteins have been retained in a variety of other functions.

Compared with Sec14p, the domain in PTP-MEG2 contains several basic residues in the putative phospholipid head-binding groove (Fig. 1E). Our point mutants suggest that these residues are important for phospholipid selection (Fig. 6). This region is highly variable among Sec14p homology domains both in topology and charge, some having a mix of basic and acidic or predominantly acidic residues, suggesting that the Sec14p homology domain is quite versatile and can evolve to bind different lipids that have complementary charges and shapes. However, charged residues may not alone account for selectivity, and a crystal structure of the Sec14p homology domain of PTP-MEG2 with bound phospholipid will probably be required for a complete understanding of the structural basis for ligand selection.

At present, it remains unclear whether the Sec14p homology domain of PTP-MEG2 binds only the head group of PtdIns(3,4,5)P$_3$, as do most other protein modules that bind phospholipids (e.g., FYVE, PH, and C2 domains), or the entire phospholipid, as yeast Sec14p does (25). The high doses of soluble Ins(1,3,4,5)P$_4$ required for competition with PtdIns(3,4,5)P$_3$ binding may argue for binding of more than just the polar head group, but this does not necessarily mean that the fatty acid moieties are involved. However, unlike Sec14p, which transports PtdIns between cellular membranes (26), the Sec14p homology domain of PTP-MEG2 seems to remain membrane bound. Thus, we have no reason to suspect that PTP-MEG2 is involved in phospholipid transport.

Our findings indicate that the high-affinity binding of PtdIns(3,4,5)P$_3$ to the Sec14p homology domain of PTP-MEG2 is important for the activity and function of PTP-MEG2 in intact cells. In the absence of PtdIns(3,4,5)P$_3$, wild-type PTP-MEG2 behaved like the catalytically inactive C515S mutant, as did the Sec14p homology domain mutant K55M/K184M. However, all these proteins were still associated with the particulate fraction and appeared to localize to the same vesicle compartment, although its morphology was changed. Thus, the Sec14p homology domain does not mediate targeting of PTP-MEG2 to the phospholipid bilayer. Rather, there must be another targeting motif in PTP-MEG2 that directs the protein to the enclosing membrane of secretory vesicles. This targeting motif appears to be present within aa 11–261, because a protein with this sequence still is in the particulate fraction (not shown). We suggest that PTP-MEG2 is a permanent resident of the secretory vesicle membrane and there acts as a sensor for PtdIns(3,4,5)P$_3$ (and perhaps also PtdIns(3,5)P$_2$). When the lipid is present, PTP-MEG2 dephosphorylates one or several substrates involved in secretory vesicle fusion and/or homeostasis. This model is supported by the recent finding that PTP-MEG2 lacking its Sec14p homology domain has a considerably higher activity than the full-length enzyme (42). Thus, the Sec14p homology domain appears to be involved in intramolecular regulation of the catalytic activity of PTP-MEG2.

A curious finding in our studies is that intracellular PtdIns(3,4,5)P$_3$ clearly colocalized only with wild-type PTP-MEG2, but not detectably with either PTP-MEG2-C515S (which still binds the lipid) or to PTP-MEG2-K55M/K184M (Fig. 7). Although the differences could be due to the detection limit of the GFP-Bik-PH related to the absence of fusion into a more concentrated compartment, it may also be that active PTP-MEG2 has an effect on the amount of PtdIns(3,4,5)P$_3$ in the secretory vesicle membrane. Such an effect could be due to stimulated PtdIns(3,4,5)P$_3$ synthesis, an inhibitory effect on the enzyme(s) that dephosphorylate PtdIns(3,4,5)P$_3$, sequestration of the inositol head group from such phosphatases, or a combination of these. Resolving these issues will require identification of the phosphoinositide kinases and phosphatases responsible for the synthesis of PtdIns(3,4,5)P$_3$ in the post-Golgi vesicles of the secretory pathway.

A recent study (43) disagrees with our findings and instead reports that the Sec14p homology domain of PTP-MEG2 binds phosphatidylserine (PtdSer). We do not understand the basis for this discrepancy, but we note that binding of phosphoinositides has been reported very recently by two other laboratories (44, 45). It may also be significant that the Sec14p domain from PTPX1, the Xenopus laevis ortholog of PTP-MEG2, in our hands bound PtdSer and PtdIns, but not phosphoinositides, in experiments conducted in parallel with those shown in Figs. 2 and 3. In contrast, PTP-MEG2 never bound any PtdSer. Although we cannot categorically exclude the possibility that PTP-MEG2 is more versatile or has a modifiable specificity, the findings we report in this study are not compatible with binding of PtdSer.

The Sec14p homology domain is apparently an ancient protein module that has evolved to fulfill numerous physiological functions in signal transduction and intracellular transport in plants, yeast, invertebrates, and vertebrates (9). A comprehensive (46) database search (A. Godzik and T. Mustelin, unpublished data) revealed many more Sec14p homology domains from several organisms, including 19 from Caenorhabditis elegans and 23 from Arabidopsis thaliana, a polyphosphoinositol transfer protein (Ssh1p) from a plant (Glycine max), ~100 protein fragments in the Drosophila melanogaster genomic database, numerous proteins from different strains of yeast, and over 20 different mammalian proteins. Like FYVE, PH, and C2 domains, Sec14p homology domains are often found in proteins involved in signal transduction, intracellular traffic, and the regulation of small GTP-binding proteins of the Ras superfamily. The presence of an N-terminal Sec14p homology domain in several GTP/GDP exchange factors for Ras family proteins is particularly intriguing. The conversion of the GTP/GDP exchange factor Db1 into a transforming oncogene occurred by loss of an N-terminal fragment that coincides with its Sec14p homology domain (47). It will be interesting to see what phospholipid or phospholipid-like molecules these proteins bind.

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References


