Localization of agonist-sensitive PtdIns(3,4,5)P_3 reveals a nuclear pool that is insensitive to PTEN expression

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Summary

Phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P_3] is a lipid second messenger, produced by Type I phosphoinositide 3-kinases (PI 3-kinases), which mediates intracellular responses to many growth factors. Although PI 3-kinases are implicated in events at both the plasma membrane and intracellular sites, including the nucleus, direct evidence for the occurrence of PtdIns(3,4,5)P_3 at non-plasma membrane locations is limited. We made use of the pleckstrin homology (PH) domain of general receptor for phosphoinositides (Grp1) to detect PtdIns(3,4,5)P_3 in an on-section labeling approach by quantitative immunogold electron microscopy. Swiss 3T3 cells contained low levels of PtdIns(3,4,5)P_3 that increased up to 15-fold upon stimulation with platelet-derived growth factor (PDGF). The signal was sensitive to PI 3-kinase inhibitors and present mainly at plasma membranes, including lamellipodia, and in a surprisingly large pool within the nuclear matrix. Comparatively little labeling was observed in endomembranes. A similar distribution of PtdIns(3,4,5)P_3 was observed in U87MG cells, which lack the PtdIns(3,4,5)P_3 phosphatase, PTEN. Re-expression of PTEN into U87MG cells ablated plasma membrane PtdIns(3,4,5)P_3, but not the nuclear pool of this lipid even when PTEN was targeted to nuclei. These data have important implications for the versatility of PI 3-kinase signaling and for the proposed functions of PTEN in the nucleus.

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Key words: Phosphoinositide 3-kinase, Phosphatidylinositol trisphosphate, Nucleus, Phosphatase, PTEN, Electron microscopy

Introduction

Stimulation of a wide range of cell surface receptors for hormones and growth factors results in the activation of Type I phosphoinositide 3-kinases (PI 3-kinases) which generate phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P_3] by phosphorylation of the D3 position of the inositol ring of phosphatidylinositol (4,5) biphosphate [PtdIns(4,5)P_2] (Cantley, 2002; Vanhaesebroeck and Alessi, 2000; Vanhaesebroeck et al., 2001). Accumulation of PtdIns(3,4,5)P_3 is usually accompanied by the production of PtdIns(3,4)P_2 (Kimber et al., 2002; Van der Kaay et al., 1999; Watt et al., 2004), due mainly to the actions of 5-phosphatases, such as SHIP-1 and SHIP-2 (Sly et al., 2003). These lipids are involved in the regulation of numerous cellular processes, such as cell survival, proliferation, growth and motility and are thought to have roles in the progression of cancer and in inflammatory and immune cell signaling. They are also thought to have important roles in diabetes due to their crucial roles in mediating many of the metabolic and transcriptional responses to insulin. As might be expected for such an important signaling molecule, PtdIns(3,4,5)P_3 is subject to tight regulatory control, both at the level of its synthesis and its subsequent metabolism. It now appears that, in many cells, the PtdIns(3,4,5)P_3 3-phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome ten) mediates the principal route of PtdIns(3,4,5)P_3 metabolism and acts to antagonize signaling through the PI 3-kinase pathway (Maehama and Dixon, 1999). PTEN is a ubiquitously distributed tumour suppressor protein and its expression is frequently abolished or reduced in a wide variety of human tumours, reinforcing the importance of PI 3-kinase signaling in malignancy (Leslie and Downes, 2004).

PtdIns(3,4,5)P_3 mediates its effects by recruiting proteins to the membrane that are capable of binding tightly and, at least in some cases, specifically to the lipid through phosphoinositide binding domains, most commonly pleckstrin homology domains (Ferguson et al., 2000; Lemmon and Ferguson, 2000). Among the proteins that bind to PtdIns(3,4,5)P_3 are protein serine-threonine kinases such as Akt/protein kinase B (PKB), tyrosine kinases such as Bruton's tyrosine kinase (Btk) and guanine nucleotide exchange factors (GEFs) for small GTPases such as the Rac-specific GEF, Tiam 1 and the Arf-GEF, Grp1 (Ferguson et al., 2000; Fleming et al., 2000). These lipid-PH domain interactions can promote membrane translocation and, in addition, may directly affect the conformation and hence activity state of their host proteins

(Fleming et al., 2000; Milburn et al., 2003). As noted above, stimulation of Type I PI 3-kinases usually causes the accumulation of both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, the latter because of the activities of one or more phosphoinositide 5-phosphatases. Several PH domains interact with both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (Ferguson et al., 2000; Lemmon and Ferguson, 2000), whereas the PH domain of TAPP1 binds only PtdIns(3,4)P_2 with high affinity (Thomas et al., 2001). By contrast, the PH domains of Grp1 and Btk are selective for PtdIns(3,4,5)P_3, at least in vitro (Ferguson et al., 2000; Lemmon and Ferguson, 2000).

Given the importance of PtdIns(3,4,5)P_3 in cell biology and disease, it is highly desirable to have reliable methods for the detection, localization and quantitation of this lipid in cells at high resolution. In this regard, green fluorescent protein (GFP)-PH domain fusions have proved particularly useful as probes for their cognate lipid ligands in cell-based experiments. For example, the PH domains of PKB, Grp1, Btk and TAPP1 all translocate to plasma membranes following growth factor receptor-mediated activation of PI 3-kinases or exposure to oxidative stress. These responses mirror the accumulation of PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 as determined biochemically, thus confirming that the specificity of these probes is retained in vivo (Gray, 1999; Kimber et al., 2002; Nore et al., 2000; Watt et al., 2004). In some cases, these approaches have implied the production of 3-phosphoinositides at spatially restricted sites, such as at the leading edge of Dictyostelium during chemotaxis (Huang et al., 2003) and in specific membrane loci in response to insulin-stimulated actin remodeling (Patel et al., 2003). Such studies, however, are incapable of resolving whether the detected lipid is at the plasma membrane itself or in sub-plasma membrane compartments, nor have they provided direct evidence for the occurrence of PtdIns(3,4,5)P_3 in intracellular membranes.

Despite this, pharmocological, molecular genetic and immunohistochemical evidence suggests PtdIns(3,4,5)P_3 is unlikely to be restricted to the plasma membrane. PI 3-kinase inhibitors have implicated 3-phosphoinositides in a wide range of membrane trafficking processes and Type I PI 3-kinases appear to be required for autophagy and possibly exocytosis (Gozuacik and Kimchi, 2004; Windmiller and Backer, 2003). PI 3-kinases have also recently been found in the nucleus along with a novel PI 3-kinase activator protein called PIKE (PI 3-kinase Enhancer) (Irvine, 2003; Martelli et al., 2004; Ye, 2006; Ye et al., 2000). Since nuclei contain a source of PtdIns(4,5)P_2 (Watt et al., 2002), these observations collectively suggest this organelle has the capacity for regulated synthesis of PtdIns(3,4,5)P_3. Enzymes that metabolize PtdIns(3,4,5)P_3 have also been identified at multiple intracellular compartments. These include a 3-phosphatase (TPIP) (Walker et al., 2001) and two distinct 5-phosphatases (SKIP and a 72 kDa enzyme) in membranes of the early secretory pathway (Gurung et al., 2003; Kong et al., 2000), which perhaps suggest the presence of PtdIns(3,4,5)P_3 in the endoplasmic reticulum (ER) and the Golgi complex.

Increasingly strong evidence suggests that endocytosed growth factor receptors are capable of recruiting signaling components including the regulatory subunit of PI 3-kinase (Wang et al., 2004), but until recently there was no evidence to establish that PtdIns(3,4,5)P_3 itself is present in the endosome compartment. More recently, however, Sato and colleagues described a fluorescent indicator for PtdIns(3,4,5)P_3 that, when targeted to endomembranes, detected growth factor-simulated responses that could be distinguished temporally from the initial production of PtdIns(3,4,5P)P_3 at the plasma membrane (Sato et al., 2003). This important advance, however, lacks the desired spatial resolution to distinguish between endosomal compartments (such as ER and the Golgi complex) and did not report on the presence of PtdIns(3,4,5)P_3 at other subcellular locations. We recently described a low temperature on-section immunolabeling procedure that exploits the ligand-binding specificity of certain PH domains to map the subcellular distributions of PtdIns(4,5)P_2 and PtdIns(3,4)P_2 (Watt et al., 2004; Watt et al., 2002). These lipids, which are, respectively, the substrate of Type I PI 3-kinases and one of the products of PtdIns(3,4,5)P_3 metabolism, were found at multiple intracellular locations. We have now applied this approach to map the distribution of PtdIns(3,4,5)P_3 itself using the PH domain of Grp1 as a selective probe. We report the occurrence of substantial, agonist-stimulatable pools of PtdIns(3,4,5)P_3 in the plasma membrane and nucleus with relatively very little of this lipid being present at intracellular membranes. Only the plasma membrane pool was significantly reduced upon overexpression of the tumour suppressor phosphatase, PTEN, implying that distinct regulatory mechanisms control the turnover of PtdIns(3,4,5)P_3 in these different compartments.

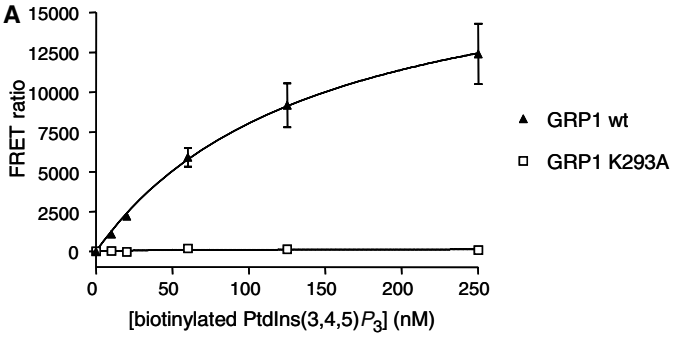
Results

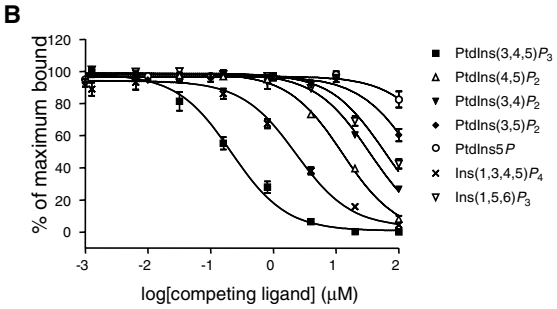
The PH domain of Grp1 is a sensitive and selective probe for PtdIns(3,4,5)P_3 present in cell membranes

We made use of the GST-tagged PH domain of Grp1 as a probe reported to have high specificity for PtdIns(3,4,5)P_3. This protein, as well as a negative control mutated PH domain (K273A), was expressed in *Escherichia coli*, purified and its ligand-binding specificity analysed in detail using a novel fluorescence resonance energy transfer (FRET)-based displacement assay (Gray et al., 2003) (Fig. 1). This revealed that short chain synthetic PtdIns(3,4,5)P_3 binds to GST-Grp1-PH with an affinity approximately 60-fold higher than that for PtdIns(4,5)P_2 and with even greater selectivity when compared with PtdIns(3,4)P_2 and other naturally occurring, low-abundance phosphoinositides, compatible with results using crude protein-lipid overlay assays (Ferguson et al., 2000; Klarlund et al., 2000). The mutated PH domain (K273A) did not bind to any of the phosphoinositides analysed. Interestingly, soluble inositol phosphates, including inositol 1,3,4,5-tetrakisphosphate [the headgroup of PtdIns(3,4,5)P_3] bound with significantly lower affinities than their respective lipid counterparts, suggesting that the glycerol moiety and/or acyl chains play some part in the binding of ligands to this PH domain. These results suggest that GST-Grp1-PH may be a suitable probe for the ultrastructural analysis of PtdIns(3,4,5)P_3 by quantitative immunoelectron microscopy when used in conjunction with the K273A mutant as a negative control.

Growth factors stimulate rapid accumulation of PtdIns(3,4,5)P_3 at multiple intracellular sites

Ultrastructural mapping of PtdIns(3,4,5)P_3 distribution was undertaken using purified, recombinant GST-Grp1-PH and applying the on-section labeling and immunogold electron microscopy approach we described recently for localisation of





C

	K_I (μM)
K_d for biotin-PIP_3 binding	0.14
PtdIns(3,4,5)P_3	0.14
PtdIns(4,5)P_2	8.07
PtdIns(3,4)P_2	21.73
PtdIns(3,5)P_2	>100
PtdIns5P	>100
Ins(1,3,4,5)P_4	1.46
Ins(1,5,6)P_3	37.66

Fig. 1. Analysis of GST-Grp1-PH binding by time-resolved FRET. (A) FRET saturation-binding isotherms. Protein (20 nM) was incubated with increasing concentrations of biotinylated PtdIns(3,4,5)P_3. (B) IC_{50}s of binding were measured by incubating FRET complexes containing 80 nM GST-Grp1-PH with increasing concentrations of the indicated unlabeled ligands. Data are presented as a mean of three independent experiments ± s.e.m. Values measured from A and B were used to calculate K_i values (see Materials and Methods) shown in C.

PtdIns(4,5)P_2 (Watt et al., 2002) and subsequently PtdIns(3,4)P_2 (Watt et al., 2004). The method employs thawed ultrathin cryosections prepared using aldehyde-fixed cells. Fig. 2 shows a montage of micrographs of control and platelet-derived growth factor (PDGF)-stimulated Swiss 3T3 cells that illustrates the range of cellular structures specifically labeled by the probe as indicated by the occurrence of electron-dense gold particles. The labeling of all compartments was substantially greater in the stimulated cells. As expected, in stimulated cells the plasma membrane was always prominently labeled, as were lamellipodia which are actin-rich plasma membrane protrusions that form rapidly in response to growth factor-stimulated activation of PI 3-kinase. Surprisingly, substantial labeling was observed also in cell nuclei, especially in growth factor-stimulated cells. Importantly, there was very limited labeling of the nuclear membrane, similar to the level observed in the ER, as most of the probe was observed in the nuclear matrix. The probe is specific for PtdIns(3,4,5)P_3 in this context because the signals were greatly stimulated by PDGF, blocked by PI 3-kinase inhibitors such as wortmannin and PI103 (data not shown) and were reduced to control levels when the K273A mutant PH domain was used (Fig. 4). Fig. 2 also shows representative examples of intracellular membranes, including the internal vesicles of a multivesicular body (MVB), Golgi stack and caveolae-like structures, all of which showed detectable labeling with the PtdIns(3,4,5)P_3-specific probe.

Fig. 3 presents a comprehensive quantitative analysis of the results illustrated by the micrographs both in terms of the proportion of the labeled PtdIns(3,4,5)P_3 observed in each intracellular compartment and in terms of the labeling density (a measure of relative concentration) in each location. Remarkably, these data show that the majority of the PtdIns(3,4,5)P_3 identified by this approach is located in two compartments, with 35-50% being present in the plasma membrane and 20-30% in the nuclear matrix (Fig. 3A). No other membrane-bound compartment exceeded 5% of the total label detected. None of these proportions changed significantly as a consequence of stimulation with PDGF for 0.5 to 30 minutes. Between 10 and 20% of the label outside of the nucleus was not associated with membrane-bound structures and is designated as cytosolic labeling. This reduced significantly after stimulation with PDGF, but this is unlikely to be because of translocation of PtdIns(3,4,5)P_3 since it merely reflects the fact that the density of labeling in the cytosol remained relatively constant whereas that in the other compartments increased substantially after stimulation (Fig. 3B).

The labeling-density measurements (Fig. 3B) also show that PtdIns(3,4,5)P_3 is concentrated in the plasma membrane. PDGF stimulated a rapid increase in plasma membrane PtdIns(3,4,5)P_3, which peaked at 15-fold over basal at 5 minutes and was still evident, though significantly reduced, for up to 30 minutes. Interestingly, PtdIns(3,4,5)P_3 appeared to be evenly distributed throughout the plasma membrane and the time-course and density of labeling in lamellipodia, a known site of PI 3-kinase signaling (Sossey-Alaoui et al., 2005), was similar to the bulk of the plasma membrane. This is consistent with previous data for PtdIns(4,5)P_2 (van Rheenen et al., 2005; Watt et al., 2002) and with the expected high rates of lateral diffusion of bilayer phospholipids, but does not support the idea that lipid signaling may be compartmentalized in discrete non-bilayer structures such as lipid rafts (Pike and Casey, 1996).

PDGF also stimulated detectable accumulation of PtdIns(3,4,5)P_3 in mitochondria and membranes of the endosomal system, including internal vesicles of MVBs,

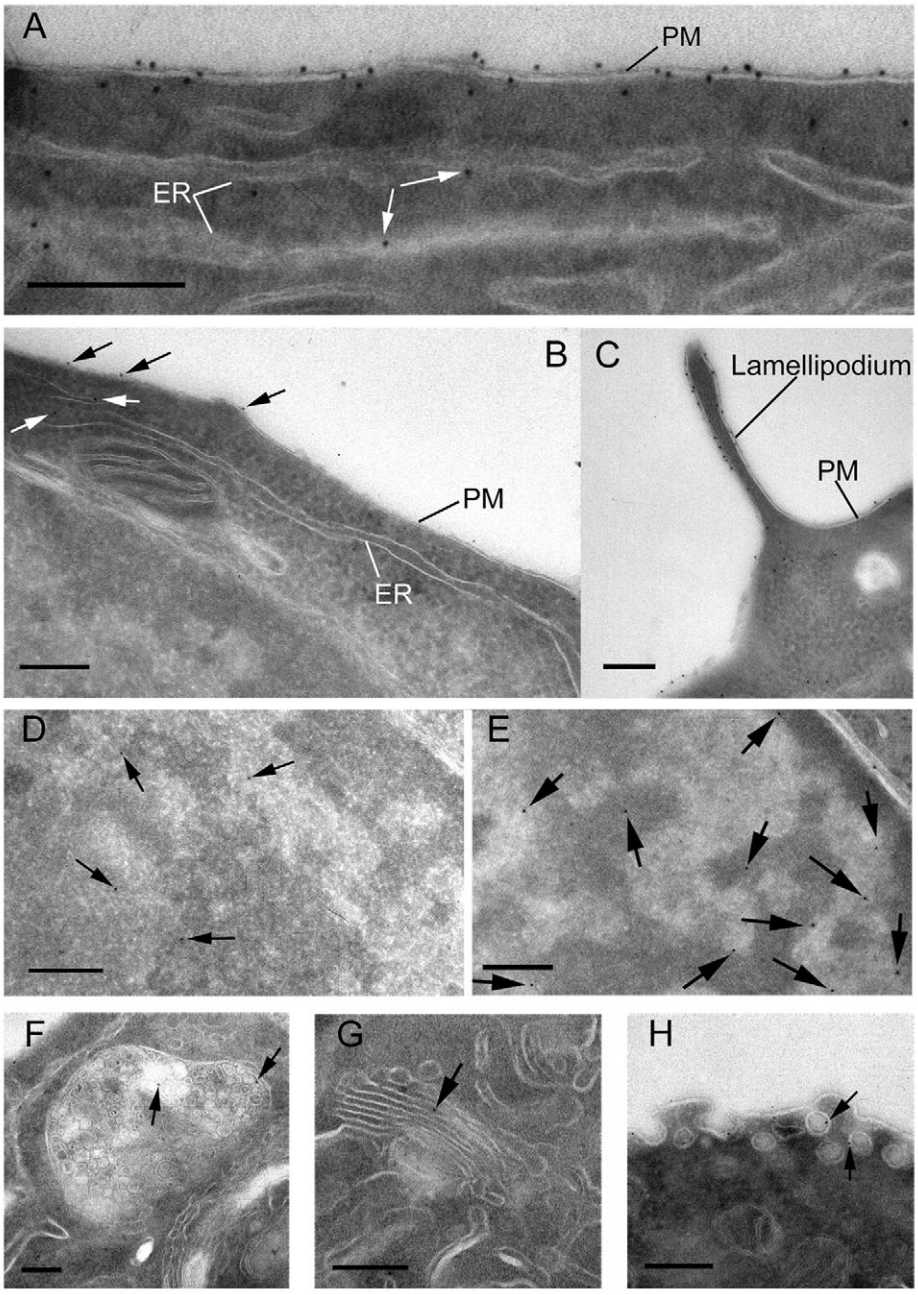


Fig. 2. GST-Grp1-PH labeling on ultrathin thawed cryosections of Swiss 3T3 cells. Ultrathin cryosections of cells stimulated with 50 ng/ml PDGF were labeled with GST-Grp1-PH as described in the Materials and Methods. GST-Grp1-PH immunogold labeling, indicated by arrows, is visualised on the plasma membrane (PM) and endoplasmic reticulum (ER) of cells stimulated with PDGF for 5 minutes (A) and unstimulated cells (B). (C) GST-Grp1-PH labeling is located on a lamellipodium structure and plasma membrane (PM) of cells stimulated with PDGF for 2 minutes. Nuclear labeling in non-stimulated and stimulated (PDGF for 5 minutes) cells is shown in D and E, respectively. Arrows in F, G and H indicate gold labeling on internal vesicles of a MVB, Golgi stack and caveolae-like structures, respectively. Bars, 200 nm.

multilamellar endosomes (MLE), the ER and nuclear membranes with similar time-courses to the response observed in plasma membranes. A striking feature of the data, however, is the very small proportion and density of the PtdIns(3,4,5)P_3 at these locations. The low proportions, for example, imply either that relatively little of the cellular Type I PI 3-kinases are active at intracellular membranes and/or that PtdIns(3,4,5)P_3 phosphatases are especially active at these sites. More importantly, PtdIns(3,4,5)P_3-binding proteins are unlikely to translocate to membranes where the interfacial concentration of this lipid is relatively low, as indicated by the low densities observed at all intracellular membrane locations.

The nuclear pool of PtdIns(3,4,5)P_3 was also growth factor-sensitive and increased two- to threefold within two minutes of exposure to PDGF (Fig. 3B, inset). The time-course of nuclear PtdIns(3,4,5)P_3 accumulation is, surprisingly, very similar to that in the plasma membrane. To address the possibility that the nuclear pool is an artifact that results from 'smearing' of unfixed lipid during sectioning, we performed the following control experiment. Unstimulated Swiss 3T3 cells were first labeled by incubating them with protein-A–gold-conjugated bovine serum albumin (BSA), which is efficiently taken up into endosomes and functions as an electron-dense marker. Unlabeled cells that had been stimulated with PDGF for 5 minutes were then mixed with the unstimulated, labeled cells and the mixed cell population fixed, frozen and sectioned for analysis of PtdIns(3,4,5)P_3. Micrographs that contained both nuclei and visible endosomes were examined. All unstimulated cells (which were identified by characteristic clusters of gold particles in the endosomes) analysed in this way retained the conspicuously low levels of nuclear PtdIns(3,4,5)P_3 noted above, whereas the stimulated cells (which lacked endosomal gold clusters) all showed relatively high levels of nuclear PtdIns(3,4,5)P_3. Thus, nuclear PtdIns(3,4,5)P_3 faithfully follows the prefixation conditions and cannot be an artifact introduced by postfixation processing (supplementary material Fig. S1).

PTEN null glioblastoma cells also contain major plasma membrane and nuclear PtdIns(3,4,5)P_3 pools

In view of the fact that the tumour suppressor lipid phosphatase PTEN is frequently found in both the cytosol and nuclei of some cells (Chung et al., 2005; Liu et al., 2005) and that its

nuclear targeting correlates with downregulation of cyclin D1 and inhibition of MAP kinase phosphorylation (Chung and Eng, 2005), we analysed the distribution of PtdIns(3,4,5)P_3 in PTEN-null U87MG glioblastoma tumour cells. Fig. 4 shows that the subcellular distribution of PtdIns(3,4,5)P_3 in U87MG cells is very similar to that in Swiss 3T3 cells. Notably, in both cell types the major pools of PtdIns(3,4,5)P_3 were in the plasma membrane and the nuclear matrix. The plasma membrane pool could be further stimulated two- to threefold by PDGF, compatible with the relatively high basal levels of PtdIns(3,4,5)P_3 that are sufficient to stimulate phosphorylation of PKB (see Fig. 5E). The nuclear pool was also stimulated by PDGF in these cells similar to that which occurred in Swiss 3T3 cells. Fig. 4 also shows that the signals observed in both cell types could be inhibited by wortmannin and were greatly reduced when the point-mutated probe (K273A) that does not bind inositides in vitro was used instead of the wild-type PH-Grp1.

PTEN selectively metabolizes the plasma membrane pool of PtdIns(3,4,5)P_3

To assess the ability of PTEN to metabolize PtdIns(3,4,5)P_3 in distinct intracellular compartments we transfected PTEN-null U87MG cells with untagged (Fig. 5A,B) or GFP-tagged (Fig. 5C-E) PTEN constructs using a baculovirus expression system with 90-100% transfection efficiency. As expected, PTEN (but not a phosphatase-dead mutant) expression caused a substantial (60-70%) reduction in the PtdIns(3,4,5)P_3 signal observed in the plasma membrane of PDGF-stimulated cells. Surprisingly, however, the small ER pool and the large nuclear pool of PtdIns(3,4,5)P_3 proved to be resistant to PTEN expression. To address the possibility that nuclear PTEN expression was too low in these cells to affect the nuclear PtdIns(3,4,5)P_3, we engineered GFP-PTEN-nls which contains an additional nuclear localization signal and was strongly localized to nuclei (Fig. 5D). Importantly, this construct was less efficient at antagonizing PKB activation (Fig. 5E) but still failed to have any detectable effect on the nuclear PtdIns(3,4,5)P_3 pool, even when sufficiently overexpressed so that it did affect the plasma membrane pool. This also confirms that the nuclear pool does not arise from the plasma membrane pool either as a 'smearing' artifact or through vesicle transport, as described for axonal transport in hippocampal neurons (Horiguchi et al., 2006).

Discussion

In order to map the distribution of PtdIns(3,4,5)P_3 within cells at an ultrastructural level of resolution, we have used an on-section labeling approach in conjunction with GST-PH-Grp1 as a highly specific probe. This approach has two key advantages over previous work that used GFP-tagged PH domains in intact cells. First, it overcomes the limited resolution of the latter technique. Second, GFP technology uses overexpression of the PH domain in the cytosol, which may alter cell function, either by sequestering lipid or by promoting and/or interfering with protein-protein interactions. An essential methodological consideration is that most lipids, including phosphoinositides, are unlikely to be immobilised by conventional aldehyde-fixation methods. We have shown previously that it is vital to perform the handling and labeling procedures at low temperatures, which prevents the diffusion

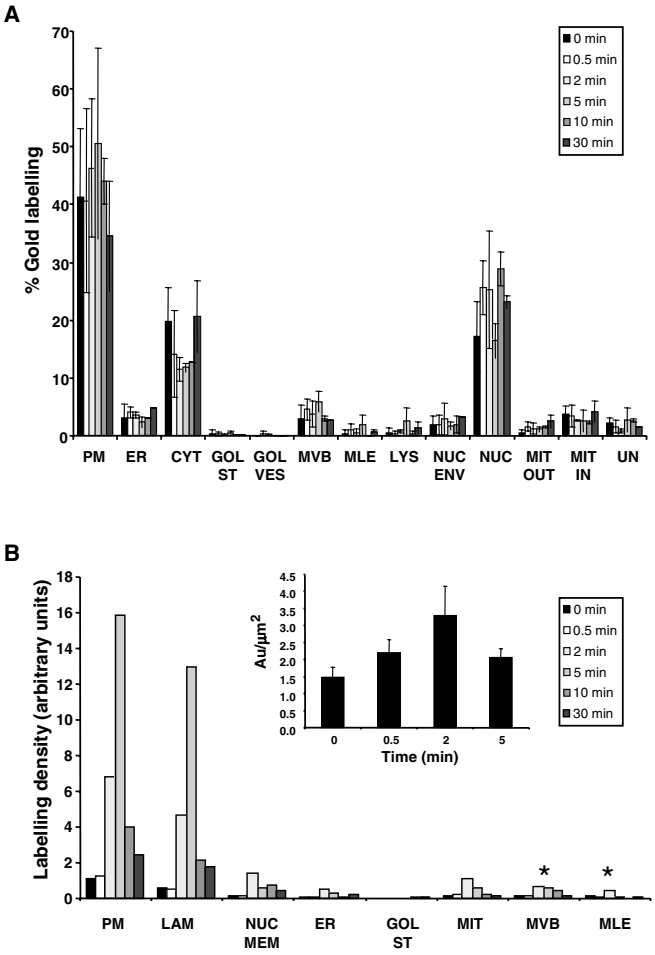


Fig. 3. Quantification of GST-Grp1-PH labeling over cellular membranes after PDGF stimulation. Ultrathin cryosections of PDGF-stimulated Swiss 3T3 cells were labeled with GST-Grp1-PH and the labeling over cellular membranes was quantified as described previously (Watt et al., 2002). The proportions of GST-Grp1-PH labeling over individual compartments by on-section labeling are shown in A as percentages of the total gold particles counted, estimated by scanning cell profiles and assigning gold particles to compartments. The results are the means for three independent experiments, in which 1350-2100 gold particles were counted per experiment. The labeling densities over membrane compartments are shown in B, in which the number of gold particles were related to the number of intersections a scanning line makes with membranes from each compartment, as described by Watt et al. (Watt, et al., 2002). The results shown are one representative data set from three individual experiments, in which 839-1643 intersections and 1598-2643 gold particles were counted per experiment. Nuclear densities from a representative experiment, expressed as the number of gold particles related to area, are shown in the inset. Data are the means from an experiment in which 1342 gold particles were counted. Asterisks over the MVB and MLE columns indicate that the data were derived using intersections through the outer membrane and adjusted appropriately, as described (Watt et al., 2004). Error bars in A and B represent s.e.m. CYT, cytosol; ER, endoplasmic reticulum; GOL ST, Golgi stack; GOL VES, Golgi vesicles; LAM, lamellipodia; LYS, lysosome; MIT IN, mitochondria inner membrane; MLE, multilamellar body; MVB, multivesicular body; MIT OUT, mitochondria outer membrane; NUC, nucleus; NUC ENV, nuclear envelope; PM, plasma membrane; UN, unassigned (gold particles associated with indistinct structures).

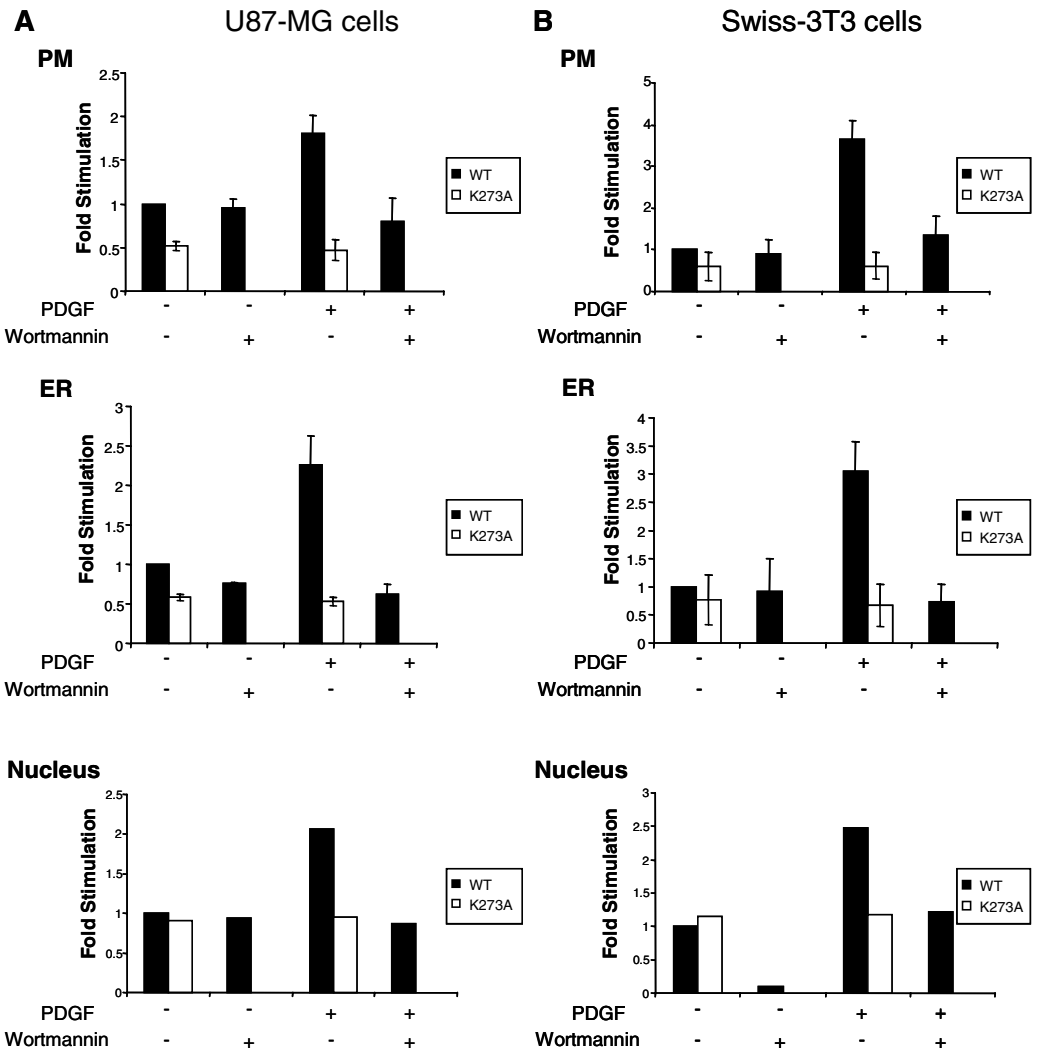


Fig. 4. GST-Grp1-PH [WT] and GST-Grp1-PH mutant [K273A] labeling in thawed cryosections of U87MG and Swiss 3T3 cells. U87MG cells (A) and Swiss 3T3 cells (B) were either treated with PDGF 50 ng/ml for 10 minutes or left unstimulated in the presence or absence of wortmannin, as indicated. The labeling densities for GST-Grp1-PH [WT] or the mutant PH domain [K273A] over the plasma membrane (PM), endoplasmic reticulum (ER) and nuclear area (nucleus) are shown. Labeling densities were calculated as golds per intersection (PM, ER) or golds per test point (nucleus), determined as described in the Materials and Methods. Data shown are expressed as fold-stimulation relative to unstimulated control cells. Results are the means of three experiments. Error bars represent s.e.m.

of lipids (Watt et al., 2002). The specificity of the probe was confirmed against a wide variety of potential ligands. Evidence that the probe detects specifically PtdIns(3,4,5)P_3 in the context of on-section labeling includes the fact that the level of the signal in the majority of compartments analysed was greatly enhanced by treatment with PDGF, was sensitive to PI 3-kinase inhibitors and was reduced when a phosphoinositide non-binding mutant PH domain was used as a control.

Perhaps unsurprisingly, the plasma membrane was the major site of PtdIns(3,4,5)P_3 accumulation, especially in stimulated and/or PTEN null cells. The probe was evenly distributed throughout the plasma membrane and its concentration in lamellipodia, known sites of PI 3-kinase signaling, was not detectably different from that in the rest of the plasma membrane. This may reflect the fact that Swiss 3T3 cells are relatively non-polarized, since other studies have provided evidence for the production of PtdIns(3,4,5)P_3 at spatially restricted sites, such as at the leading edge of Dictyostelium during chemotaxis and in specific membrane loci in response to insulin-stimulated actin remodeling (Huang et al., 2003; Patel et al., 2003). These differences may reflect the cell and/or receptor types involved. Alternatively, it is possible that such structures and hence focal localization of lipid signals is somehow lost during the preparation and/or decoration of cryosections.

More importantly, the proportion of PtdIns(3,4,5)P_3 and its density was extremely low in all intracellular membranes examined. A previous report that used a FRET-based probe for PtdIns(3,4,5)P_3 that was targeted to distinct compartments when expressed in live cells showed that PtdIns(3,4,5)P_3 accumulates rapidly in the plasma membrane in response to stimulation with PDGF, and then, after a short delay of approximately 1 minute, in endomembranes. Further work established that PtdIns(3,4,5)P_3 was generated in situ within

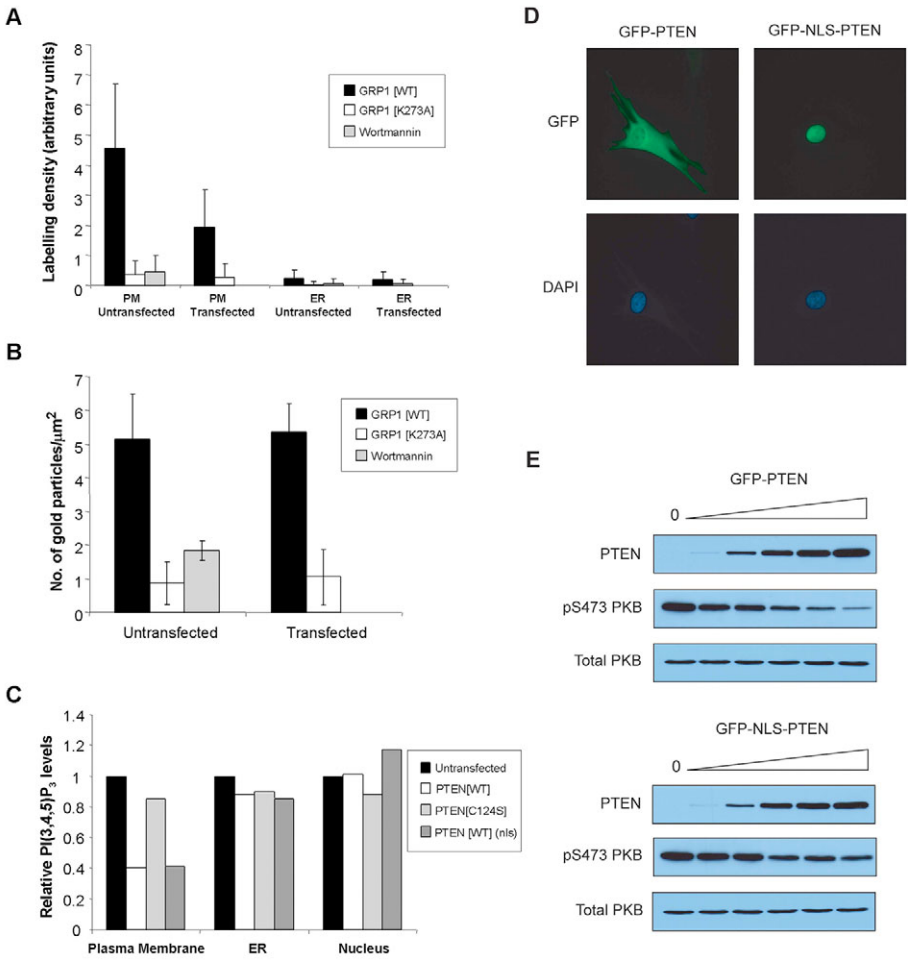


Fig. 5. Effect of exogenous PTEN expression on GST-Grp1-PH labeling density after PDGF stimulation in U87MG cells. Ultrathin cryosections of both untransfected and GFP-PTEN-transfected U87MG cells, stimulated with 50 ng/ml PDGF for 10 minutes, were labeled with GST-Grp1-PH. (A) The labeling density over the plasma membrane and endoplasmic recticulum (ER) compartments in cells expressing PTEN [WT] is shown (densities obtained as described in Fig. 2 legend). (B) Nuclear GST-Grp1-PH labeling of cells transfected with PTEN [WT] is expressed as the number of gold particles related to area. Results are the means from a single experiment in which 616 intersections (PM and ER) or 1614 gold particles (nucleus) were counted. Error bars in A and B represent s.e.m. (C) The GST-Grp1-PH labeling for plasma membrane, ER and nuclear compartments in cells transfected with various PTEN constructs (WT; C124S phosphatase dead; or PTEN nls, containing an engineered nuclear localization signal). Data are normalized labeling densities from a representative data set of two similar experiments. (D) The subcellular localization of GFP-PTEN and GFP-NLS-PTEN in U87MG cells. Whereas GFP-PTEN is observed throughout the cytoplasm and nucleus, GFP-NLS-PTEN appears to be exclusively localized to the nucleus. (E) The effects of increasing expression of GFP-PTEN and GFP-NLS-PTEN on pS473-PKB phosphorylation in U87MG cells. The expression of each construct was titrated by adding increasing volumes of baculovirus supernatant to cells (0-1.0 ml in 10 ml of medium). After 24 hours, cells were lysed and the PTEN expression and pS473-PKB phosphorylation analysed by western blotting. The blots shown are representative of three independent experiments.

the endomembrane compartment and required clathrin-mediated endocytosis of the PDGF receptor (Sato et al., 2003). Several other lines of evidence have emphasized the likely importance of persistent signaling by endocytosed growth factor receptors (Gonzalez-Gaitan, 2003; Miaczynska et al., 2004; Wang et al., 2004). Our data are compatible with these conclusions, but suggest that, in quantitative terms, PI 3-kinase signaling in endosomes (or other compartments identified in these studies) is negligible by comparison with either the plasma membrane or indeed, the nucleus (see below).

The nuclear pool of PtdIns(3,4,5)P_3 identified in this study is remarkable for two reasons. The first is that the signal was observed in the nuclear matrix in the absence of any discernible membrane structures. Its distribution was far from random, however, and appeared to be concentrated towards the edges of electron-dense regions of heterochromatin. This observation is similar to previous work that identified non-membrane pools of PtdIns(4,5)P_2 in the nuclear matrix, much of which is associated with electron-dense nuclear speckles involved in mRNA processing (Irvine, 2003; Martelli et al., 2004; Osborne et al., 2001). The second is that, in quantitative terms, the nuclear matrix appears to be a major site for the accumulation of this lipid signal. There is a substantial body of indirect evidence suggesting important roles for Type I PI 3-kinase-dependent signaling in the nucleus, including effects on chromatin structure, pre-mRNA splicing, cell cycle progression and nuclear responses to DNA damage (Deleris et al., 2006). A previous report claimed to detect radioloabelled PtdIns(3,4,5)P_3 biochemically in isolated nuclei (Neri et al., 2002), but our data are the first to provide direct evidence that the nuclear matrix is a major site of PtdIns(3,4,5)P_3 production. As this response was completely blocked by wortmannin (Fig. 5B), it is not a product of the nuclear inositol polyphosphate multikinase that has been reported to exhibit wortmannin-insensitive PI 3-kinase activity (Resnick et al., 2005). It is, however, compatible with the well-documented intra-nuclear occurrence of Type I PI 3-kinases (Kim, 1998; Lu et al., 1998; Martelli et al., 2000; Metjian et al., 1999), a regulatory GTPase called PIKE (Ye, 2006), PtdIns(3,4,5)P_3-specific phosphatases, SHIP2 and PTEN (Deleris et al., 2003), as well as the substrate lipid, PtdIns(4,5)P_2.

The potential role of PTEN in the nucleus is currently the subject of intensive research. Our results show that nuclear sequestration of PTEN reduces its ability to antagonize PKB-dependent signaling and suggest that positive nuclear functions of PTEN are likely to be PtdIns(3,4,5)P_3 independent. They

also suggest either that PtdIns(3,4,5)P_3 itself must be exported from nuclei in order to be metabolized efficiently or that other enzymes, such as members of the 5-phosphatase family, must perform this function in nuclei. Note, however, that in a previous study we did not detect the expected 5-phosphatase product [PtdIns(3,4)P_2] in the nuclear matrix (Watt et al., 2004). The results may also suggest that PTEN is only active as a lipid phosphatase when targeted to plasma membranes. Indeed, PTEN has been shown to bind plasma membranes when in the open conformation that results from dephosphorylation of the C-terminal tail (Das et al., 2003) and this targeting also appears to require an N-terminal acidic lipid-binding site (Vazquez et al., 2006). We have also shown that the activity state of PTEN is highly sensitive to the composition of substrate-containing lipid vesicles, with high activity requiring an acidic character like that of the inner leaflet of the plasma membrane (McConnachie et al., 2003).

In summary, we report the first ultrastructural analysis of the subcellular distribution of the key lipid second messenger, PtdIns(3,4,5)P_3. Rapid, agonist-dependent synthesis of PtdIns(3,4,5)P_3 occurs in two major cellular compartments, the plasma membrane and the nuclear matrix. There is no evidence for the focal accumulation of this lipid within membrane microdomains in Swiss 3T3 cells, and intracellular membranes accounted for no more than 10-20% of the signal observed. Importantly, at least in Swiss 3T3 cells by this analysis, endosomes do not appear to be important sites of PI 3-kinase signaling. A similar distribution of PtdIns(3,4,5)P_3 was seen in PTEN null glioblastoma cells, including a substantial nuclear pool that was resistant to PTEN re-expression, showing that these lipid pools are metabolically segregated and suggesting that nuclear targeting of PTEN has some function other than to antagonize PI 3-kinase signaling in the nucleus.

Materials and Methods

U87MG cells and mouse fibroblast Swiss 3T3 cells were obtained from the European Cell Culture Collection. Cell culture reagents were all obtained from Life Technologies.

Anti-GST antibody was obtained from Chemicon International (Temecula, CA, USA), anti-PTEN-A2B1 antibody from Santa Cruz, and anti-pS473-PKB and anti-total-PKB antibodies from Cell Signalling Technology.

Expression and purification of GFP-fusion proteins

The Grp1 PH domain (amino acids 263-380) was PCR cloned from a mouse brain cDNA library (Stratagene) as described previously (Gray et al., 1999) and expressed as a GST-fusion protein from the vector pGEX 4T1 (Amersham Pharmacia). The expression vector pGEX 4T1 Grp1-PH K273A was produced by PCR mutagenesis using the oligonucleotide K273A-S 5′-gaaggctggctgctggcgctgggggtctggtg-3′ and its reverse complement. These proteins were expressed in *E. coli* BL21 cells and affinity purified on glutathione-Sepharose 4B (Amersham Pharmacia) using the manufacturer's standard protocols.

Assessment of protein-lipid binding specificity

To assess the phosphoinositide-binding properties of the GST-fusion proteins a protein lipid overlay assay was performed as described previously using dipalmitoylphosphoinositides (Cell Signals, Columbus, OH, USA) (Dowler et al., 2000). A more quantitative assessment of lipid-binding specificity was obtained using the time-resolved-fluorescence resonance energy transfer (TR-FRET) method described previously (Gray et al., 2003). K_i values were determined for competing ligands using the relationship defined by Cheng and Prusoff (Cheng and Prusoff, 1973).

Cell culture, stimulation and transfection

U87MG cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1× non-essential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin. Mouse fibroblast Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. For stimulation of cells with PDGF, the cells were grown to approximately 80% confluency on 10-cm diameter plastic culture dishes. The cells were washed in KREBS (modified) buffer and incubated for 1 hour in KREBS at 37°C. Recombinant rat PDGF (B-chain homodimer; Sigma) was then added directly to the buffer at a final concentration of 50 ng/ml and the incubation was continued for up to 30 minutes, as required. Where indicated, cells were pre-treated for 30 minutes with 100 nM wortmannin (Sigma), prior to stimulation. After the desired period of stimulation, the medium was removed and cells were fixed by addition of 2% (v/v) glutaraldehyde (Agar Scientific, Stansted, UK) in 0.2 M Pipes, pH 7.2, for 1 hour at 4°C.

Expression vectors encoding untagged PTEN and GFP-PTEN (GFP-PTEN$^{[WT]}$ and GFP-PTEN$^{[C124S]}$), have been previously described (Leslie et al., 2001). GFP-PTEN$^{[WT]}$NLS (nuclear localization signal) was produced by introducing three copies of the SV40 large T antigen NLS between the GFP and PTEN, by PCR cloning from the vector pEF-myc-Nuc (Invitrogen). Immunoprecipitated GFP-NLS-PTEN had very similar enzymatic activity to GFP-PTEN when assayed in vitro against Ins(1,3,4,5)P_4. PTEN proteins were expressed in U87MG cells growing at low density (<70% confluency) using a baculovirus delivery system adapted for mammalian expression as described in Leslie et al. (Leslie et al., 2001). At 24 hours after plating, viral supernatants were added to the culture medium, up to 1 ml of viral supernatant to 10 ml of medium. Where indicated, cells were treated with PDGF, as described above, after confirmation of PTEN expression.

Cell lysis and western blotting

U87MG cells were seeded into 100 mm dishes and transfected with GFP-PTEN or GFP-NLS-PTEN baculovirus supernatants, using up to 1 ml of each in 10 ml of medium. After 24 hours, the cells were washed with chilled PBS and lysed on ice in NP-40-based lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8.0), 1 mM sodium β-glycerophosphate, 1 mM sodium orthophosphate, 10% glycerol, 0.1% NP-40] containing complete protease inhibitors (Roche). Lysates were clarified by centrifugation at 20,000 ***g*** for 10 minutes at 4°C, and protein concentrations were determined by the Bradford assay using BSA as standard. An amount of each sample (10 μg) was resolved by SDS-PAGE on 10% Tris-glycine gels and transferred to PVDF membrane for western blotting by standard protocols. PTEN, total PKB and pS473-PKB antibodies were all used at a 1:500 dilution.

Fluorescence microscopy

U87MG cells were seeded onto glass coverslips in 35 mm dishes and transfected with 100 μl of GFP-PTEN or GFP-NLS-PTEN baculovirus supernatant in 1 ml of medium. After 24 hours, the cells were washed with PBS and fixed with 3% paraformaldehyde for 15 minutes at room temperature. Coverslips were washed with PBS and nuclei were counterstained with 0.5 μM DAPI for 5 minutes. The coverslips were then mounted with hydromount (National Diagnostic Laboratories) and examined using a Leica inverted fluorescence microscope with a Hammamatsu ORCA-ER charge coupled device (CCD) camera attached. Images were exported to Adobe Photoshop.

On-section labeling and quantification of PtdIns(3,4,5)P_3 using GST-Grp1

Cell fixation, cryosectioning and labeling for immunoelectron microscopy were performed as described previously (Watt et al., 2002), but using recombinant GST-Grp1 for the specific detection of PtdIns(3,4,5)P_3. Determination of the proportions and densities of gold labeling over cell membranes was achieved using the grid-scanning technique described previously (Lucocq, 1994).

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