Detection of novel intracellular agonist responsive pools of phosphatidylinositol 3,4-bisphosphate using the TAPP1 pleckstrin homology domain in immunoelectron microscopy

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PtdIns(3,4) P_2 , a breakdown product of the lipid second messenger PtdIns(3,4,5) P_3 , is a key signalling molecule in pathways controlling various cellular events. Cellular levels of PtdIns(3,4) P_2 are elevated upon agonist stimulation, mediating downstream signalling pathways by recruiting proteins containing specialized lipid-binding modules, such as the pleckstrin homology (PH) domain. A recently identified protein, TAPP1 (tandem-PH-domain-containing protein 1), has been shown to interact *in vitro* with high affinity and specificity with PtdIns(3,4) P_2 through its C-terminal PH domain. In the present study, we have utilized this PH domain tagged with glutathione S-transferase (GST–TAPP1-PH) as a probe in an on-section immunoelectron microscopy labelling procedure, mapping the subcellular distribution of PtdIns(3,4) P_2 . As expected, we found accumulation of

PtdIns(3,4) P_2 at the plasma membrane in response to the agonists platelet-derived growth factor and hydrogen peroxide. Importantly, however, we also found agonist stimulated PtdIns(3,4) P_2 labelling of intracellular organelles, including the endoplasmic reticulum and multivesicular endosomes. Expression of the 3-phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) in PTEN-null U87MG cells revealed differential sensitivity of these lipid pools to the enzyme. These data suggest a role for PtdIns(3,4) P_2 in endomembrane function.

Key words: electron microscopy, immunogold, localization, phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4) P_2], phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5) P_3], phosphoinositide, stereology.

INTRODUCTION

Receptor stimulation with growth factors and hormones activates the phosphoinositide 3-kinase (PI3-kinase) family of lipid kinases to produce lipid second messengers. These trigger downstream signalling pathways for the regulation of numerous cellular processes, such as growth and survival, by recruiting proteins capable of binding tightly and specifically to these lipids [1]. Type I PI3-kinases generate PtdIns(3,4,5) P_3 by phosphorylation of PtdIns(4,5) P_2 at the D3 position of the inositol ring. PtdIns(3,4) P_2 is mainly produced by dephosphorylation of PtdIns(3,4,5) P_3 by the action of 5-phosphatases, such as the Src homology 2 (SH2)-containing inositol phosphatases, SHIP1 and SHIP2. Since the majority of PtdIns(3,4) P_2 is generated via the metabolism of PtdIns(3,4,5) P_3 , PtdIns(3,4) P_2 is also a minor phosphoinositide [typically 500-fold less abundant than PtdIns(4,5) P_2] [2]. Alternative pathways for PtdIns(3,4) P_2 synthesis that are independent of type I PI3-kinase have also been described. These involve the actions of type II PI3-kinases [3] and 4-phosphorylation of PtdIns3P in response to integrin receptor cross-linking in platelets [4].

At the plasma membrane, PtdIns(3,4) P_2 is now emerging as an important player in signalling cascades via its interactions with lipid-binding domains. PKB (protein kinase B)/Akt, a key mediator of insulin signalling, possesses a pleckstrin homology (PH) domain, binding both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 .

Studies on bone-marrow-derived mast cells from SHIP-knockout mice indicate that both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 are needed for PKB function [5]. Given that the levels of PtdIns(3,4) P_2 reach a peak long after the initial burst of PtdIns(3,4,5) P_3 has subsided [6], this pattern of binding could extend the time for which PKB can function. Another protein binding both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 is DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositides) [7]. Upon PDGF (platelet-derived growth factor) stimulation, DAPP1 translocates to the plasma membrane where tyrosine phosphorylation by Src family kinases occurs. Interestingly, after translocation, DAPP1 is internalized into the endosomal system, although the cause and function of this trafficking event is unclear [8]. A third protein, TAPP1 (tandem-PH-repeat-containing protein 1), has a PH domain with narrow specificity for PtdIns(3,4) P_2 [and not PtdIns(3,4,5) P_3] [9,10]. TAPP1 protein and TAPP1-PH domain translocate from cytosol to the plasma membrane in PDGF- or H₂O₂-stimulated Swiss 3T3 cells [11].

Phosphoinositides are of growing importance for functions of endomembranes. PtdIns4P, PtdIns(4,5) P_2 and PtdIns3P have all been implicated in the regulation of membrane traffic and/or the structural integrity of the Golgi and/or endocytic pathway [12–15]. However, at present, there are very few data on intracellular pools of PtdIns(3,4) P_2 . This lipid has been implicated in phagosome targeting of p47^{phox} [16], a key cytosolic subunit in the activation of NADPH oxidase. This enzyme catalyses the

Abbreviations used: COPII, coat protein complex II; DAPP1, dual adaptor for phosphotyrosine and 3-phosphoinositides; DMEM, Dulbecco's modified Eagle's medium; EM, electron microscopy; ER, endoplasmic reticulum; FBS, foetal bovine serum; FSG, cold-water-fish-skin gelatin; GFP, green fluorescent protein; GST, glutathione S-transferase; IGF, insulin-like growth factor; MVB, multivesicular endosome; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SHIP, Src homology 2-containing inositol phosphatase; TAPP1, tandem-PH-domain-containing protein 1; TTBS, Tris/Tween 20-buffered saline; YFP, yellow fluorescent protein.

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transfer of electrons from NADPH to oxygen to form superoxide, a precursor of microbicidal oxidants [17], and recent evidence implicates PtdIns(3,4) P_2 in specific roles in NADPH oxidase assembly *in vitro* [18]. There is also a single report of localization of PtdIns(3,4) P_2 to nuclear envelope membranes [19], an observation consistent with studies localizing appropriate lipid kinases and phosphatases on internal cellular membranes. These include PI3-kinases in endosomes and Golgi [20–22], and PI4-kinases in Golgi, endoplasmic reticulum (ER) and endosomes [23,24]. A 3-phosphatase and two 5-phosphatases that can utilize PtdIns-(3,4,5) P_3 as substrate have also been localized to membranes of the early secretory pathway, suggesting the presence of relevant phosphoinositide substrates on ER or Golgi membranes [25–27].

Given the significance of phosphoinositides at intracellular sites, we have embarked on an investigation of the intracellular distribution of these lipids. To discover the precise distribution of phosphoinositides, we recently developed techniques for localization of lipid at the electron-microscopy (EM) level using tagged PH domains as specific probes on ultrathin sections [28]. The on-section immunoEM labelling approach avoids over-expression of GFP (green fluorescent protein)-tagged constructs, which can sequester endogenous lipid and possibly disrupt cell function [29]. It also avoids artifactual increases in membrane signals, linked to changes in cell morphology, that are seen with fluorescence techniques [30]. By carrying out procedures at near to 0 °C, we can limit the problem of lipid extraction from the sectioned membrane seen previously when the labelling procedure was carried out at room temperature (approx. 21 °C) (S. A. Watt and J. M. Lucocq, unpublished work), allowing the high-resolution EM study and quantification of the signal. In the present study, we have used the C-terminal PH domain of TAPP1 tagged with glutathione S-transferase (GST) as a probe to identify cellular pools of PtdIns(3,4) P_2 . We show (i) major increases in GST–TAPP1 labelling at the plasma membrane in response to PDGF or H₂O₂ stimulation, (ii) agonist-responsive pools of PtdIns(3,4) P_2 on the ER and on membranes of multivesicular endosomes (MVBs), and (iii) data that are consistent with distinct mechanisms of enzymic PtdIns(3,4) P_2 regulation in ER, MVBs and plasma membrane.

MATERIALS AND METHODS

Materials

FuGENE™ 6 transfection reagent and Complete Protease Inhibitor Cocktail Tablets were from Roche (Mannheim, Germany), PDGF was from Calbiochem (Nottingham, U.K.) and H₂O₂ was from Sigma (Poole, Dorset, U.K.). Insulin-like growth factor-1 (IGF1), microcystin-LR, foetal bovine serum (FBS) and other cell-culture reagents were obtained from Life Technologies (Paisley, Scotland, U.K.). Inositol phosphates and phosphoinositides were from Cell Signals (Lexington, KY, U.S.A.). Anti-GST antibody was obtained from Chemicon International (Temecula, CA, U.S.A.).

Buffers

Buffer A comprised 1 mM EGTA, 1 mM EDTA, 1 % (w/v) Triton X-100, 50 mM Tris/HCl, pH 7.5, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 μ M microcystin-LR, 0.1 % (v/v) 2-mercaptoethanol, 0.27 M sucrose and Complete Protease Inhibitor Cocktail (one tablet per 25 ml). Buffer B comprised 10 mM 2-mercaptoethanol, 50 mM Tris/HCl,

pH 7.5, 0.1 mM EGTA and 0.27 M sucrose. TTBS (Tris/Tween 20-buffered saline) comprised 50 mM Tris/HCl, pH 8.0, 0.1 % (v/v) Tween 20 and 150 mM NaCl.

Expression and purification of GST–PH domains

The pGEX-4T-1 constructs encoding the PH domains of TAPP1 and TAPP1 [R211L] (TAPP1 with a Arg²¹¹ → Leu mutation) were transformed into BL21 *Escherichia coli* cells and a 500 ml culture was grown at 37 °C in Luria–Bertani broth containing 100 μ g/ml ampicillin until the D_{600} was 0.6. Isopropyl β -D-thiogalactoside (0.25 mM) was added, and the cells were cultured for a further 16 h at 26 °C. The cells were resuspended in 25 ml of ice-cold buffer A, freeze–thawed once, and lysates were prepared by sonication. The lysates were clarified by centrifugation (20 000 *g* for 30 min at 4 °C) and the supernatant was filtered through a 0.44 μ m filter and incubated for 60 min on a rotating platform with 1 ml of glutathione–Sephadex pre-equilibrated in buffer A. The beads were collected by centrifugation (1 min at 3000 *g*), and were washed three times with 15 ml of buffer A containing 0.5 M NaCl, then a further ten times with 15 ml of buffer B. GST–proteins were eluted from the beads with 2 ml of buffer B containing 20 mM glutathione, and the beads were removed by filtration through a 0.44 μ m filter. The eluate was divided into aliquots, snap-frozen in liquid nitrogen and stored at –80 °C.

Cell culture, stimulation and transfection

Mouse fibroblast Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin, and were cultured on 10 cm diameter plastic dishes at 37 °C in a humidified atmosphere of 5 % CO₂. For live-cell imaging, cells were grown on 42 mm diameter glass coverslips, transfected with 1 μ g of pEYFP-TAPP1 using FuGENE™ 6 transfection reagent following the manufacturer's protocol, left for 10 h at 37 °C then serum-starved overnight. Coverslips were mounted on a heated chamber on a Zeiss 410 laser scanning confocal microscope, in 1 ml of pre-warmed serum-free medium (DMEM plus 25 mM Hepes, minus Phenol Red) and 2 $\times$ agonist in 1 ml of medium added (100 ng/ml PDGF or 2 mM H₂O₂), to give final concentrations of 50 ng/ml and 1 mM respectively. Images were collected every 60 s over a 30 min period.

U87MG cells were obtained from the European Cell Culture Collection and were maintained in MEM (minimal essential medium), 2 mM glutamine, 1 $\times$ non-essential amino acids, 1 mM sodium pyruvate and 10 % FBS. Wild-type GFP–PTEN (phosphatase and tensin homologue deleted on chromosome 10) was expressed in U87MG cells growing at low cell density (< 70 % confluence) using a baculovirus delivery system adapted for mammalian expression as described in [31]. At 24 h after plating, viral supernatants were added to culture medium, at 1 ml of viral supernatant in 10 ml of medium. Where indicated, cells were stimulated with 50 ng/ml recombinant rat PDGF (B-chain homodimer; Sigma) and fixed in 2 % (v/v) glutaraldehyde (Agar Scientific, Stansted, Essex, U.K.) in 0.2 M Pipes, pH 7.2, for 30 min at room temperature. Fixed cells were stored in PBS (150 mM NaCl and 10 mM sodium phosphate, pH 7.4) at 4 °C until further processing.

Protein–lipid overlay assay

The phosphoinositide-binding properties of the GST-tagged PH domains used were determined using a protein–lipid overlay assay

as described previously [32]. A 1 μ l aliquot of lipid solution, dissolved in chloroform/methanol/water (1:2:0.8 by vol.), and containing various amounts of the indicated lipids (between 3 and 50 pmol) was spotted on to Hybond C nitrocellulose membrane and was allowed to dry at room temperature for 1 h. The membrane was blocked in 2 % (w/v) fatty-acid-free BSA in TTBS for 1 h, then incubated overnight at 4 °C with gentle stirring in the same solution containing 0.2 μ g/ml of the indicated GST-fusion protein. The membranes were then washed three times over 15 min in TTBS followed by incubation for 1 h with anti-GST monoclonal antibody (1:1000; Sigma). Membranes were washed as before, then incubated for 1 h with anti-(mouse IgG)–horse-radish peroxidase conjugate (1:5000; Pierce, Chester, U.K.). Finally, the membranes were washed four times over 20 min. GST-fusion proteins bound to the membranes were then visualized by enhanced chemiluminescence (ECL[®]; Amersham Biosciences, Little Chalfont, Bucks., U.K.). Competition experiments were carried out as above, except that 20 μ M of the indicated inositol headgroup was added along with the GST-fusion protein.

On-section labelling of PtdIns(3,4) P_2 using GST-TAPP1

Swiss 3T3 cells were cultured in 10 cm diameter plastic dishes to approx. 80 % confluence, stimulated with the indicated agonists as above, before being fixed by addition of 2 % (v/v) glutaraldehyde in 0.2 M Pipes, pH 7.2, for 30 min at room temperature. For wortmannin inhibition experiments, wortmannin was dissolved in DMSO at a concentration 1000-fold higher than that used, or the equivalent volume of DMSO as a control was added to the tissue culture medium 10 min before stimulation. Cells were scraped from the dish using a rubber policeman and pelleted at 12 000 g for 30 min before being infiltrated in 2.1 M sucrose at 4 °C overnight. Small fragments of pellets were mounted on iron panel pins and frozen in liquid nitrogen before cutting ultrathin cryosections on a Leica EM FCS ultracut UCT microtome. Sections were cut at –110 °C and section retrieval was performed using a modification to the pick-up method described by Watt et al. [28]. The frozen droplet of pick-up solution was allowed to thaw at just above 0 °C at the bottom of a deep ice-box, then transferred directly on to a carbon/pioloform[®]-coated cooper support grid on a plastic dish on ice. The subsequent immunolabelling procedure was carried out entirely on ice with pre-cooled solutions. The grids were first incubated on 0.1 M NH_4Cl in PBS (10 min), on PBS containing 0.5 % cold-water-fish-skin gelatin (FSG; Sigma) for 10 min and then on 10 μ l droplets of GST-tagged PH domain (approx. 3 μ g/ml) in 0.5 % FSG/PBS for 30 min. Following three washes over 15 min in PBS, the GST was localized using anti-GST antibodies and Protein A–gold essentially as described in [33]. Sections were then contrasted using methylcellulose/uranyl acetate (1.8 % and 0.3 % respectively) and viewed on a JEOL 1200EX electron microscope at 80 kV. For competition experiments, the indicated inositol phosphate at a final concentration of 1 mM was pre-mixed with the appropriate concentration of GST-tagged PH domain for 1 h before cryosection incubation.

Quantification of immunolabelling

Determination of the proportions and densities of gold labelling over cell membranes was achieved using the grid-scanning technique described previously [28]. Between 200 and 300 intersections through cell membranes were counted for each grid using the top edge of a clearly defined feature on the viewing screen at a primary magnification of $\times 20\,000$ (final magnification $\times 200\,000$). Gold particles were assigned to specific compart-

ments to give the percentage over each compartment, and were related to the number of intersections over each membrane to give the labelling density. In order to calculate the specific labelling density over each compartment, separating stimulated labelling from background labelling, we used the following formula:

$$\text{Labelling density} = (\text{stimulated } \Sigma \text{gold} / \Sigma \text{intersections}) \\ - (\text{unstimulated } \Sigma \text{gold} / \Sigma \text{intersections})$$

where the sum (Σ) is that of three experiments. For stereological reasons, it can be problematic to obtain gold density data on MVBs, because the size of the internal structures is small compared with the thickness of cryosections and the visualization of these structures was often not ideal. We therefore used micrographs of epoxy-resin-embedded sections taken at $\times 20\,000$ to estimate the length of internal membrane profiles. A square lattice grid with 0.2 μ m spacing was overlaid on the structure and the line intersection counts with the limiting membrane and internal vesicles were made. This showed inner vesicles to have 2.7-fold more intersections than the outer membrane (coefficient of error, 6.7 %; $n = 8$ micrographs). This ratio could then be used to estimate the relative profile length (i.e. number of intersection counts) of internal MVBs from intersection counts on the limiting membrane in cryosections. Gold density on cryosections was calculated by dividing gold on inner vesicles by outer membrane intersections multiplied by 2.7.

To obtain amounts of labelling (from which proportions over different compartment could be calculated), the specific labelling density for each compartment was multiplied by the mean number of intersections for three experiments, through each compartment:

$$\text{Labelling} = (\text{stimulated golds} / \text{intersections}) \\ - (\text{unstimulated golds} / \text{intersections}) \\ \times \text{mean intersections}$$

For the estimation of gold-labelling density over nucleoplasm and cytosol, micrographs were taken at a magnification of $\times 12\,000$ and the images from phosphorimager plates were scanned on a Databis micron plate reader (Birkenfeld, Germany). On-screen images were overlaid with a 0.5 μ m square lattice grid in Adobe Photoshop 5.5 and grid corners (points) falling over compartments of interest were counted, with areas estimated from the product of the sum of these point hits and the real area of each grid lattice square (0.25 μm^2) [34].

Structures were identified according to morphological criteria. Caveolae-like structures were identified as small vesicles of approx. 40–50 nm, usually in groups at the periphery of the cell or connected to the plasma membrane, and had no identifiable coat (structures previously seen to label with an anti-caveolin-1 antibody in other cell types) (S. Watt and J. Lucocq, unpublished work). Golgi vesicles were defined as elongated or circular structures situated close to *cis*, *trans* or lateral aspects of the Golgi stack. Putative peripheral endosomes were identified as irregular or elongated profiles of approx. 80–200 nm in length and 30–80 nm in diameter, and were situated close to the plasma membrane. MVBs were defined as round or oval sac-like profiles with a single limiting membrane containing three, or more, smaller internal vesicles.

RESULTS

TAPP1 PH domain as a specific probe for PtdIns(3,4) P_2

Previous work in mouse fibroblast Swiss 3T3 cells demonstrated elevated levels of both PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 in

Figure 1 Demonstration of specificity of GST-TAPP1-PH

The lipid-binding specificity of GST-TAPP1-PH was assessed in a protein-lipid overlay assay (A). Serial dilutions of eight known phosphoinositide species were spotted on to nitrocellulose membranes and incubated with GST-TAPP1-PH. Membranes were washed, and bound protein was detected using rabbit anti-GST antibodies as described in the Materials and methods section. Numbers on the left-hand side indicate the amount of lipid used (pmol). The results of on-section labelling of Swiss 3T3 cells with GST-TAPP1-PH are shown in (B)–(D). Serum-starved cells were treated with 50 ng/ml PDGF for 10 min, 1 mM H₂O₂ for 15 min or 100 ng/ml IGF1 for 10 min. Where +WT is indicated, cells were pre-treated for 10 min before addition of 100 nM wortmannin. The number of gold particles counted over the plasma membrane of ultrathin cryosections labelled with GST-TAPP1-PH was related to the number of intersections that a scanning line makes with the plasma membrane (see the Materials and methods section). For each experiment, 200–300 intersections were counted. Results are means $\pm$ S.E.M. ($n=3$).

response to several stimuli, including PDGF and H₂O₂ [35,36]. Subsequently, we showed that, in response to H₂O₂ or PDGF stimulation, the PtdIns(3,4)P₂-binding PH domain of TAPP1, tagged with YFP (yellow fluorescent protein), underwent a rapid translocation from the cytosol to the plasma membrane, as detected by fluorescence and immunoEM [11]. This observation combined with the specific binding to PtdIns(3,4)P₂ observed in protein-lipid overlay assays [9], and the selectivity for PtdIns(3,4)P₂ shown by resolution of the three-dimensional crystal structure [10], indicated that the C-terminal PH domain of TAPP1 can be utilized as a probe in order to determine the subcellular localization of PtdIns(3,4)P₂.

We first confirmed the translocation to the cell periphery using TAPP1-PH domain tagged with YFP upon PDGF or H₂O₂ stimulation (results not shown). However, in order to study the high-resolution subcellular localization of PtdIns(3,4)P₂, we wanted to avoid overexpression of fluorescent protein chimaeras, which may interfere with endogenous lipids, disturb normal cellular functions and produce signals that are dependent on changes in

morphology [29,30]. To this end, we used our *in situ* immunogold-labelling procedure on ultrathin cryosections of non-transfected Swiss 3T3 cells, as previously described [28]. We generated a GST-tagged TAPP1 PH domain as a probe for PtdIns(3,4)P₂ and applied it to ultrathin cryosections, then detected the bound protein using an anti-GST antibody followed by Protein A complexed to colloidal gold.

As a test of the lipid specificity, we applied GST-TAPP1-PH in a protein-lipid overlay assay, which showed that of the eight known phosphoinositide species, GST-TAPP1-PH binds strongly to PtdIns(3,4)P₂ only (Figure 1A). Stimulation with PDGF or H₂O₂ results in a production of PtdIns(3,4)P₂ at the plasma membrane; therefore we treated Swiss 3T3 with either of these agonists and labelled with GST-TAPP1-PH. As can be seen from Figure 2(A), this resulted in an accumulation of gold labelling on the plasma membrane. The density of gold labelling was quantified by systematically scanning cell profiles and counting intersections through, and gold associated with, the plasma membrane, as described previously [28]. After 10 min of treatment with PDGF

Figure 2 GST-TAPP1-PH immunogold labelling on ultrathin thawed cryosections of Swiss 3T3 cells

(A) GST-TAPP1-PH labelling on the plasma membrane of cells stimulated with 50 ng/ml PDGF for 10 min is indicated by arrows. Arrows in (B) and (C) indicate gold labelling on ER membrane and on internal vesicles of a MVB respectively. Scale bars are 200 nm.

(50 ng/ml), we observed a 2–2.5-fold increase in GST-TAPP1-PH labelling over the plasma membrane as compared with non-stimulated control cells (Figure 1B), with an even more pronounced increase (approx. 4-fold) after 15 min of treatment

with 1 mM H_2O_2 (Figure 1C). In both cases, this increased labelling was inhibited by a 10 min pre-incubation with 100 nM of the PI3-kinase inhibitor, wortmannin. As an additional test of whether the signals were related to $\text{PtdIns}(3,4)\text{P}_2$ rather than to $\text{PtdIns}(3,4,5)\text{P}_3$, we stimulated cells with 100 ng/ml IGF1, a treatment which is known to produce a large increase in $\text{PtdIns}(3,4,5)\text{P}_3$ levels, but relatively little $\text{PtdIns}(3,4)\text{P}_2$ [35]. Stimulation with this agonist produced only a moderate elevation in GST-TAPP1-PH labelling compared with control levels, with PDGF stimulation causing an almost 2-fold greater increase compared with that induced by IGF1 (Figure 1D).

Labelling with TAPP1 PH domain, but not a non-lipid-binding mutant, is strongly inhibited by the inositol headgroup $\text{Ins}(1,3,4)\text{P}_3$

In order to show that the labelling was related to the lipid-binding capacity of the PH domain, we employed a non- $\text{PtdIns}(3,4)\text{P}_2$ -binding mutant, GST-TAPP1-PH [R211L], which has an leucine residue substituted for an essential arginine residue. This tagged protein, which does not bind $\text{PtdIns}(3,4)\text{P}_2$ on protein–lipid overlay assays (Figure 3B), was applied to ultrathin cryosections of both unstimulated and H_2O_2 -stimulated Swiss 3T3 cells as before. Consistent with the previous results, H_2O_2 stimulated a substantial increase in GST-TAPP1-PH labelling on the plasma membrane, whereas the density of labelling with GST-TAPP1-PH [R211L] was unaffected by H_2O_2 treatment and stayed close to control levels (Figure 3A). Similar results were obtained using the R211L mutant protein with PDGF-stimulated cells (Figure 3C).

The specificity of the labelling was also tested using a competition experiment of the type used previously to inhibit PH-domain-labelling of lipids [28]. Here, we pre-incubated GST-TAPP1-PH with the soluble inositol headgroup, $\text{Ins}(1,3,4)\text{P}_3$, contained in $\text{PtdIns}(3,4)\text{P}_2$. When used in a protein–lipid overlay assay, $\text{Ins}(1,3,4)\text{P}_3$ prevented binding of the PH domain to $\text{PtdIns}(3,4)\text{P}_2$, but pre-incubation with either $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$ had no effect (Figure 3B). We then assessed whether or not $\text{Ins}(1,3,4)\text{P}_3$ would inhibit GST-TAPP1-PH labelling on cryosections of PDGF-stimulated Swiss 3T3 cells. As seen in Figure 3(C), $\text{Ins}(1,3,4)\text{P}_3$ inhibited the majority of the labelling over the plasma membrane. However, when the non-lipid-binding mutant, GST-TAPP1-PH [R211L], was pre-incubated with $\text{Ins}(1,3,4)\text{P}_3$, no decrease in labelling was seen (Figure 3C). In addition, the non-cognate headgroup, $\text{Ins}(1,4,5)\text{P}_3$, had no effect on the labelling of the plasma membrane with GST-TAPP1-PH, in agreement with results of the protein–lipid overlay assay [gold density, 0.58 ± 0.02 particles/intersection using $\text{Ins}(1,4,5)\text{P}_3$, compared with 0.13 ± 0.02 particles/intersection using $\text{Ins}(1,3,4)\text{P}_3$; $n = 3$ independent experiments]. Results here and elsewhere are means $\pm$ S.E.M.

Stimulation of Swiss 3T3 cells with PDGF leads to increased levels of $\text{PtdIns}(3,4)\text{P}_2$ in ER and other endomembranes

Although the majority of gold labelling was present on the plasma membrane (Figure 2A), smaller amounts were evident on other membrane-bound compartments, such as ER and MVBs (Figures 2B and 2C). To assess whether this gold labelling represented previously undetected hormone-responsive pools, we repeated the PDGF stimulation experiments and quantified labelling over intracellular membranes using the same scanning technique as used for plasma membrane in Figure 1 (see the Materials and methods section). Again we found a marked increase in the density of gold labelling on the plasma membrane after stimulation with PDGF (both 10 and 30 min) (Figures 4A and 4B) that was inhibited by pre-treatment with wortmannin (results not

Figure 3 Immunogold labelling with a non-lipid-binding TAPP1 mutant and inhibition of GST-TAPP1-PH labelling with inositol headgroups

Cryosections of H₂O₂-stimulated cells were labelled with a non-lipid-binding TAPP1 mutant, GST-TAPP1-PH [R211L], and gold-labelling densities over the plasma membrane compared with GST-TAPP1-PH (A). The effect of inositol phosphate headgroups on GST-TAPP1-PH binding to PtdIns(3,4)P₂ was assessed by performing a protein-lipid overlay assay (B). Serial dilutions of PtdIns(3,4)P₂ were spotted on to nitrocellulose membrane and incubated with GST-TAPP1-PH, the non-lipid-binding mutant GST-TAPP1-PH [R211L], or GST-TAPP1-PH pre-mixed with various inositol headgroups. Membranes were washed and bound protein detected using antibodies to GST. Numbers on the left indicate amount of lipid used (pmol). The effect of inositol headgroup inhibition on the plasma membrane labelling of PDGF-stimulated cells is shown in (C). GST-TAPP1-PH and GST-TAPP1-PH [R211L] were pre-incubated for 1 h with Ins(1,3,4)P₃, then used to label cryosections. Results in (A) and (C) are means $\pm$ S.E.M. ($n = 3$).

Figure 4 Quantification of GST-TAPP1-PH labelling over cellular membranes after PDGF stimulation

Ultrathin cryosections of PDGF-stimulated cells were labelled with GST-TAPP1-PH as described in the Materials and methods section. The number of gold particles over cellular compartments was related to the number of intersections that a scanning line makes with membranes from each compartment. (A) and (B) show the density of gold labelling over each compartment in unstimulated and PDGF-stimulated cells. (C) and (D) show the density and proportion of stimulated gold labelling over each compartment respectively. Asterisks over the MVB inner vesicle columns indicate that the data has been derived using intersections through the outer membrane and adjusted appropriately, as described in the Materials and methods section. PM, plasma membrane; GOL ST, Golgi stack; GOL VES, Golgi vesicles; PER TUB, peripheral tubules (putative early endosomes); MVB OM, MVB outer limiting membrane; MVB IV, MVB inner vesicles; NUC ENV, nuclear envelope; CAV, caveolae-like structures; MIT OM, mitochondria outer membrane; MIT CRIS, mitochondria cristae. Results in (A) and (B) are means $\pm$ S.E.M. ($n = 3$).

Table 1 Specificity of labelling over intracellular compartments assessed using headgroup inhibition

Cells were stimulated with PDGF for 30 min, fixed, embedded, sectioned and labelled using GST-TAPP1-PH in the presence of either Ins(1,3,4)P₃ or Ins(1,4,5)P₃ (see the Materials and methods section). Labelling density was assessed as gold/intersection (MVBs and ER) or gold particles/μm² (nucleus and cytosol). Because of the small size of the MVB vesicles, the inner vesicle intersections were estimated indirectly using line intersection counts made on epoxy resin sections (see the Materials and methods section). For MVBs and ER, *n* = 3; for nucleus and cytosol, *n* = 5. Results are means ± S.E.M. ND, not determined.

Compartment	Labelling density		
	No headgroup	Ins(1,3,4)P ₃	Ins(1,4,5)P ₃
MVBs			
Inner vesicles	0.22 ± 0.03	0.02 ± 0.017	0.22 ± 0.03
Limiting membrane	0.095 ± 0.015	0.026 ± 0.02	0.1 ± 0.02
ER	0.16 ± 0.008	0.04 ± 0.029	0.16 ± 0.04
Nucleus	0.48 ± 0.12	0.456 ± 0.12	ND
Cytosol	0.384 ± 0.16	0.8 ± 0.12	ND

shown). Increases in gold-labelling density after stimulation were also apparent on intracellular organelles, including ER, MVBs, nuclear envelope and mitochondria.

As previous work was unable to detect PtdIns(3,4)P₂ in Swiss 3T3 cells in the absence of stimulation [36], the labelling we observed in unstimulated cells is likely to be explained by non-specific sticking of reagents to the section. Therefore, to interpret the labelling patterns more easily, we estimated specific label generated by stimulation. This was calculated by subtracting the gold density of non-stimulated cells from that estimated in stimulated cells, over each compartment (see the Materials and methods section). The specific gold density data presented in Figure 4(C) reveals those compartments that contain detectable agonist-stimulated labelling. Combining the intersection counts with the gold density, we were able to estimate the amounts (and therefore the proportions) of specific agonist-stimulated label over intracellular compartments.

The highest density of specific GST-TAPP1-PH labelling was found on the plasma membrane. A substantial concentration was also seen on the inner vesicles of MVBs, which showed a 4.8-fold decrease in labelling density when wortmannin treatment preceded 30 min of PDGF stimulation. Inner vesicle gold-particle density was estimated using intersections with the limiting membrane of MVBs, as detailed in the Materials and methods section. This labelling was also inhibitable by addition of Ins(1,3,4)P₃ (Table 1). The outer, limiting membrane of MVBs also showed an increase in gold labelling upon 30 min of PDGF stimulation, with a density of labelling lower than that seen on the inner vesicles, but still approx. 6-fold greater than unstimulated levels. This labelling was also seen to be inhibitable by inositol headgroup competition (Table 1) and by pre-treatment with wortmannin (0.04 ± 0.02 golds/intersection with wortmannin compared with 0.095 ± 0.015 golds/intersection with no wortmannin; *n* = 3).

Of the other compartments, ER and nuclear envelope showed the most marked ligand-dependent labelling. Labelling on ER was wortmannin-sensitive, with 2.7-fold less gold labelling when 30 min PDGF-stimulated cells were pre-treated with wortmannin. In addition, the signal seen on ER was inhibited by Ins(1,3,4)P₃, but not Ins(1,4,5)P₃ (Table 1). Detectable, but weak, signals were also found over Golgi apparatus, peripheral tubules (putative peripheral endosomes) and mitochondria, whereas caveolae-like structures contained no stimulated increase in labelling density.

Labelling was also detected in the cytosol and nucleus, but this was neither responsive to PDGF stimulation (nucleus, 0.676 ± 0.28 gold/μm² for control compared with 0.48 ± 0.25 gold/μm²

and 0.4 ± 0.12 gold/μm² for 10 and 30 min PDGF stimulations respectively; cytosol, 0.44 ± 0.4 gold/μm² compared with 0.16 ± 0.136 gold/μm² and 0.32 ± 0.19 gold/μm² for 10 and 30 min PDGF stimulations respectively; *n* = 5 for each), nor inhibitable by Ins(1,3,4)P₃ (Table 1).

The data in Figure 4(D) show the proportions of specific labelling, giving an indication of the relative amounts of labelled PtdIns(3,4)P₂ over cellular compartments. As would be expected, approx. 70% of specific labelling was found at the plasma membrane after both 10 and 30 min stimulations. The proportion of gold over other labelled compartments was smaller, with the largest pools situated over inner vesicles of MVBs (13.5 and 14.5%) and ER (5.7 and 8.2%; 10 and 30 min respectively).

Stimulatable pools of PtdIns(3,4)P₂ on ER and other endomembranes after H₂O₂ treatment

In Swiss 3T3 cells, exposure to oxidative stress by treatment with H₂O₂ is known to induce a large increase in PtdIns(3,4)P₂ levels, but only a small and transient increase in PtdIns(3,4,5)P₃ [36]. Treatment with 1 mM H₂O₂ produced similar patterns of increased GST-TAPP1-PH labelling as seen with PDGF. Figure 5(A) reveals that the plasma membrane and ER showed large increases in labelling density and this was also inhibitable by wortmannin treatment (results not shown). This was also the case for MVB inner vesicles, in which H₂O₂ stimulation induced a 4.4-fold increase over unstimulated cells and 5.3-fold higher labelling compared with wortmannin-treated cells. Specific stimulated GST-TAPP1-PH labelling (Figure 5B) was found over plasma membrane and also ER, with substantial densities over the inner vesicles of MVBs. Other structures with lower densities included peripherally situated tubules that probably represent endosomal structures, nuclear envelope and mitochondria. The signal detected over the nucleoplasm and cytosol was non-responsive to H₂O₂ (nucleus, 0.512 ± 0.08 gold/μm² for control compared with 0.52 ± 0.34 gold/μm² for stimulated; cytosol, 0.44 ± 0.27 gold/μm² for control compared with 0.16 ± 0.21 gold/μm² after stimulation; *n* = 5).

In terms of the relative proportions of stimulated labelling over different membranes, >60% was found on the plasma membrane, with fairly substantial amounts on the ER and MVB inner vesicles (11.4 and 12.3% respectively) and much smaller pools detected on peripherally located tubules (putative peripheral endosomes), nuclear envelope and mitochondrial membranes (<5%) (Figure 5B).

U87MG cells expressing PTEN show differential down-regulation of TAPP1 labelling in different endomembranes

U87MG cells, which lack the 3-phosphatase tumour suppressor PTEN, were cryosectioned and immunolabelled with GST-TAPP1-PH. An increase in gold labelling was observed after PDGF stimulation over the same structures seen to have stimutable pools in Swiss 3T3 cells. We found a 2.5-fold increase in labelling density over both plasma membrane (0.42 ± 0.064 golds/intersection compared with 0.17 ± 0.024 golds/intersection; *n* = 3) and MVB inner vesicles (0.22 ± 0.047 golds/intersection compared with 0.09 ± 0.029 golds/intersection; *n* = 3), and an approx. 6-fold increase over ER (0.19 ± 0.005 golds/intersection compared with 0.03 ± 0.011 golds/intersection; *n* = 3). An increase in labelling was also observed on the limiting membrane of MVBs.

To test the sensitivity of PtdIns(3,4)P₂ to PTEN, we transfected U87MG cells with GFP-PTEN using a baculoviral delivery system. This results in substantial expression in 90–100% of the cells

Figure 5 Quantification of GST-TAPP1-PH labelling over cellular membranes after H₂O₂ stimulation

Ultrathin cryosections of H₂O₂-treated cells were labelled with GST-TAPP1-PH and the density and proportion of gold labelling over cellular compartments was quantified as for PDGF-stimulated cells. Data in (A) compare the gold density between unstimulated and H₂O₂-stimulated cells. The density of stimulated labelling is shown in (B) and the proportion of stimulated gold labelling over each compartment is seen in (C). Asterisks indicate that the data for MVB inner vesicles have been estimated from outer membrane intersections and adjusted appropriately (see the Materials and methods section). PM, plasma membrane. See the legend to Figure 4 for other abbreviations. Results in (A) are means $\pm$ S.E.M. ($n = 3$).

and produces roughly 5–10-fold increased levels of PTEN protein expression compared with other cell lines (N. R. Leslie, unpublished work). Cryosections of these cells, stimulated with PDGF for 10 min, were labelled with GST-TAPP1-PH. In the absence of PTEN, the plasma membrane, ER and MVB inner vesicles all showed increases in labelling after stimulation with PDGF. However, after PTEN transfection, the stimulated labelling over plasma membrane and inner vesicles of MVBs was reduced to approx. 40% and 50% respectively (Figure 6). However, labelling over the ER appeared to remain at similar levels to those seen

Figure 6 Effect of exogenous PTEN expression on GST-TAPP1-PH labelling density after PDGF stimulation in U87MG cells

Ultrathin cryosections of both untransfected and GFP-PTEN-transfected U87MG cells, stimulated with 50 ng/ml PDGF, were labelled with GST-TAPP1-PH. The data shown indicate the amount of stimulated gold labelling seen in U87MG cells expressing PTEN as a percentage of the stimulation level seen in untransfected cells. Data are shown for the plasma membrane, ER and MVB inner vesicles, i.e. compartments that show an increase in GST-TAPP1-PH labelling upon PDGF stimulation. Results are the means for two independent experiments with three EM grids counted for each experiment. Bars represent the range.

in non-transfected cells (overall reduction in stimulated labelling was 4%). These data therefore indicate a differential sensitivity of the stimulated ER pool to exogenous PTEN compared with labelling of plasma membrane and MVBs. In addition, they provide strong evidence for the validity of the PH domain probe and the on-section labelling system.

DISCUSSION

We have used an *in situ* high-resolution lipid-labelling approach to identify agonist-responsive pools of PtdIns(3,4) P_2 in cell membranes, demonstrating the C-terminal PH domain of TAPP1 to be an effective probe for this lipid and identifying previously undetected pools of PtdIns(3,4) P_2 . The labelling method used utilizes the PH domain from the C-terminus of TAPP1 as a high-affinity, highly specific probe for the detection of PtdIns(3,4) P_2 . This membrane-binding module has previously been demonstrated to be narrowly specific for PtdIns(3,4) P_2 [9], and *in vitro* binding studies here reiterate its specificity for this lipid over other known phosphoinositide species. Here, with a GST tag, it serves to affinity-label ultrathin cryosections followed by detection with anti-GST antibodies and electron-dense Protein A–gold, in a procedure described previously [28]. The rationale for this approach was to detect PtdIns(3,4) P_2 directly in untransfected cells, as an alternative approach to previous *in vivo* studies indicating production at the plasma membrane as detected by YFP-TAPP1-PH recruitment from the cytosol upon agonist stimulation [11].

The specificity of the labelling was established in a number of ways. First, we found that on stimulation of Swiss 3T3 cells with agonists known to increase levels of PtdIns(3,4) P_2 , there was a marked increase in plasma membrane labelling. H₂O₂ produced a 2-fold greater increase in labelling signal compared with PDGF, consistent with previous data showing that H₂O₂ stimulates PtdIns(3,4) P_2 levels that are 3-fold higher than those seen after PDGF [36]. Secondly, the cognate inositol phosphate headgroup, Ins(1,3,4) P_3 , specifically inhibited plasma membrane labelling, whereas the non-cognate Ins(1,4,5) P_3 did not. Thirdly, a non-lipid-binding mutant TAPP1-PH domain produced labelling that was unresponsive to agonists and was not inhibited by cognate headgroups. Fourthly, the agonist-stimulated labelling

was sensitive to pre-treatment with the PI3-kinase inhibitor, wortmannin. Finally, GST-TAPP1-PH is highly selective for PtdIns(3,4) P_2 in a protein–lipid overlay assay. By these criteria, we conclude that the on-section labelling obtained with GST-TAPP1-PH after agonist stimulation is selective for PtdIns(3,4) P_2 .

How sensitive/quantitative is the labelling? PtdIns(3,4) P_2 reputedly comprises only 0.2% of total cellular phosphatidylinositol bisphosphates in agonist-stimulated cells, with PtdIns(4,5) P_2 comprising > 99% [2], a molar ratio of 500:1. Compared with our previous results using the PH domain of phospholipase C δ_1 [28], the PtdIns(3,4) P_2 labelling generated by H₂O₂ stimulation is about one quarter of that seen for PtdIns(4,5) P_2 in astrocytoma cells. Clearly this difference is not as pronounced as might be expected with respect to their cellular concentrations. This may be explained by the following: (i) differences in the labelling affinities of the two probes; the TAPP1 C-terminal PH domain has a K_d for PtdIns(3,4) P_2 of approx. 20 nM [9] compared with 1.7 μ M for PLC δ_1 binding to PtdIns(4,5) P_2 [37], (ii) a substantial portions of PtdIns(4,5) P_2 may be masked by interactions with PH domains of PtdIns(4,5) P_2 effector proteins, and (iii) compartmentalization of PtdIns(4,5) P_2 into tightly packed microdomains could mask inositol headgroups, making them too crowded for each molecule to bind to the PH domain [38,39].

We found only one previous study showing evidence for PtdIns(3,4) P_2 in membranes other than the plasma membrane [19], and no designated roles have been attributed to this lipid other than signalling from the plasma membrane. It is interesting therefore that agonist-stimulated labelling was present in a variety of intracellular membranes, with a significant pool evident on MVB internal membranes and ER, after both PDGF and H₂O₂ stimulation.

The PtdIns(3,4) P_2 of MVBs may be generated locally in these organelles or, alternatively, the lipid could be functionally imported via endocytosis. The latter possibility is supported by work on the PtdIns(3,4) P_2 /PtdIns(3,4,5) P_3 -binding protein, DAPP1. DAPP1 undergoes a PI3-kinase-dependent translocation to the plasma membrane that is then followed by internalization [8], suggesting uptake of the lipid into the endocytic system. Local generation would require the concentration of the appropriate lipid kinases/phosphatases in endosomal compartments. One pathway of PtdIns(3,4) P_2 generation might involve production of PtdIns3P and then action of a PI4-kinase. Type III PI3-kinase (vps34 homologue) and its product PtdIns3P have been detected on MVBs [21,40], but the only endosomal PI4-kinase known (type II isoform) does not show detectable activity on phosphatidylinositol monophosphates [3]. Interestingly, however, studies in human platelets have indicated a pathway whereby PtdIns3P is converted to PtdIns(3,4) P_2 via the actions of a novel PI4-kinase [4]. An alternative pathway may be via PtdIns(3,4,5) P_3 dephosphorylation by the PtdIns(3,4,5) P_3 -specific 5-phosphatase, SHIP, an enzyme known to negatively regulate phagocytosis [41,42]. An additional possibility for generating PtdIns(3,4) P_2 would be phosphorylation of PtdIns4P by PI3-kinase, and the localization of type I and II PI3-kinase isoforms at high resolution will therefore be crucial.

The presence of PtdIns(3,4) P_2 labelling on ER membranes is consistent with the localization of enzymes that use 3-phosphoinositides as substrates. PtdIns(3,4,5) P_3 has not thus far been detected on ER, although the ER localization of the 3-phosphatase TPPI α [TPTE (trans-membrane phosphatase with tensin homology) and PTEN homologous inositol lipid phosphatase) and the PtdIns(3,4,5) P_3 5-phosphatase SKIP (skeletal muscle- and kidney-enriched inositol phosphatase) [25,26] would suggest the presence of PtdIns(3,4,5) P_3 and possible pathways for generation of phosphatidylinositol bisphosphates. Studies using liposomes

have identified a requirement for anionic phospholipids such as PtdIns(4,5) P_2 in the recruitment of components of the COPII coat protein complex [43], suggestive of a role in COPII budding from the ER, although PtdIns(3,4) P_2 was not tested. Generation of PtdIns(3,4) P_2 at the ER via lipid kinases appears less likely as, firstly, no ER-located PI3-kinase has been detected, and secondly, PtdIns3P has not been found at the ER [40], thereby arguing against 4-phosphorylation by ER-located PI4-kinases [23,44]. We also detected signal for PtdIns(3,4) P_2 over the nuclear envelope, consistent with previous labelling experiments using PtdIns(3,4) P_2 monoclonal antibodies although in that study the phosphoinositides in other organelles may have been lost due to the use of detergent during sample preparation [19].

Interestingly, an inositol 5-phosphatase known to hydrolyse PtdIns(3,4,5) P_3 has been localized to the Golgi [27], where PI4-kinases and PI3-kinases [22,23] have also been localized, but we saw no significant amount of labelling over the Golgi with either PDGF or H₂O₂ treatment. This may have been due to PtdIns(3,4) P_2 levels being too low or transient for detection. Specific labelling within the nucleus meanwhile did not appear to be agonist-responsive, an interesting observation considering the prior indications for nuclear pools of phosphoinositides such as PtdIns(3,4,5) P_3 and PtdIns(4,5) P_2 [45–48]. Finally, the localization of a type II 5-phosphatase to mitochondria-like structures suggests that 3-phosphoinositides may reside here [49]; however, the signal we found over the mitochondria would appear to be largely non-specific, as it was not abolished with Ins(1,3,4) P_3 .

The results of GST-TAPP1 labelling in PTEN-null U87MG cells are particularly intriguing. First, the decrease in stimulated labelling seen on the plasma membrane in cells treated to express PTEN compared with untreated cells strongly supports the validity of the PH domain as a probe for PtdIns(3,4) P_2 . Secondly, the PDGF-stimulated pools that were sensitive to expression of PTEN were found on the plasma membrane and MVBs, but not ER. Again, this supports further the specificity of the PH domain as a probe in this labelling system. The apparent lack of down-regulation of PDGF-stimulated labelling on the ER suggests that the pool of lipid here may be regulated by alternative mechanisms to the other pools, suggesting that PtdIns(3,4) P_2 present on ER membranes is regulated via a mechanism independent of PTEN. An alternative explanation could be aberrant targeting of PTEN to other membranes, but this appears unlikely since PTEN is mainly cytosolic, with only a fraction present on the plasma membrane [50]. Future studies using manipulation of lipid kinases and phosphatases will be important to clarify the nature of lipid synthesis in different cellular pools.

In summary, we have demonstrated for the first time, using high-resolution microscopic techniques and specific lipid binding probes, the existence of previously undetected agonist-responsive pools of PtdIns(3,4) P_2 in cellular membranes other than the plasma membrane. The majority of PtdIns(3,4) P_2 labelling was found on the plasma membrane and future studies will be important to elucidate whether this is contained within specialized microdomains/rafts, particularly given recent data suggesting the presence of PtdIns(3,4,5) P_3 in rafts [51]. The localization of PtdIns(3,4) P_2 to organelles such as ER and MVBs raises intriguing questions as to its roles at these sites and its method of generation. Indeed, our results raise the possibility that the pool of PtdIns(3,4) P_2 on the ER may be regulated by an alternative mechanism to those at the plasma membrane and endosomes. The utilization of powerful tools, such as PH domains, and the high-resolution localization of the enzymes responsible for PtdIns(3,4) P_2 synthesis in these membranes should help us to answer these questions.

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