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A Novel HPLC-Based Approach Makes Possible the Spatial Characterization of Cellular PtdIns5P and Other Phosphoinositides

Deborah Sarkes and Lucia E. Rameh*

Boston Biomedical Research Institute, 64 Grove Street, Watertown, MA 02472, USA

SYNOPSIS

Phosphatidylinositol-5-phosphate (PtdIns5P) was discovered in 1997 (Rameh, L.E.; Tolias, K.F.; Duckworth, B.C. and Cantley, L.C. 1997, *Nature* 390, 192–196) but very little is known about its regulation and function. Hitherto, studies of PtdIns5P regulation have been hindered by the inability to measure cellular PtdIns5P using conventional HPLC, owing to poor separation from PtdIns4P. Here we present a new HPLC method for resolving PtdIns5P from PtdIns4P, which makes possible accurate measurements of basal and inducible levels of cellular PtdIns5P in the context of other phosphoinositides. Using this new method, we found that PtdIns5P is constitutively present in all cells examined (epithelial cells, fibroblasts and myoblasts, among others) at levels typically 1% to 2% of PtdIns4P levels. In the β -pancreatic cell line BTC6, which is specialized in insulin secretion, PtdIns5P levels were higher than in most cells (2.5% to 4% of PtdIns4P). Using subcellular fractionation, we found that the majority of the basal PtdIns5P is present in the plasma membrane, but it is also enriched in intracellular membrane compartments, especially in smooth endoplasmic reticulum (SER) and/or Golgi, where high levels of PtdIns3P were also detected. Unlike PtdIns3P, PtdIns5P was also found in fractions containing very low-density vesicles. Knockdown of PtdIns5P 4-kinase (PIP4k) leads to accumulation of PtdIns5P in light fractions and fractions enriched in SER/Golgi, while treatment with Brefeldin A results in a subtle, but reproducible, change in PtdIns5P distribution. These results indicate that basal PtdIns5P and the PtdIns5P pathway for PtdIns(4,5)P₂ synthesis may play a role in Golgi-mediated vesicle trafficking.

Keywords

phosphoinositides; PtdIns5P; PtdIns5P 4-kinases; HPLC; subcellular fractionation; vesicle transport

INTRODUCTION

Phosphoinositides (PIs) have long been known to participate in basal cellular functions such as vesicle transport and cytoskeleton dynamics, as well as responses triggered by extracellular cues including proliferation, differentiation and chemotaxis [1]. While phosphatidylinositol-4-phosphate (PtdIns4P) and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) are abundant in cells, the phosphoinositide 3-kinase (PI3k) lipid products phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P₂) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) are virtually absent in quiescent cells, but can be rapidly stimulated by extracellular factors.

*To whom correspondence should be addressed: Lucia E. Rameh, rameh@bbri.org, (617) 658-7777 (phone), (617) 972-1761 (fax).

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D.S. and L.E.R. designed, performed and analyzed the experiments. L.E.R. conceived the project and wrote the manuscript.

Phosphatidylinositol-3-phosphate (PtdIns3P), on the other hand, is constitutively present in eukaryotic cells, but is also regulated in specific subcellular locations.

Phosphatidylinositol-5-phosphate (PtdIns5P) is the last member of the PI family to be discovered and very little is known about its regulation and function [2]. Like PI3k lipid products, PtdIns5P levels are low in abundance, but can be up-regulated by extracellular stimuli. PtdIns5P levels increase in response to stress signals [3], insulin [4] or T cell receptor stimulation [5], after thrombin-stimulated platelet aggregation [6] or during cell cycle progression [7].

Cellular PtdIns5P was also shown to increase during bacterial invasion due to the catalytic activity of the virulence factors IpgD from *Shigella flexneri* [8] or SigD/SopB from *Salmonella sp* [9], indicating that PtdIns5P may play a role in membrane and cytoskeleton events that facilitate pathogen invasion. Two new phosphatases capable of generating PtdIns5P have been recently identified; viz. PtdIns(4,5)P₂ 4-phosphatase types I and II, which are mammalian analogs of IpgD that can generate PtdIns5P from the dephosphorylation of PtdIns(4,5)P₂ *in vitro* [10]. PtdIns5P levels are negatively regulated by PIP4k (also known as PIPk type II), which are a family of 4-kinases that specifically use PtdIns5P as a substrate to generate PtdIns(4,5)P₂ [11]. A PTEN-related PI 5-phosphatase (PLIP) is also a potential negative regulator of cellular PtdIns5P [12,13].

Despite the identification of several enzymes involved in the regulation of PtdIns5P, many questions remain regarding the synthesis and degradation of basal and inducible cellular PtdIns5P. It is not clear yet whether PtdIns5P can only be generated by phosphatases or whether a PtdIns-specific 5-kinase exists. The role of different PIP4k isoforms on the regulation of basal or stimulated PtdIns5P is also unclear. PIP4k type II β , for instance, is present in the nucleus and is phosphorylated and inactivated in response to stress signals, leading to an increase in nuclear PtdIns5P [3,14–17]. This isoform interacts with the EGF and TNF α receptors [18, 19] and modulates early insulin responses [20], suggesting that PtdIns5P is also present at the plasma membrane. In addition, the type II α isoform translocates to the cytoskeleton in response to platelet aggregation [21]. Based on this evidence, many have suggested that different enzymes or cues regulate distinct subcellular pools of PtdIns5P [22]. However, the subcellular distribution of this lipid has never been fully examined.

PtdIns5P studies have been hindered by the inability to measure PtdIns5P levels using conventional HPLC, owing to poor separation from PtdIns4P. For this reason, most studies thus far have used an enzymatic assay based on the ability of PIP4k to use PtdIns5P as a substrate [6]. This approach, however, does not allow for measurements of PtdIns5P in the context of the other cellular PIs and is susceptible to interference by PIP4k inhibitors in the assay, such as its own product PtdIns(4,5)P₂.

Here we present a new HPLC method to measure cellular PtdIns5P levels in the context of the other PIs. This allows sensitive and accurate detection of basal PtdIns5P levels and changes in response to extracellular factors. Using this method, we found that all cells examined thus far have detectable basal levels of PtdIns5P, which are typically around 1% of that of PtdIns4P. The insulinoma cell line BTC6 had higher levels of PtdIns5P than other cells. Using cellular fractionation combined with HPLC measurements of PIs, we defined the subcellular localization of basal PtdIns5P in HeLa and BTC6 cells, which was previously impossible due to the lack of PtdIns5P-specific probes. This approach revealed that the majority of PtdIns5P resides in various intracellular vesicles and plasma membrane, but are particularly enriched in light microsomal and smooth endoplasmic reticulum (SER)/Golgi-containing fractions. PtdIns3P was also found to be specifically concentrated in SER/Golgi-enriched fractions, but in contrast to PtdIns5P, it was completely absent from light microsomal fractions.

Knockdown of PIP4ks resulted in accumulation of PtdIns5P in the Golgi-enriched fractions and Brefeldin A treatment resulted in the redistribution of PtdIns5P, indicating that PtdIns5P may play a role in Golgi-mediated intracellular trafficking.

MATERIALS AND METHODS

Cell lines, maintenance and manipulations

HeLa and BTC6 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Retroviruses carrying the pSuper. retro.puro shRNA vectors (OligoEngine) were generated by transiently transfecting 293T cells using Lipofectamine Plus (Invitrogen). Stable knockdown cells were generated by infection with pSuper. retro.puro-derived retrovirus carrying the target sequence for PIP4k II α , PIP4k II β , PIP4k II γ or a control sequence, which consisted of the target sequences with 4–6 bases mismatched (C1). Infected cells were selected by puromycin treatment.

Metabolic labeling of phosphoinositides

Cells were metabolically labeled with 200 μ Ci/ml inorganic [32 P] for 1.5–4 hrs in phosphate-free DMEM or with 10 μ Ci/ml [3 H]inositol for 24–72 hrs in inositol-free DMEM supplemented with dialyzed fetal calf serum (Gibco) and 200 mM L-glutamine.

Subcellular fractionation

Cells plated in 150 mm tissue culture dishes and labeled or not for the time indicated were washed in PBS, rinsed with cytosol buffer (0.2 M sucrose; 25 mM HEPES, pH 7; 125 mM potassium acetate; 1 mM dithiothreitol; 1 mM sodium orthovanadate; 2 mg/ml sodium fluoride; 2 mg/ml β -glycerophosphate; 1 mM phenanthroline; 1 mM benzamidine and protease inhibitor cocktail from Sigma) and scraped from the dish. The cell suspension was passed through a 29 1/2 gauge needle 12 times and centrifuged at 100 g for 10 min. The supernatant (microsomal fraction) was loaded on top of a discontinuous sucrose gradient (20–65% sucrose in cytosol buffer) and centrifuged for 4.5 hrs at 55,000 rpm in a TL100 centrifuge, using a TLS55 rotor (Beckman). Six fractions were collected from the top of the gradient and designated microsomal fractions 1 (lightest) through 6 (densest). The pellet containing unbroken cells, nuclei and associated membranes was re-suspended in cytosol buffer, frozen and thawed 3 times to break any remaining intact cells, and centrifuged at 500g for 10 min. The supernatant was designated fraction X. The pellet was re-suspended in cytosol buffer containing 1% Triton X100 and centrifuged at 14,000 g for 10 min to separate the pellet containing the nuclear fraction from the supernatant containing the Triton-soluble membranes. For lipid analysis, 2 M HCl was added to each fraction for a final concentration of 1 M and the lipids were extracted with 1:1 methanol: chloroform (vol:vol). For protein analysis, each fraction was mixed with SDS-containing loading buffer and analyzed by Western-blot.

HPLC method for phosphoinositide analysis

Cellular phosphoinositides were extracted and deacylated as described [23]. Deacylated lipids were separated by anionic-exchange HPLC (Agilent 1200) using two partisphere SAX columns (Whatman) in tandem and a four-step gradient of ammonium phosphate pH 6.0 (10 mM to 40 mM over 60 min; 40 mM to 150 mM over 5 min; 150 mM isocratic for 20 min and 150 mM to 650 mM over 25 min). Radiolabeled eluate was detected by an online flow scintillation analyzer (Perkin-Elmer) and quantified using ProFSA software (Perkin-Elmer).

Western blot analysis

Protein lysates were prepared from either intact cells or subcellular fractions. Total protein lysates were obtained by lysis in buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl,

1 mM EDTA, 1% Triton-X100 and 10% glycerol as well as protease and phosphatase inhibitors. Lysates were centrifuged at 14,000×g to remove the insoluble fraction and were normalized based on total protein content measured by Bradford Assay (BioRad). Total cellular protein or protein from various subcellular fractions were mixed with SDS-loading buffer, boiled 5 min and separated by 10% SDS-PAGE. The proteins were transferred onto nitrocellulose membrane, which was blocked with 5% milk dissolved in Tris-buffered saline (TBS) plus 1 mM sodium orthovanadate. Membranes were probed overnight with the appropriate primary antibody. After washing, membranes were incubated for 60 min with secondary antibodies conjugated to IR680 (Rockland and Molecular probes) or IR800 (Rockland). The membranes were washed in TBS-Tween and bound antibodies were detected and quantified using the Odyssey Infrared Imaging System (LICOR). Antibodies against β 1-integrin, GM130, ATP synthase, caveolin and tubulin were from BD Transduction Laboratories. Antibodies against S6-kinase, calnexin, phospho-histone and EEA1 were from Cell Signaling Technology. Antibodies against lamin A/C and Erk were from Santa Cruz, against β COP was from ABR, and against 58k Golgi was from Abcam. Antibodies against Rab5 were from BD Transduction Laboratories and from Cell Signaling Technology. Antibody against PIP4k II β was a gift from Dr. M. Chao and antibody against PIP4k II γ was generated in rabbits by injection of a GST-PIP4k II γ N-terminal fusion protein.

RESULTS

1) Separation and detection of cellular PtdIns5P using a novel HPLC method

In order to study PtdIns5P levels in cells, we developed a new HPLC method for separation of PtdIns5P from cellular PtdIns4P. Deacylated PIs from [3 H]inositol-labeled HeLa cells expressing the bacterial phosphatase IpgD were used to test various protocols, since they have elevated levels of PtdIns5P. Using a conventional anionic-exchange column and shallow ammonium phosphate gradient for the elution of phosphoinositides, the PtdIns5P peak eluted 0.5 to 1.0 min after the PtdIns4P peak, which was not sufficient for a complete separation of the PtdIns5P peak from the declining base of the much larger cellular PtdIns4P peak. Since decreasing the slope of the gradient had little or no effect on the separation of these two isomers, we decided to test whether increasing the bed volume of the column would improve separation. For this purpose, we attached two 250 mm Whatman Partisphere SAX columns in tandem, which improved the separation of PtdIns5P from PtdIns4P by one minute. We tested various pHs for ammonium phosphate and determined pH 6.0 to be the optimal pH for separation. The combination of the larger bed volume and higher pH resulted in a 4-minute separation between PtdIns4P and PtdIns5P and complete separation of the bases of the two peaks in cells expressing IpgD (Figure 1A). Using this new method we were also able to detect basal levels of PtdIns5P in control HeLa cells, without interference from the PtdIns4P peak (Figure 1B).

To validate this new HPLC method, we measured the levels of PtdIns5P in cells treated with insulin or hydrogen peroxide, which increase PtdIns5P levels as measured by enzymatic assay [4] [24]. In CHO-IR cells labeled with [32 P], insulin induced PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 (not shown) as expected, and also increased PtdIns5P levels 2.5 fold (Figure 1C) without affecting the levels of PtdIns4P or PtdIns(4,5) P_2 (not shown). In HeLa cells labeled with [3 H] inositol, basal PtdIns5P levels were around 1% of the levels of PtdIns4P (Figure 1B and Table I) and approximately 30% of the levels of PtdIns3P. Hydrogen peroxide treatment of HeLa cells significantly increased PtdIns5P levels, and to a lesser extent, PtdIns4P and PtdIns(4,5) P_2 levels (Figure 1D). PtdIns5P levels in hydrogen peroxide-treated cells were equal to or higher than PtdIns3P levels. After normalization with PtdIns4P, hydrogen peroxide treatment increased PtdIns5P by 2.5 fold (Figure 1D, inset). These results are comparable to the results obtained by other groups using the enzymatic assay for PtdIns5P detection [4,24] and

demonstrate that our new HPLC method allows us to consistently and accurately measure small levels of cellular PtdIns5P in the context of other PIs.

2) Basal PtdIns5P levels are present in various cell lines

Using this new HPLC method, we compared the levels of basal PtdIns5P in various cell lines labeled with [^3H]inositol. As PtdIns4P is one of the most abundant PIs and is constitutively present in all cells, we chose to normalize the data using PtdIns4P, in order to compare the levels of PtdIns5P between cell lines with different rates of PI metabolism. Table I shows that HeLa (human cervical cancer), CHO (Chinese hamster ovary), NIH-3T3 (mouse fibroblast) and 3T3-L6 (rat myoblast) cells have similar levels of PtdIns5P (around 1% of PtdIns4P), whether they were labeled in high or low serum media. In fact, from a panel of more than 20 cell lines including cancer cells and normal cell lines, PtdIns5P was detected in all cells examined at levels ranging from 0.5 to 2.0 % of PtdIns4P (table I and unpublished data). Therefore, unlike the PI3k lipid products, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, which are absent or below detection levels in most cells, PtdIns5P is constitutively present in all cells examined. Notably, the mouse insulinoma cell line BTC6 had higher levels of PtdIns5P than other cell lines tested (2.5 to 4% of PtdIns4P). In these cells the level of incorporation of [^3H]inositol into phosphatidylinositol (PtdIns) was similar to the level in HeLa, but the levels of [^3H]inositol incorporation into PtdIns4P and PtdIns(4,5)P₂ were significantly lower, likely owing to a slower PI turnover. For example, after 48 hours labeling, the levels of [^3H]PtdIns4P in BTC6 cells were half that of the levels in HeLa cells and the levels of [^3H]PtdIns(4,5)P₂ were 5 to 10 times lower, while [^3H]PtdIns-3,5-P₂ was completely undetectable. Notwithstanding, the levels of [^3H]-PtdIns5P in BTC6 cells were higher than in HeLa cells and did not correlate with the levels of [^3H]inositol labeling of PtdIns(4,5)P₂. This raises the possibility that basal PtdIns5P is generated from phosphorylation of PtdIns rather than dephosphorylation of PtdIns(4,5)P₂ or PtdIns(3,5)P₂ as previously suggested [25].

3) Subcellular distribution of phosphoinositides and protein markers

In order to investigate the distribution of PIs within the cell, we used this new HPLC method combined with biochemical fractionation of cellular organelles. HeLa and BTC6 cells were labeled with [^3H]inositol for 48–72 hours, then cell lysates were fractionated using differential centrifugation to generate a low spin pellet fraction (P1) and a supernatant fraction (S1). The P1 fraction was further fractionated into unbroken cells, nuclei and membranes associated with nuclei, while S1 was further fractionated by sucrose density gradient to generate six distinct fractions, as described in Methods. The protocol was designed to minimize loss of material, volume of samples, and time between lysis and lipid extraction. From each cellular lysate, nine fractions of equal volume were generated and analyzed for either PIs or protein content. As no portion of the lysate was wasted (there are no washes involved, for example) we were able to calculate the total content of each lysate by adding up the contents from each fraction. Figure 2A shows that in BTC6 cells, 72% of the cellular PIs (solid bars) were present in the supernatant (S1), where cytosol as well as microsomes are expected to be present, and 28% in the low spin pellet (P1). The majority of the PIs present in the low spin pellet were triton-soluble (23% out of 28%) and only 2% were triton-insoluble. In contrast, over 60% of the PIs from HeLa cells (Figure 2B) were in the triton-soluble P1 fraction, 9% were triton-insoluble and only 16% were present in the supernatant (S1 fraction).

The triton-soluble P1 fraction was rich in β 1-integrin (white bars), an integral membrane protein, and in calnexin (not shown), an endoplasmic reticulum (ER) protein. The nuclear proteins Lamin A/C and phospho-histone (grey bars) were most abundant in the triton-insoluble P1 fraction, as expected. These results confirm that the triton-soluble P1 fraction contains ER membranes associated with the nucleus, while the triton-insoluble fraction contains the nucleus per se. As expected, the cytosolic protein Erk (hatched bars) and the membrane protein β 1-

integrin (white bars) were found in the S1 fraction, confirming that S1 contains the microsomal and cytosolic portions. The contrasting distribution of PIs between S1 and P1 fractions in HeLa and BTC6 cells indicates that the nucleus-associated ER network is much more prominent in HeLa cells than in BTC6 cells.

Figure 3 shows the molecular characterization of the six fractions obtained after sucrose density gradient separation of the S1 fractions from HeLa (A, B and C) and BTC6 cells (D). Measurements of the sucrose density and distribution of total PIs along the gradient are shown in Figure 3A. Fraction 1 of the gradient had very few proteins, while fraction 2 contained the bulk of the proteins (based on Ponceau staining, not shown) and was enriched in cytosolic proteins, such as Erk (Figure 3B and D). Fraction 3 of the gradient contained Golgi and SER markers, which are relatively light organelles, while fractions 4, 5 and 6 contained mitochondria and rough endoplasmic reticulum (RER) markers, which are denser organelles (Figure 3B, C and D). Plasma membrane markers were found to localize sharply in fraction 4 (Figure 3B and D). This distribution is consistent with the expected density of each of these organelles in sucrose [26]. Remarkably, in BTC6 cells, where the S1 fraction contained visible amounts of microsomes (based on the turbidity of the solution), discrete opaque bands were seen in fractions 2, 3 and 4. In summary, this characterization shows that our approach allows us to generate subcellular fractions enriched in plasma membrane or in organelles that are either lighter than plasma membrane (SER and Golgi) or heavier than plasma membrane (RER and mitochondria). The small error bars highlight the reproducibility of these experiments (note also the similarities between the two cell lines).

4) Overall PI distribution after differential centrifugation

Table II shows the distribution of each PI into P1-derived fractions and S1. In BTC6 cells, the microsomal fraction (S1) contained the majority of all PIs, while in HeLa cells, the nuclear-associated ER fraction (triton-soluble P1) contained the majority of all PIs. Although each PI followed the same pattern, a higher portion of PtdIns, as compared to other phosphorylated PIs (especially PtdIns(4,5) P_2), was found at the nuclear-associated ER fraction. This is consistent with the fact that PtdIns is primarily synthesized at the ER, where it can be phosphorylated into other PI forms or transported to other membrane compartments.

From the sum of the absolute counts from each individual PI peak in the various fractions (not shown) we were able to determine that the ratio between all PIs were within the expected range, indicating that very little loss, if any, occurred due to post-lytic dephosphorylation. For example, in three independent experiments where BTC6 cells were fractionated, the total counts for the PtdIns5P peak in each experiment were 8.7×10^3 , 2.9×10^3 and 7.9×10^3 , and for PtdIns4P they were 3.6×10^5 , 1.1×10^5 and 2.3×10^5 , respectively. In HeLa cells the total counts for the PtdIns5P peak in three independent experiments were 7.6×10^3 , 11.7×10^3 and 13.5×10^3 , and for PtdIns4P they were 4.1×10^5 , 13.2×10^5 and 9.2×10^5 , respectively. Therefore, the total PtdIns5P content after BTC6 cell fractionation was 2.4, 2.6 and 3.4 % of PtdIns4P, and the total content for HeLa cells was 1.8, 0.9 and 1.4 % of PtdIns4P, which are within the expected range for these cells (compare to Table I).

5) Distribution of microsomal PtdIns5P

Figure 4 shows the distribution of microsomal (S1) PtdIns5P and PtdIns over the density gradient (A for HeLa and B for BTC6). The majority of the microsomal PtdIns5P (black squares) was found in fraction 4, which is enriched in plasma membrane. Importantly, a significant portion of PtdIns5P was also found in fraction 3 and in the triton-soluble P1 fraction (Table II), which are enriched in SER/Golgi markers. In BTC6 cells, 30% of the microsomal PtdIns5P was in fraction 3, while only 20% of PtdIns was present in this fraction. In HeLa cells, PtdIns5P was almost equally distributed between fractions 3 and 4, in a pattern that

deviated from the distribution of PtdIns. Fraction 3 was the only fraction in which PtdIns5P distribution was higher than PtdIns distribution in both cells, suggesting that PtdIns5P is specifically enriched in SER/Golgi.

To confirm this, we determined the concentration of PtdIns5P (relative to PtdIns) in each individual fraction and compared it to its concentration in the total cell. Figure 4C shows that in HeLa cells a higher relative concentration of PtdIns5P is found in all microsomal fractions, but especially in fraction 3, where the local concentration of this lipid was more than twice its concentration in the total cell (dashed line). In BTC6 cells (Figure 4D), the PtdIns5P enrichment in fraction 3 was also evident (1.35 fold enrichment). The relative PtdIns5P concentration in P1 fractions, which include membranes associated with the nucleus and nuclear fraction, was lower than in the total cell. These results confirm that, although PtdIns5P is most abundant in plasma membrane, it is specifically enriched in SER and/or Golgi.

In order to determine the subcellular fractions where PtdIns5P is converted into PtdIns(4,5) P_2 , we generated PIP4k II α , II β and II γ knockdown HeLa cells by infection with retrovirus carrying pSuper vectors in which the shRNA sequence for targeting each PIP4k isoform was introduced. In all three PIP4k knockdown cell lines, the basal PtdIns5P levels were higher than in the control cell line, but in the PIP4k II γ knockdown, PtdIns5P levels were highest (1.8 to 2 fold higher than control, not shown). In these cells, the PIP4k II γ protein is 90% knocked-down and PIP4k II β is 50% knocked-down (Figure 5A). Figure 5B shows that in PIP4k II γ knockdown cells (open bars) the PtdIns5P concentration increased in the lighter fractions of the gradient, especially in fraction 3, which had almost twice as much PtdIns5P as the control cells (black bars). These results indicate that the PtdIns5P pathway for PtdIns(4,5) P_2 synthesis is active in SER/Golgi.

6) Distribution of microsomal PtdIns4P and PtdIns(4,5) P_2

As with PtdIns5P, PtdIns4P accumulated in fraction 3 of the gradient when compared to PtdIns (especially in BTC6 cells, Figure 6B, triangles), but it was most abundant in plasma membrane. Although our results are contrary to the common understanding that the majority of PtdIns4P is present in the Golgi, they are consistent with immunocytochemistry findings reported by Hammond and colleagues, where the majority of PtdIns4P was detected at the plasma membrane [27].

In our analysis, PtdIns(4,5) P_2 distribution in HeLa cells was similar to the distribution of PtdIns4P (Figure 6A, diamonds). In BTC6 cells, however, PtdIns(4,5) P_2 was found sharply in fraction 4 (Figure 6B, diamonds). The percentage of PtdIns(4,5) P_2 in fraction 4 was almost 70%, while the percentages of PtdIns and other lipids in this fraction were around 50%, indicating that plasma membranes are enriched in PtdIns(4,5) P_2 . In fact, the pattern of PtdIns(4,5) P_2 distribution closely resembled the distribution of β 1-integrin in these cells (Figure 3D, black triangles). Hammond and colleagues also report that PtdIns(4,5) P_2 is predominantly at the plasma membrane [27].

7) Distribution of microsomal PtdIns3P

Unexpectedly, we found that in HeLa cells the majority of the PtdIns3P (more than 50%) was in fraction 3, where only 20% of PtdIns is found (Figure 6A). In BTC6 cells (Figure 6B), although most PtdIns3P is in fraction 4, a significantly higher percentage of this lipid, as compared to PtdIns, was distributed into fraction 3 (33% versus 21%). When the relative concentration of PtdIns3P was examined in various fractions, we found that the levels of PtdIns3P in HeLa cells were almost 4 times higher in fraction 3 than in the total cell (Figure 6C). In BTC6 cells, PtdIns3P levels were almost 2 times higher in fraction 3 than in the total cell (Figure 6D). Although PtdIns3P enrichment was only observed in fraction 3, we also found

this lipid to be present in fractions 4, 5 and 6, where the endosome marker Rab5 is present (Figures 3C and 7A). In contrast to PtdIns5P, PtdIns3P was virtually absent or very low in fraction 1, which is the lightest fraction of the gradient. PtdIns3P distribution in HeLa cells closely matched the distribution of the COPI vesicle marker β COP (compare to Figure 3B). These results indicate that PtdIns3P is most abundant in SER and/or Golgi, contrary to the belief that the majority of PtdIns3P is found in endosomes.

8) Brefeldin A affects the distribution of PtdIns5P and PtdIns3P

Since PtdIns3P and PtdIns5P are enriched in fraction 3 of the gradient, we decided to thoroughly characterize this fraction using various Golgi and ER markers. Figure 7A shows that fraction 3 is enriched in SER and Golgi markers such as calnexin (70%), GM130 (55%) and β COP (40%), while it only contains 10% of the endosomal markers Rab5 or EEA1. Although EEA1 is an early endosomal marker that binds to PtdIns3P, the majority of this protein was found in fraction 2 of the gradient, where cytosolic proteins are found. This is probably due to a weak association of EEA1 with PtdIns3P and Rab5, which may not be strong enough to last through the gradient centrifugation. The presence of mitochondrial and RER markers in fraction 3 were equal to or lower than 10% (not shown and Figures 3C and D). Interestingly, over 30% of the PIP4k II γ protein was found in fraction 3 (black diamonds), consistent with the findings that knockdown of this enzyme increased PtdIns5P in this fraction (Figure 5B).

Next, we decided to examine the effect of pharmacological disruption of Golgi in the distribution of PtdIns3P and PtdIns5P. After 2 hrs of Brefeldin A treatment, the Golgi markers 58K and GM130 were completely dispersed into small vesicles throughout the cytosol, as determined by immunocytochemistry (data not shown). Figure 7B shows that after Brefeldin A treatment, there was a subtle, but reproducible decrease in the distribution of PtdIns5P and PtdIns3P into denser microsomal fractions and an increase in the presence of these lipids in lighter microsomal fractions (compare open shapes with solid shapes). Brefeldin A treatment did not affect the distribution of PtdIns4P or PtdIns(4,5)P₂ (not shown). These results suggest that disruption of the Golgi interferes with the distribution of PtdIns3P and PtdIns5P within the cells, confirming that these lipids are present in internal membranes involved in intracellular trafficking.

DISCUSSION

The studies of PI function in cells have been revolutionized by the use of fluorescent probes that recognize specific PIs and allow subcellular imaging of these lipids in real-time. These analyses rely on the assumption that a particular probe can bind to its lipid partner regardless of the microenvironment where that lipid resides within the cell. It is clear, however, that the association between a PI and its protein probe *in vivo* can be influenced by several factors [28]. PI-binding probes can simultaneously bind to lipids and proteins (reviewed by [28]). Often, a PI probe can bind to more than one PI, and although it may have a preferential partner, the binding *in vivo* will be affected by the abundance of weaker partners. For instance, the PHD domain of ING2, which was shown to bind to PtdIns5P, can also bind to PtdIns3P and PtdIns4P [29], which will likely affect the localization of this domain, since they are far more abundant than PtdIns5P in cells. In addition, the specificity of a probe may change depending on the chemical environment of a particular compartment. In order to avoid some of the pitfalls associated with live cell imaging, some groups have utilized lipid-binding probes to determine the cellular distribution of PI in fixed cells [30]. However, if a PI is bound to its protein partner in a specific subcellular compartment, it may not be available for binding to its probe. Such is the case for PtdIns(4,5)P₂, which binds to many cytoskeletal proteins [31]. Thus, analyses of the subcellular localization of PI-binding probes cannot on their own provide a full

understanding of the distribution of the various PIs in cells. Here we have combined subcellular fractionation techniques with HPLC analysis of cellular PIs to study the distribution of cellular PIs, without the complications of using PI probes. A newly developed HPLC method described here makes possible the study of the distribution of cellular PtdIns5P, which has not been previously assessed. The similarities between our findings on PtdIns4P localization and the ones recently reported by Hammond and colleagues using immunostaining with anti-PtdIns4P antibodies [27] highlight the reliability of our approach and the importance of using direct methods for measuring PI distribution in cells concomitantly with cellular probes.

While studying PtdIns5P localization, we made the surprising observation that a significant portion of cellular PtdIns3P is found in SER and/or Golgi. Supporting our findings is the report that Beclin/PtdIns 3-kinase complex localizes to the trans-Golgi network [32]. Although most FYVE domains specifically localize to endosomes in live or fixed cells [30], one FYVE domain-containing protein, DFCP1, was found associated with the Golgi [33]. It is possible that in SER/Golgi PtdIns3P is tightly bound to unidentified proteins, making it inaccessible to its probes. Whether the subpopulation of PtdIns3P associated with SER and Golgi plays a role in endosomal and/or autophagosomal biogenesis or in a different membrane trafficking process needs to be further investigated.

Our data provides strong evidence that PtdIns5P is enriched in intracellular vesicles of the ER and Golgi and therefore it may have a role in intracellular trafficking. In fact, the portion of microsomal PtdIns5P that accumulated in SER/Golgi-containing fractions was higher than the portion of PtdIns4P, which is a known Golgi resident (compare Figures 4B, squares, with 6 B, triangles). A role for PtdIns5P in basic cell function, such as intracellular trafficking, is supported by our observation that PtdIns5P is present at basal levels in all cells examined (Table I).

Lecompte and collaborators have predicted a role for PtdIns5P in membrane trafficking, based on analysis of the phylogenetic distribution of proteins implicated in PtdIns5P metabolism [25]. They hypothesize that PtdIns5P is involved in trafficking from late endosomes to the plasma membrane. Indeed, our data show that PtdIns5P is found in several intracellular vesicles and plasma membrane, but in contrast to Lecompte's prediction, PtdIns5P seems to be particularly enriched in microsomal vesicles that are lighter than plasma membrane vesicles. This observation is consistent with PtdIns5P being particularly enriched in SER/Golgi-derived vesicles rather than endosomal vesicles. Therefore, we propose a role for PtdIns5P in Golgi-mediated transport (from or to ER, to plasma membrane or to late endosomes). Consistent with this model is the fact that PIP4k II γ was found to localize at the ER [34] and at the Golgi ([35]; Figure 5B and Sarkes and Rameh, unpublished). In addition, the BTC6 insulinoma cell line, which is specialized in insulin secretion, was found to have the highest PtdIns5P levels of all the cells analyzed (Table I) and also to have high levels of PIP4k II γ expression (not shown). Interestingly, the *Dictyostelium* phosphatase PLIP, which was shown to preferentially use PtdIns5P as a substrate, localizes in the Golgi, suggesting the presence of PtdIns5P in the Golgi of *Dictyostelium* cells [12].

Several groups have now suggested a role for PtdIns5P in signaling. PtdIns5P has been implicated in insulin-induced PI3k signaling and Akt activation [20] and in TCR-activated phosphorylation of Dok-1 and Dok-2 [5], responses that occur at the plasma membrane level. PtdIns5P has also been implicated in stress-induced p53 acetylation mediated by ING2 in the nucleus [3]. It is unclear at this point whether PtdIns5P present at the ER/Golgi plays a role in the cellular responses to extracellular signals. We are currently studying the subcellular localization of inducible PtdIns5P, which will help us answer this and other questions regarding the biological function of PtdIns5P in cells.

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Abbreviations

PI	phosphoinositide
PtdIns	phosphatidylinositol
P	phosphate
PIP4k	phosphatidylinositol-5-phosphate 4-kinase
PI3k	phosphoinositide 3-kinase
SER	smooth endoplasmic reticulum
RER	rough endoplasmic reticulum
PM	plasma membrane
HPLC	high performance liquid chromatography
shRNA	short hairpin RNA
FBS	fetal bovine serum

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FIGURE 1.

PtdIns5P measurements using a novel HPLC method. (A) HPLC profile of $[^3\text{H}]$ inositol-labeled HeLa cells expressing IpgD. (B) HPLC profile of $[^3\text{H}]$ inositol-labeled HeLa cells transfected with control vector. (C) PtdIns5P levels (relative to total PI levels) in CHO-HIR cells before and after insulin stimulation (10 nM for 5 min), measured using $[^{32}\text{P}]$ -labeled cellular lipids and the novel HPLC method for PtdIns5P detection. Data is the average and error bars are the standard error of three independent labelings. (D) HPLC profile of $[^3\text{H}]$ inositol-labeled HeLa treated with H_2O_2 (500 μM for 15 min). Insert shows PtdIns5P levels (relative to total PtdIns4P levels) in HeLa cells before and after H_2O_2 treatment. Data is the average and error bars are the standard error of four independent labelings.

FIGURE 2.

Subcellular distribution of protein markers and lipids after differential centrifugation.

Distribution of the membrane marker integrin $\beta 1$ (white bars), the cytosolic proteins Erk 1/2 (hatched bars), the nuclear markers lamin A/C (HeLa cells) or phospho-histone (BTC6 cells) (grey bars) and total phosphoinositides (black bars) after differential centrifugation of BTC6 (A) or HeLa (B) cell lysates. S1 equals the sum of all 6 fractions from the sucrose gradient. The data shown represent the percentage distribution against the sum of all fractions and is representative of more than 3 separate experiments.

FIGURE 3.

Distribution of protein markers and lipids after fractionation of the cytosolic/microsomal fraction through a 20%–65% sucrose gradient (A) Distribution of [3 H]-total phosphoinositides from HeLa cells (solid line) detected after chloroform/methanol extraction of lipids from various fractions. Data shown is the average and range from two separate experiments. Also plotted is the sucrose density (dashed lines) measured from each fraction using a refractometer. (B and D) Distribution of the cytosolic protein Erk 1/2 (crosses) the Golgi protein β COP (circles), the membrane protein integrin β 1 (triangles) and the ribosomal protein S6 (squares) from HeLa cells (B) or BTC6 (D). (C) Distribution of the Golgi protein 58K (crosses), the membrane protein caveolin (circles), the mitochondrial protein ATP synthase (triangles) and the endosomal protein Rab5 (squares) from HeLa cells.

FIGURE 4.

Distribution and relative concentration of PtdIns5P through the various subcellular fractions. [^3H]-PtdIns5P (squares, solid line) and [^3H]-PtdIns (stars), from HeLa (A and C) or BTC6 (B and D) cells were detected and quantified after chloroform/methanol extraction, deacylation and HPLC analysis of lipids from each fraction. In A and B, the amount of lipid present in each fraction is expressed as a percentage of its total from all fractions. In C and D, the concentration of PtdIns5P is expressed as percentage of PtdIns. Data shown is the average and standard error from 3 separate experiments. Due to the low lipid content in the sucrose fractions 1 and 6 and in the nuclear fraction of BTC6 cells (see Figures 2A and 4B), relative PtdIns5P determinations were subject to a larger error in these fractions.

FIGURE 5.

Relative concentrations of PtdIns5P in each subcellular fraction in control or PIP4k II γ knockdown HeLa cells. (A) Western blot showing the protein levels of PIP4k II β and PIP4k II γ in control HeLa cells or HeLa cells infected with pSuper-II α , pSuper-II β or pSuper-II γ . Tubulin was used as loading control. (B) PtdIns5P levels in various subcellular fractions (relative to total phosphoinositide levels in each fraction) from HeLa control (black bars) or pSuper-II γ infected HeLa cells (white bars). Data shown are the average and standard error of 3 (for control cells) and 4 (for PIP4k II γ knockdown cells) independent experiments.

FIGURE 6.

Distribution of phosphoinositides and relative concentration of PtdIns3P through the various subcellular fractions. [³H]-PtdIns3P (circles), [³H]-PtdIns4P (triangles), [³H]-PtdIns(4,5)P₂ (diamonds) and [³H]-PtdIns (stars) were detected after chloroform/methanol extraction of lipids from each fraction, deacylation and HPLC analysis. In A and B the amount of lipid present in each fraction is expressed as a percentage of its total from all fractions. In C and D, the concentration of PtdIns3P is expressed as percentage of PtdIns. Data shown is the average and standard error from 3 separate experiments.

FIGURE 7.

Distribution of protein markers for ER, Golgi and endosomes after fractionation of the BTC6's cytosolic/microsomal fraction through a 20%–65% sucrose gradient (A) and effect of Brefeldin A on PtdIns5P and PtdIns3P distribution (B). (A) Distribution of the early endosomal protein EEA1 (stars) and Rab5 (open circles), the COPI protein β COP (black circles), the Golgi marker GM130 (black squares), the ER protein calnexin (triangles) and PIP4k II γ (diamonds) from BTC6 cell lysates. The amount of each protein present in each fraction is expressed as a percentage of its total from all fractions. (B) Distribution of PtdIns5P (squares) and PtdIns3P (circles) before (black squares and circles) or after (open square and circles) treatment with Brefeldin A (2 μ g/ml for 2 hrs). The amount of each lipid present in each fraction is

expressed as a percentage of its total from all fractions. Data is the average and bars represent the standard error of two experiments (except for GM130 which is from one experiment).

TABLE I

Basal PtdIns5P levels in various cell lines. Cells were labeled with [³H]inositol in medium containing or not FBS, as indicated, for the time indicated. PtdIns5P levels were determined as described in Methods and normalized against PtdIns4P. Data shown is a representative measurement or the average from 2–4 independent experiments. SE represents the standard error.

Cell line	FBS in growth media (%)	labeling time (hr)	PtdIns5P (% of PtdIns4P) $\pm$ SE
HeLa	0.5–1.0	24	0.98
		48	1.16 $\pm$ 0.03
		72	1.32 $\pm$ 0.18
HeLa	10	24	0.65 $\pm$ 0.08
		48	1.00 $\pm$ 0.17
		72	1.41 $\pm$ 0.12
CHO-HIR	0	24	1.57
		48	0.92
		72	1.32
NIH 3T3	1	48	1.36
		72	1.2
3T3-L6	10	24	1.17
		72	1.18
BTC-6	10	24	2.93
		48	3.08 $\pm$ 0.23
		72	2.53 $\pm$ 0.5

Distribution of PIs into S1 and P1-derived fractions. The percentage of various [³H]-labeled PIs present in each fraction was measured after differential centrifugation, as described. S1 equals the sum of all 6 fractions from the sucrose gradient. The data shown represent the percentage distribution against the sum of all fractions and is the average and standard error from three (BTC6) or two (HeLa) separate experiments. (*) Indicates that the measurements were above background in only one out of three experiments.

TABLE II

Cell line	PI	P1			S1
		triton-soluble	triton-insoluble	unbroken	
BTC6	PtdIns	23.4 -/+ 0.4	1.1 -/+ 0.3	3.8 -/+ 0.5	71.7 -/+ 1.0
	PtdIns3P	16.8 -/+ 2.0	2.5 (*)	3.7 -/+ 0.2	80.0 -/+ 3.9
	PtdIns4P	18.0 -/+ 1.7	2.5 (*)	4.1 -/+ 0.4	78.5 -/+ 3.7
	PtdIns5P	19.8 -/+ 0.6	3.7 (*)	4.3 -/+ 0.5	76.1 -/+ 2.5
	PtdIns(4,5)P ₂	8.1 -/+ 0.4	0.7 (*)	1.3 -/+ 0.7	90.4 -/+ 0.4
HeLa	PtdIns	66.9 -/+ 2.7	9.0 -/+ 1.4	6.1 -/+ 0.6	17.9 -/+ 0.7
	PtdIns3P	48.8 -/+ 5.3	8.8 -/+ 3.4	5.6 -/+ 1.8	36.9 -/+ 0.1
	PtdIns4P	49.4 -/+ 9.9	7.9 -/+ 2.1	6.1 -/+ 2.3	36.6 -/+ 5.5
	PtdIns5P	48.8 -/+ 10.0	8.4 -/+ 3.4	8.1 -/+ 3.8	34.7 -/+ 2.8
	PtdIns(4,5)P ₂	43.5 -/+ 12.2	4.6 -/+ 0.3	4.6 -/+ 2.1	47.4 -/+ 10.4