Phosphatidylinositol 5-Phosphate Biosynthesis Is Linked to PIKfyve and Is Involved in Osmotic Response Pathway in Mammalian Cells*

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The cellular functions, regulation and enzymology of phosphatidylinositol (PtdIns) 5-P, the newest addition to the family of phosphoinositides (PI), are still elusive. Whereas a kinase that uses PtdIns-5-P as an intracellular substrate has been assigned, a kinase that produces it remained to be identified. Here we report that PIKfyve, the enzyme found to synthesize PtdIns-5-P in vitro and PtdIns-3,5-P2 in vitro and in vivo, is responsible for PtdIns-5-P production in a cellular context. Evidence is based on examination of two groups of cell types by two independent approaches. First, [32P]orthophosphate-labeled cells (SF9, 3T3-L1 fibroblasts, and 3T3-L1 adipocytes) that show a high pressure liquid chromatography (HPLC)-detectable peak of the PtdIns-5-P head group at basal conditions demonstrated a 20–50% increase in radioactive PtdIns-5-P amounts upon expression of PIKfyveWT. Second, cell types (HEK293), in which the basal levels of radioactive PtdIns-5-P were undetectable by HPLC head group analysis, demonstrated higher in vitro type II PIP kinase-directed conversion of the endogenous PtdIns-5-P pool into PtdIns-4,5-P2 when induced to express PIKfyveWT. Conversely, a decrease by 60% in the conversion of PtdIns-5-P to PtdIns-4,5-P2 was associated with induced expression of the dominant-negative kinase-deficient PIKfyveK183E mutant in HEK293 cells. When 3T3-L1 fibroblasts and 3T3-L1 adipocytes were subjected to osmotic shock, levels of PtdIns-5-P measured by both approaches were found to decrease profoundly upon a hypo-osmotic stimulus. Together, these results identify PIKfyve as an enzyme responsible for PtdIns-5-P biosynthesis and indicate a role for PtdIns-6-P in osmotic response pathways in mammalian cells.

It is now well recognized that dynamic changes in the phosphorylation status of intracellular phosphatidylinositol (PtdIns) regulate diverse cellular processes such as cell signaling, membrane trafficking, cytoskeletal reorganization, DNA synthesis, and cell cycle. In mammalian cells, a wide spectrum of phosphoinositide kinases and phosphatases produce seven phosphorylated metabolites of PtdIns, known so far, i.e. PtdIns-3-P, PtdIns-4-P, PtdIns-5-P, PtdIns-3,4-P2, PtdIns-3,5-P2, PtdIns-4,5-P2, and PtdIns-3,4,5-P3 (for recent reviews see Refs. 1–8). In most cases, phosphorylated metabolites of PtdIns, collectively called PIs, were found to serve as regulatory membrane-localized signals that recruit/activate protein effectors when their downstream function is required. Downstream proteins recognize PI signals through PI-specific binding domains, including the pleckstrin homology domain and several newly identified modules such as the FYVE finger (from the first letters of Fab1p, YOTB, Vac1p, and EEA1), the FERM (Four-point-one-Ezrin-Radixin-Moesin), the ENTH (Epsin N-terminal homology), and the PX (Phox homology) domains (for recent reviews see Refs. 9–12).

Given the pleiotropic functions of PIs, it is apparent that the kinases and phosphatases responsible for their synthesis and turnover play an essential role in cell regulation. Whereas the phosphatases and their contribution to the interconversion of the PIs are less well characterized (6–8), phosphoinositide kinases have been identified for most PIs (1–4). Studies with purified mammalian enzymes divide PIs into a number of classes differing by the phosphorylation of specific hydroxyl groups in the inositol ring: PI-3-Ks, PI-4-Ks, and PI-5-Ks (1–4).

PI 3-kinases, which catalyze the phosphorylation at position D-3 in PtdIns, PtdIns-4-P, PtdIns-4,5-P2, and likely PtdIns-5-P, are further subdivided into three classes, i.e. IA, IB, II, and III, based on their structure, in vitro substrate specificity, and mode of activation. PI 4-Ks are also represented by several types. Some of them display restricted substrate specificity strictly directed toward position D-4 of PtdIns, but not of PIs, and are called PtdIns 4-Ks. Position 4 in D-3-phosphorylated PtdIns, and to a lesser extent, in D-3-phosphorylated PtdIns, can be attacked by the enzymatic activity of another subclass of PI 4-kinases, known as type II PIPKs or PIP 4-Ks. Finally, position 5 can be phosphorylated by two subclasses of enzymes: PI 5-Ks (or type I PIPKs; Ref. 13) and PIKfyve (Refs. 14 and 15 and reviewed in Ref. 16), which display preferences for D-4- and D-3-phosphorylated PtdIns, respectively. Both enzymes are capable of converting PtdIns to PtdIns-5-P in vitro (13–15). It should be emphasized that often in vitro determined substrate specificity does not reflect that in living cells, where factors such as substrate availability, accessibility and presentation, or enzyme regulation may alter the enzyme specificity. This, combined with the fact that each PI (with the exception of PtdIns-3,4,5-P3) could be produced by dephosphorylation of higher phosphorylated PIs, indicates that PI biosynthesis and turnover are exceedingly complex.

Although studies with purified enzymes revealed PtdIns-5-P synthesis is supported by two enzymes, the kinase(s) responsible for PtdIns-5-P production in a cellular context remained unknown. The identification of this 5-phosphorylated PtdIns

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‡ The abbreviations used are: PtdIns, phosphatidylinositol; GroPIns, glycerophosphoinositol; HPLC, high pressure liquid chromatography; PI, phosphoinositide; K, kinase; HA, hemagglutinin; P, phosphate; P2, bisphosphate; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; HEK, human embryonic kidney.
metabolite, the latest addition to the PI family, was somewhat delayed because of its poor chromatographic separation from PtdIns-4-P on HPLC columns (17). Moreover, when detected in mammalian cells, PtdIns-5-P was found to represent only a minor fraction of PIs (17), thus making cellular studies even more problematic. PtdIns-5-P is a substrate for type II PIPKs (17) and, thus far, has been reported to occur naturally in resting mouse NIH3T3 fibroblasts in culture (17), human platelets (18), and Chlamydomonas cells (19). Negative results were reported in yeast (20, 21), several mammalian cell types in culture (22), and Arabidopsis (23). Little is known about the intracellular regulation of PtdIns-5-P. In platelets, levels of PtdIns-5-P have been shown to increase acutely upon thrombin stimulation, whereas in Chlamydomonas cells PtdIns-5-P was up-regulated by hyperosmotic stress (18, 19). Clearly, although the natural occurrence of PtdIns-5-P in the higher eukaryotic cells may play an important role in different cellular processes, the intracellular production, regulation of metabolism, and physiological functions of PtdIns-5-P are largely unknown. Therefore, to define the enzyme involved in PtdIns-5-P biosynthesis and a possible regulation of PtdIns-5-P levels, we have examined various cell types, in which accumulated radioactive PtdIns-5-P during $^{32}P$orthophosphate cell labeling is readily detected by HPLC head group analysis. In addition, mammalian cells, previously shown negative for radioactive PtdIns-5-P by HPLC (22), were examined in vitro for type II PIPK-directed conversion of PtdIns-5-P to PtdIns-4,5-P$_2$. We report here that PIKfyve is responsible for PtdIns-5-P biosynthesis and that the osmoregulatory response in mammalian cells involves a robust change in PtdIns-5-P levels.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—**Conditions for maintaining mouse 3T3-L1 fibroblasts and their differentiation into insulin-sensitive adipocytes were previously described (14). Human embryonic kidney (HEK) 293 cells were maintained in DMEM, containing 10% fetal bovine serum, and the above antibiotics. SF9 insect cells were maintained in a complete Grace’s medium supplemented with 10% fetal bovine serum and 100 µg/ml gentamycin.

**Generation of Stable Cell Lines—**Stably transfected doxycline-inducible (Tet-On) cell lines expressing PIKfyve WT (clone 9) or PIKfyve, K1013E (clone 5) were generated following the Tet-Off/Tet-On gene expression System manual (Clontech). Briefly, PIKfyve WT or PIKfyve, K1013E cDNA, released by Xba1/SflI from pBluescript IKS+ (14), together with an HA-encoding adapter (flanked with BamHI and Xba1 restriction sites) were cloned into the BamHI-SalI sites of the pTRE2hyg vector. The expected organization of the constructs was confirmed by restriction mapping. The new pTRE2hyg-based vectors linearized by SalI were used to transfect a HEK293 Tet-On cell line (Clontech) by LipofectAMINE as a transfection reagent. Transfected cells were selected by hygromycin treatment at 125 µg/ml. Individual cell clonal lines were isolated by the help of cloning cylinders, propagated and then probed for a doxycycline-inducible expression of recombinant PIKfyve proteins by Western blot analysis with anti-HA polyclonal antibodies (a kind gift by Mike Czech) as described elsewhere (14, 15). Experiments were carried out with cells seeded on collagen IV pre-coated plates to promote attachment.

**Baculoviral and Adenoviral Infections—**Baculovirus expressing PIKfyve WT was generated and characterized previously (24). SF9 cells seeded on 75-cm$^2$ flasks were infected with recombinant PIKfyve WT baculovirus or control baculovirus and processed for $^{32}P$orthophosphate labeling 5 days post-infection. Recombinant adenoviruses, expressing HA-tagged PIKfyve WT and GFP (AdPikfyve), or GFP alone (AdEmpty) were generated by the AdEasy system (25) as described elsewhere (22). Viral stocks were purified by ultracentrifugation in two discontinuous CsCl gradients and subsequent passage through a Nap 10 column (Sephadex G-25, Amersham Biosciences). Purified viral stocks were titered, and the lowest dilution resulting in 100% infection of HEK293 cells (5 h post-infection) was defined as a multiplicity of infection of 1. 3T3-L1 fibroblasts, 3T3-L1 adipocytes, or HEK293 cells were infected with AdPikfyve or AdEmpty at a multiplicity of infection equal to 40, 40, and 1, respectively. These conditions yielded ~25–30% (3T3-L1 fibroblasts or adipocytes) and 100% infection efficiency (HEK293 cells) at day 3 and day 1 post-infection, respectively, when cells were processed for $^{32}P$ labeling.

**Labeling of Cellular Phospholipids with $^{32}P$Orthophosphate and Lipid Extraction—**Infected SF9 cells were transferred from 75-cm$^2$ flasks into 50-ml Falcon tubes and then washed twice with a phosphate-free Grace’s medium. Following 30 min of incubation, the medium was replaced with a phosphate-free Grace’s medium supplemented with 0.5% bovine serum albumin and 1 mM $^{32}P$orthophosphate (PerkinElmer Life Sciences). After 2 h of incubation at 25 °C, the cells were washed in PBS, and the lipids were extracted as described below. Basal or adenosvirused-infected 3T3-L1 and HEK293 cells or stably transduced HEK293 cells were washed in phosphate-free DMEM and then labeled for 2.5 h at 37 °C in phosphate-free DMEM supplemented with 0.5% bovine serum albumin and 0.8 mM $^{32}P$orthophosphate as described previously (22, 24). Cells were washed with ice-cold PBS, containing protease (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 µg/ml pepstatin) and phosphatase inhibitors (50 mM NaF, 10 mM sodium pyrophosphate, 25 mM sodium β-glycerophosphate, and 2 mM sodium metavanadate), and scraped with CH$_3$OH, 1 M HCl (1:1) in the presence of 5 mM EDTA and 5 mM tetrabutylammonium sulfate. Extracted radiolabeled lipids were dry-converted as described previously (15, 22) and analyzed by HPLC. In some experiments, prior to lipid extraction, cells were subjected to treatment with different osmolytes.

**Cell Treatment—**Two days after seeding of 3T3-L1 fibroblasts (cDNA was a kind gift by Richard Anderson; Ref. 27) or 8–12 days following initiation of the differentiation program in these cells to acquire the adipocyte phenotype, and in some cases following $^{32}P$orthophosphate labeling, cells were incubated at 37 °C for 10 min in DMEM (control stimulus), in DMEM, diluted to one-quarter strength with water (hyperosmotic stimulus), or in DMEM containing either 0.6 M sorbitol or 0.2 M NaCl (hyperosmotic stimulus). Cells were plated on ice and washed once with PBS supplemented with protease and phosphatase inhibitors listed above. Cells were then scraped with CH$_3$OH, 1 M HCl (1:1) containing 5 mM tetrabutylammonium sulfate and 5 mM EDTA, and the lipids were extracted as described above. Lipids were then dried under N$_2$ and stored at −77 °C for PtdIns-5-P conversion assay or deacylated for HPLC analysis.

**PtdIns-5-P Conversion Assay—**PtdIns-5-P conversion assay was performed as described by Morris et al. (18). Briefly, PI$\alpha$s were isolated from cell lipid extracts on neomycin-coated glass beads prepared according to procedures published previously (26) using glyceryl-1,2,3-triacetate treated glass pore beads, 200–400 mesh (Sigma), stored at 25 °C in water/methanol (1:1, v/v). Twenty five µl of packed neomycin beads equilibrated at room temperature in 500 µl of chloroform, methanol, 425 mM ammonium formate (5:10:2; v/v) in glass microtubes were incubated for 1 h with duplicate samples of extracted lipids redissolved in 500 µl of chloroform, methanol, 425 mM ammonium formate (5:10:2; v/v). All samples contained 5 nmol of PtdIns-5-P as carrier. After two 500-µl washes in chloroform, methanol, 425 mM ammonium formate (5:10:2; v/v), PI$\alpha$s were washed twice for 20 min with 250 µl of water/methanol (1:1, v/v) and twice with 250 µl of chloroform, methanol, 2 M aqueous triethylamine bicarbonate (2:6:3; v/v). PtdIns (20 nmol) was added as carrier to all samples before drying them in a SpeedVac at low medium heat setting for 2–3 h and storing at −80 °C or proceeding further for PtdIns-5-P conversion assay using bacterial produced and purified recombinant His-tagged type II PI(PK (CDNA was a kind gift by Richard Anderson, Ref. 27). Samples were vortexed vigorously with 45 µl of His PI(PK assay buffer consisting of 50 mM Tris-HCl, pH 7.4, 80 mM KCl, 10 mM magnesium acetate, 2 mM EGTA, and 0.01% sodium deoxycholate. After addition of 1 µl of recombinant type II PI(PK (5 µg) and preincubation at 30 °C for 5 min, 5 µl of 50 µl of [γ-$^{32}P$]ATP (5 µCi) was added, and incubation was continued for 30 min at 30 °C. The reaction was stopped with 20 µl of 60 °C TLC plate (Whatman, PE SIL G, 250 µm), which was then developed in 65:35 (v/v) n-propyl alcohol, 2 M acetic acid. Dried plates were exposed 8–24 h and then visualized by autoradiography. To detect PtdIns-4,5-P$_2$ product derived from PtdIns-5-P, standard lipids labeled with 1–5 pmol of PtdIns-5-P were processed similarly. Control samples with no enzyme and/or no lipids were run in each experiment. The PtdIns-4,5-P$_2$ radioactive spot was confirmed by HPLC following lipid extraction from the silica scrapings and deacylation.

**HPLC Analysis and Data Quantitation—**HPLC analysis of PtdIns-5-P was performed following HPLC by guest on July 25, 2018http://www.jbc.org/Downloaded from
Radioactive lipids extracted from [32P]orthophosphate-labeled 3T3-L1 fibroblasts (see Fig. 6A) or 3T3-L1 fibroblasts (Fig. 1). Quantitation from eight independent 32P labeling experiments indicated that accumulated basal levels of [32P]PtdIns-5-P in this cell type were quite substantial, corresponding to as much as 11.5 ± 3% of the [32P]PtdIns-4-P levels. When detected in other cell types by the same methodology, the amounts of PtdIns-5-P were found to be comparable with PtdIns-3-P amounts (Ref. 17 and see below). Intriguingly, in 3T3-L1 adipocytes accumulated amounts of radiolabeled PtdIns-5-P were found to profoundly exceed that of [32P]PtdIns-3-P by 4–6-fold (Fig. 1).

Similarly to 3T3-L1 adipocytes, HPLC analyses of deacylated radioactive lipids extracted from [32P]orthophosphate-labeled liver from 3T3-L1 fibroblasts (Fig. 2A) or 3T3-L1 fibroblasts (Fig. 2A) revealed a 32P-labeled peak whose elution time coincided with that of the [3H]GroPIns-5-P internal standard. Quantitation from three independent 32P labelings for either S9 cells or 3T3-L1 fibroblasts indicated that accumulated radiolabeled PtdIns-5-P amounts represented 12.6 ± 4.0 and 3.2 ± 0.5% of PtdIns-4-P, respectively. As opposed to wt 3T3-L1 adipocytes, the PtdIns-5-P levels in S9 cells and 3T3-L1 fibroblasts were comparable with the basal PtdIns-3-P amount in these cells (not shown).

[32P]PtdIns-5-P Amounts Are Increased upon Heterologous Expression of PIKfyveWT—By having identified cell types capable of accumulating HPLC-detectable basal PtdIns-5-P upon cell labeling with inorganic 32P, we next examined the effect of expression of PIKfyveWT on levels of this lipid. Cells were transduced with PIKfyveWT or control viruses, and subsequent labeling with [32P]orthophosphate and extraction of the radioactive lipids, HPLC analyses were performed with deacylated products. As demonstrated in Fig. 2, S9 cell infection with baculovirus encoding PIKfyveWT or 3T3-L1 adipocyte transduction with adenovirus encoding PIKfyveWT resulted in accumulation of significantly higher amounts of [32P]PtdIns-5-P peak that eluted identically with the [3H]GroPIns-5-P internal standard. A similar increase of basal [32P]GroPIns-5-P was also observed in 3T3-L1 fibroblasts, which expressed recombinant PIKfyveWT delivered by adenovirus-mediated gene transfer (not shown). Quantitation performed as detailed under “Experimental Procedures” and based on three independent experiments for each cell line revealed that this increase corresponded to 40 ± 10, 23 ± 8, and 20 ± 5% over the basal radioactive PtdIns-5-P detected in control S9 cells, 3T3-L1 adipocytes, and 3T3-L1 fibroblasts, respectively, that were transduced with control viruses. Given the 25–30% infection efficiency of the PIKfyveWT adenovirus in 3T3-L1 fibroblasts and 3T3-L1 adipocytes, as judged by the fluorescence detection of the GFP reporter, the calculated PtdIns-5-P increase due to expression of PIKfyveWT is apparently underestimated by at least 3-fold. In agreement with our previous in vivo and in vitro studies (15, 22), expression of PIKfyveWT in the above cell types resulted in an increase of the PtdIns-3,5-P2 radioactive levels as well, consistent with the ability of PIKfyve enzyme activity to utilize PtdIns-3-P substrate. Together, these results demonstrate increased radioactive PtdIns-5-P amounts associated with PIKfyveWT expression and are consistent with the notion that intracellular PtdIns-5-P production is dependent on the PIKfyve enzyme.

Effect of PIKfyveWT Heterologous Expression in Cells with HPLC-undetectable Basal [32P]PtdIns-5-P Levels—Whereas basal 3T3-L1 fibroblasts and adipocytes show well defined radioactive peaks migrating identically with the [3H]GroPIns-5-P standard upon separation by HPLC, similar analysis in other mammalian cell types in culture such as COS-7, CHO-T and HEK293 cells failed to detect this lipid (22). Moreover, in contrast to 3T3-L1 fibroblasts and adipocytes, expression of high levels of PIKfyveWT in COS-7, CHO-T, and HEK293 cells (at
radioactive PtdIns-4,5-P₂ formed upon incubation of cell lipid extracts with \(^{[γ-32P]}\)ATP and recombinant type II PIPK (18). Because PtdIns-3-P but not PtdIns-4-P could be utilized to some extent as an additional substrate for type II PIPK, a subsequent HPLC head group analysis confirms the PtdIns-4,5-P₂ product formed. Recent application of this assay documented PtdIns-5-P production in human platelets, murine erythrocytes, and *Chlamydomonas* cells (18, 19, 28). We have applied this assay to examine the basal and a plausible PIKfyve-dependent PtdIns-5-P synthesis in HER293 cell lines that have been transfected to stably express PIKfyveWT (clone 9) upon doxycycline induction. Compared with endogenous PIKfyve, PIKfyveWT expression increased by \(-7\)-fold 18–48 h post-induction as documented by Western blot analysis with anti-PIKfyve antibodies (not shown and see Fig. 4A). PtdIns-5-P-conversion assay revealed substantial amounts of basal PtdIns-5-P in this cell type (Fig. 3B), which was calculated to be in the range of \(10–30\) pmol/mg protein. More importantly, our data demonstrated that the induction of PIKfyveWT expression in HER293 cells resulted in a significant rise of cellular PtdIns-5-P levels (Fig. 3B). This was evidenced by the observed \(1.9 \pm 0.3\)-fold increase of the chromatographed PIP₂ radioactive spot (Fig. 3C), confirmed by HPLC head group analysis to be composed of PtdIns-4,5-P₂ but not PtdIns-3,4-P₂ (Fig. 3D). In contrast, doxycycline treatment of the parental HER293 cells resulted in no change of the basal PtdIns-5-P to PtdIns-4,5-P₂ conversion, as judged by the detection of PtdIns-4,5-P₂ radioactive spots with similar intensity upon TLC (Fig. 3, B and C). Together, these results indicate, first, that whereas a \([^{32P}]\)PtdIns-5-P peak is undetectable by HPLC analysis in radiolabeled HER293 cells, this 5-phosphorylated metabolite naturally occurs in this cell type, and, second, that similarly to 3T3-L1 fibroblasts and adipocytes, the route for PtdIns-5-P biosynthesis in HER293 cells may entirely or partially rely on PIKfyve.

**Dominant-negative Kinase-deficient PIKfyveK1831E Decreases PtdIns-5-P Production**—If the contribution of PIKfyve enzymatic activity to the PtdIns-5-P cellular pool is physiologically significant, one would expect that expression of kinase-deficient mutants of PIKfyve with a dominant-negative effect (22) should arrest PtdIns-5-P production. To test this possibility we have generated a HER293 stable cell line inducibly expressing PIKfyveK1831E, Western blot analysis with anti-HA antibodies of the cell lysates derived from candidate PIKfyveK1831E-expressing clones selected one clone (clone 5) that demonstrated a significant induction of the mutant protein expression after 18 h of cell growth in the presence of doxycycline (Fig. 4A). The level of expression of PIKfyveK1831E in this clone was similar to that observed for PIKfyveWT-inducible expression of clone 9, exceeding by \(-7\)-fold the endogenous PIKfyve levels (Fig. 4A and data not shown). Importantly, PtdIns-5-P conversion assay revealed that the induction of PIKfyveK1831E expression was associated with a significant reduction of cellular PtdIns-5-P levels as manifested by the \(60 \pm 4\%\) decrease of the chromatographed PtdIns-4,5-P₂ radioactive spot versus non-induced cells (Fig. 4, B and C). This result is consistent with the idea that in the cellular context a substantial portion of PtdIns-5-P is due to PIKfyve enzymatic activity.

**PtdIns-5-P Dramatically Decreases upon Hypo-osmotic Shock**—A condition shown to affect the turnover of PIs in yeast, plant, and mammalian cells is osmotic stress (19, 23, 29–32). Related to PtdIns-5-P levels, a hypoosmotic increase has been reported in plants (19). To examine possible osmotically regulated changes in levels of PtdIns-5-P in mammalian cells, we have exposed 3T3-L1 fibroblasts or 3T3-L1 adipocytes for 10 min to DMEM containing non-permeant osmolytes in the form

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**FIG. 2.** Expression of PIKfyveWT in SF9 cells and 3T3-L1 adipocytes results in accumulation of substantially higher amounts of \([^{32P}]\)PtdIns-5-P. SF9 cells (A and B) and differentiated 3T3-L1 adipocytes (C and D) were infected with recombinant baculovirus expressing control LacZ and PIKfyveWT (SF9) or with adenovirus encoding empty virus or PIKfyve WT (3T3-L1 adipocytes) as indicated. Five (SF9 cells) or 3 days (3T3-L1 adipocytes) post-infection, cells were labeled with \([^{32P}]\)orthophosphate as detailed under *Experimental Procedures.* Lipids were extracted, deacylated, and co-injected with \([^{3}H\]GroPIns-5-P and \([3H]\)GroPIns-4-P internal standards. Fractions were collected every 0.25 min and monitored for \(3H\) and \(32P\) radioactivity by liquid scintillation counting. Shown are representative graphs of the HPLC elution profiles of the regions covering GroPIns-5-P and GroPIns-4-P elution time out of three independent experiments for each cell line with similar results.

\(~100\%\) cell infection efficiency) did not result in an accumulation of a quantifiable, clear-cut \(^{32}\)P-radioactive peak corresponding to the elution time of GroPIns-5-P (22) (Fig. 3A). These results may indicate that PIKfyve lipid substrate preferences for PtdIns-3-P versus PtdIns vary among different mammalian cells. Alternatively, or additionally, technical limitations associated with a poor resolution of GroPIns-5-P from mammalian cells. Alternatively, or additionally, technical limitations associated with a poor resolution of GroPIns-5-P from technical limitations associated with a poor resolution of GroPIns-5-P from these considerations are the data from the HPLC runs demonstrating a small shoulder with migration properties of PtdIns-3-P that manifests by the \(60\)–\(80\%\)glycerol migration of GroPIns-5-P (22) (Fig. 3B). This was evidenced by the observed \(1.9 \pm 0.3\)-fold increase of the chromatographed PIP₂ radioactive spot (Fig. 3C), confirmed by HPLC head group analysis to be composed of PtdIns-4,5-P₂ but not PtdIns-3,4-P₂ (Fig. 3D). In contrast, doxycycline treatment of the parental HER293 cells resulted in no change of the basal PtdIns-5-P to PtdIns-4,5-P₂ conversion, as judged by the detection of PtdIns-4,5-P₂ radioactive spots with similar intensity upon TLC (Fig. 3, B and C). Together, these results indicate, first, that whereas a \([^{32P}]\)PtdIns-5-P peak is undetectable by HPLC analysis in radiolabeled HER293 cells, this 5-phosphorylated metabolite naturally occurs in this cell type, and, second, that similarly to 3T3-L1 fibroblasts and adipocytes, the route for PtdIns-5-P biosynthesis in HER293 cells may entirely or partially rely on PIKfyve.

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**PtdIns-5-P Dramatically Decreases upon Hypo-osmotic Shock**—A condition shown to affect the turnover of PIs in yeast, plant, and mammalian cells is osmotic stress (19, 23, 29–32). Related to PtdIns-5-P levels, a hypoosmotic increase has been reported in plants (19). To examine possible osmotically regulated changes in levels of PtdIns-5-P in mammalian cells, we have exposed 3T3-L1 fibroblasts or 3T3-L1 adipocytes for 10 min to DMEM containing non-permeant osmolytes in the form.
of non-ionic or ionic molecules (hyperosmotic treatment, 0.6 M sorbitol, or 0.2 M NaCl) or to DMEM made hypo-osmotic by 75% dilution with water. The lipids were then extracted and subjected to PtdIns-5-P conversion assay in the presence of [\(\gamma^{32}\)P]ATP and recombinant type II PIPK. As illustrated in Fig. 5, treatment of both cell types with a hypo-osmotic solution induced a robust decrease of PtdIns-5-P intracellular production as evidenced by the 8.4 ± 3.5-fold (3T3-L1 fibroblasts) and 5.5 ± 1.5-fold reduction (3T3-L1 adipocytes) of the intensity of the PtdIns-4,5-P\(_2\) spot (confirmed by HPLC head group analysis; data not shown). Treatment with osmotically active compounds, however, did not induce statistically significant changes in the cellular PtdIns-5-P levels in four experiments for each cell type (Fig. 5). Together these results demonstrate a robust reduction of cellular PtdIns-5-P levels upon 3T3-L1 cell exposure to a hypo-osmotic solution and suggest a role of PtdIns-5-P as a regulatory intermediate in the osmotic response pathway in mammalian cells.

PtdIns-4-P Levels Remain Unchanged upon Hypo-osmotic Shock in 3T3-L1 Cells—Whereas the dramatic decrease of the type II PIP kinase-directed PtdIns-4,5-P\(_2\) formation by hypo-osmotic shock was clearly documented, it is still possible that this reduction affects the PtdIns-4-P rather than the PtdIns-5-P cellular levels. This is due to the fact that type II PIPK could still use PtdIns-4-P to make PtdIns-4,5-P\(_2\) although at a rate 100 times less effective than PtdIns-5-P (18). Therefore, to confirm that PtdIns-4,5-P\(_2\) was formed from PtdIns-5-P, but not from PtdIns-4-P, and to verify the conclusions for the robust decrease of the PtdIns-5-P cellular levels upon hypo-osmotic shock, we have examined the accumulation of radioactive PtdIns-4-P in these cells by HPLC. Following a 10-min hypo-osmotic treatment of [\(^{32}\)P]orthophosphate-labeled 3T3-L1 fibroblasts, and a subsequent lipid extraction, deacylated samples were subjected to HPLC separation along with [\(^{3}H\)]GroPIns-5-P and [\(^{3}H\)]GroPIns-4-P internal standards. As demonstrated in Fig. 6, cell exposure to hypo-osmotic shock did not change the accumulated [\(^{32}\)P]GroPIns-4-P. Its amounts were similar to that detected in non-treated cells. Conversely, and consistent with the results obtained with PtdIns 5-P-conversion assay, hypo-osmotic shock decreased the accumulated [\(^{32}\)P]GroPIns-5-P amounts. In fact, hypo-osmotic shock induced a complete disappearance of the [\(^{32}\)P]PtdIns-5-P peak seen in the control cells (Fig. 6A). These results demonstrate that whereas radiolabeled PtdIns-5-P amounts are decreased in 3T3-L1 cells, PtdIns-4-P remain unchanged upon hypo-osmotic shock, implying that the dramatic decrease of the in vitro PtdIns-4,5-P\(_2\) formation by type II PIPK under this condition is due to a selective decrease in PtdIns-5-P levels.

Hypo-osmotic shock of 3T3-L1 fibroblasts induced a substan-

![Fig. 3. Basal PtdIns-5-P production and its increase upon PIKfyve\(^{WT}\) expression in HEK293 cells determined by PtdInsP conversion assay.](image-url)

A, HEK293 cells were infected with adenovirus encoding empty virus or PIKfyve\(^{WT}\) as indicated. One day post-infection, cells were labeled with [\(^{32}\)P]orthophosphate for 2 h, and deacylated lipids were analyzed by HPLC along with [\(^{3}H\)]GroPIns-5-P and [\(^{3}H\)]GroPIns-4-P internal standards as detailed in the legend to Fig. 2. Shown is an overlay of HPLC runs demonstrating that although a PtdIns-5-P peak was not detected, more [\(^{32}\)P] radioactivity within the fractions with the elution time of [\(^{3}H\)]GroPIns-5-P was found in the AdPIKfyve\(^{WT}\) sample. B and C, parental HEK293 cells or HEK293 cells stably expressing HA-PIKfyve\(^{WT}\) (clone 9) were seeded (60-mm dishes) in the presence or absence of doxycycline as described under “Experimental Procedures.” Twenty hours following induction of the protein expression cells were washed and the lipids extracted. PIs were isolated on neomycin-coated glass beads and subjected to enzymatic conversion for 1 h at 30 °C in the presence of [\(^{32}\)P]ATP with (lanes 2–5) or without type II PIP kinase (lane 1). [\(^{32}\)P]-Labeled lipids were separated by TLC and visualized by autoradiography. Shown is a representative autoradiogram (B) and quantitation from five independent experiments in duplicates presented as a percent of PtdIns-4,5-P\(_2\) formed in PIKfyve\(^{WT}\)-stable cells with no induction (C). D, [\(^{32}\)P]PtdIns-4,5-P\(_2\) spot shown in B was scraped from the TLC plate and, following lipid extraction and deacylation, was subjected to HPLC analysis together with [\(^{3}H\)]GroPIns-4,5-P\(_2\) standard (data not shown). Eluted is a single PIP2 peak with migration properties of GroPIns-4,5-P\(_2\) (arrowhead).
30 min at 37°C in the presence of [γ-32P]ATP and type II PIP kinase. 32P-Labeled products were separated by TLC and visualized by autoradiography (B). Shown is a chemiluminescence detection of a representative immunoblot (A), an autoradiogram of a representative TLC plate (B), and quantification of the TLC autoradiograms from three independent experiments presented as a percent of PtdIns-4,5-P2 formed in PIKfyveK1831E stable cells with no induction (C).

Figure 5. Decrease of PtdIns-5-P synthesis upon hypo-osmotic shock of 3T3-L1 cells. 3T3-L1 fibroblasts (A) or differentiated 3T3-L1 adipocytes (B) were incubated in DMEM alone (control), DMEM made hypo-osmotic by 75% dilution with water (Hypo), DMEM supplemented with 200 mM NaCl, or DMEM supplemented with 600 mM sorbitol for 10 min at 37°C as indicated. Cells were then washed with PBS, and the lipids were extracted as described under “Experimental Procedures.” PIs were isolated on neomycin-coated glass beads and subjected to conversion by type II PIP kinase for 1 h at 30°C in the presence of [γ-32P]ATP, followed by analysis of HPLC elution characteristics. Shown is a representative autoradiogram (A and B) and quantitation from five (C) and three (D) independent experiments for each cell type presented as a percent of the PtdIns-4,5-P2 formed from PIP of control cells.

**Discussion**

Since the discovery of PtdIns-5-P as a substrate for type II PIPK and its natural occurrence in NIH3T3 fibroblasts (17), studies related to this PtdIns metabolite have been surprisingly sparse. As a result, we know almost nothing about the intracellular synthesis, roles, and regulation of PtdIns-5-P. This lack of information is mainly due to the fact that PtdIns-5-P is only a minor fraction of PIs whose HPLC elution characteristics are very similar to that of the abundant PtdIns-4-P metabolite. As a result, most of our knowledge about PtdIns-5-P is derived from studies related to this PtdIns metabolite (17).

PIKfyve Activity In Vitro Remains Unchanged Upon Osmotic Shock—The results presented above demonstrating a PIKfyve-dependent route in PtdIns-5-P biosynthesis and an osmoregulatory control of the intracellular PtdIns-5-P synthesis suggest the hypothesis that PIKfyve enzymatic activity may be affected by changes in osmolarity. To test this, we have examined PIKfyve lipid kinase activity in vitro by subjecting PIKfyve immunoprecipitates, derived from osmotically stimulated or control 3T3-L1 fibroblasts and adipocytes, to incubation in the presence of PtdIns and [γ-32P]ATP and a subsequent TLC separation of the extracted lipids. The data demonstrated no changes in the *in vitro* formed PtdIns-5-P and PtdIns-3,5-P2 in response to either hypo-osmotic or ionic/non-ionic hyperosmotic shock (data not shown). These results indicate that if the intracellular PIKfyve is subject to osmotic regulation, these changes do not result in a sustained alteration in the PIKfyve lipid kinase activity.
was typically seen as a small peak within the trailing edge of PtdIns-4-P long before the latter reaches the base line (Refs. 13, 15, 17, and this study). Apparently, low amounts of PtdIns-5-P are likely to be hidden within the PtdIns-4-P tail. Recent studies in yeast identifying Vac14 protein has been first suggested by the data demonstrating a powerful role of PIKfyve in PtdIns-5-P cellular production has been documented upon hypo-osmotic shock in mouse 3T3-L1 cells implicating PtdIns-5-P as a regulatory intermediate in the osmotic response pathway in mammalian cells.

The role of PIKfyve in PtdIns-5-P cellular production has been first suggested by the data demonstrating a powerful ability of a purified PIKfyve protein to support the conversion of PtdIns to PtdIns-5-P (14, 15). Subsequent in vivo studies, however, both in yeast and several mammalian cell types heterologously expressing high levels of PIKfyveWT, failed to detect intracellular PtdIns-5-P production (21, 22). Because an increase of PtdIns-3,5-P2 was possible to be documented under these conditions, PIKfyve has been implicated in PtdIns-3,5-P2 biosynthesis. However, the cell types tested so far all showed undetectable accumulation of radioactive PtdIns-5-P at resting conditions in vivo (21, 22). This implies that limitations of technical or other nature may have been preventing PtdIns-5-P production and/or detection. Among those, one could envision an upstream regulator(s) of PIKfyve PtdIns-5-P-synthesizing activity, which becomes a limiting factor particularly under conditions of PIKfyve overexpression in certain cells. Recent studies in yeast identifying Vac14 protein as an upstream activator of the yeast PIKfyve ortholog Fab1p lipid kinase (33, 34) indicate that this assumption may be correct. Another group of limiting factors may include a rapid

**Fig. 6.** Whereas the [32P]PtdIns-5-P peak disappears, the [32P]PtdIns-4-P amounts remain unchanged upon hypo-osmotic shock of radiolabeled 3T3-L1 fibroblasts. 3T3-L1 fibroblasts (60-mm dishes) were labeled with [32P]orthophosphate for 2 h in phosphate-serum-free DMEM. The medium was then replaced with DMEM alone (control) or DMEM made hypo-osmotic by 75% dilution with water (Hypo) as indicated. Cells were then washed with PBS and lipids extracted as described under “Experimental Procedures.” Deacylated lipids co-injected with [3H]GroPIns-5-P, [3H]GroPIns-4-P, and [3H]GroPIns-4,5-P2 standards were analyzed by HPLC. Fractions, collected every 0.25 min, were monitored for 3H (peak indicated by arrow) and 32P radioactivity by liquid scintillation counting. Shown is a representative HPLC elution profile of PIP2s out of two independent experiments with similar results (A) and the quantitation of the indicated radioactive peaks presented as percent of control values (B).

**Fig. 7.** Increase in accumulated [32P]PtdIns-4,5-P2 amounts upon hyperosmotic shock of radiolabeled 3T3-L1 fibroblasts. 3T3-L1 fibroblasts (60-mm dishes) were labeled with [32P]orthophosphate for 2 h in phosphate-serum-free DMEM. The medium was then replaced with DMEM (control) or DMEM containing 0.6 M sorbitol (Hyper). Cells were then washed with PBS and lipids extracted as described under “Experimental Procedures.” Deacylated lipids co-injected with [3H]GroPIns-4,5-P2 internal standard were analyzed by HPLC. Fractions, collected every 0.25 min, were monitored for 3H (peak indicated by arrow) and 32P radioactivity by liquid scintillation counting. Elution times of [32P]GroPIns-3,5-P2 and [32P]GroPIns-3,4-P2 standards (arrows) were determined from a parallel HPLC run. Shown is a representative HPLC elution profile of PIP2s out of two independent experiments with similar results.
PtdIns-5-P turnover coupled with uneven specific activity of the $^{[32P]}$ATP pool during cell labeling and/or unfavorable PtdIns-5-P versus PtdIns-4-P detection under HPLC analysis, discussed above. Overcoming these restrictions was made possible in the present study, at least in part, by applying two strategies. First, usage of cell types in which the accumulation of radioactive PtdIns-5-P peak upon HPLC head group analysis was evident at resting conditions, and second, usage of the PtdIns-5-P enzymatic conversion in cell types with undetectable levels of PtdIns-5-P under HPLC analysis. Both strategies unequivocally demonstrated an increase of PtdIns-5-P production associated with expression of high levels of PIKfyve WT in both mammalian and insect cells, thus establishing the biosynthetic pathway in PtdIns-5-P metabolism. That this pathway is likely physiologically relevant is further substantiated in the present study by the data demonstrating a marked decrease of PtdIns-5-P levels by inducing the expression of the dominant-negative kinase-deficient PIKfyve K1831E mutant. Because PIKfyve is an evolutionarily conserved protein found also in plants (16) where a substantial basal PtdIns-5-P production has been recently documented (up to 18% of the PtdIns-4-P amounts; Ref. 19), we suggest a biosynthetic mechanism that operates due to plant PIKfyve in these cells. It remains to be re-examined by PtdIns-5-P conversion assay whether the reported lack of PtdIns-5-P production in yeast cells (20, 21) is associated with technicalities in detection or truly reflects a more restricted substrate specificity of Fab1p toward 3-phosphorylated PtdIns. In any case, Fab1p is able to convert PtdIns into PtdIns-5-P in vitro (21).

One important aspect of the present studies is the observation that the intracellular PIKfyve lipid kinase activity synthesizes two lipid products. Thus, we reproducibly detected a simultaneous increase of both PtdIns-5-P and PtdIns-3,5-P2 production upon transduction of $^{32P}$-labeled 3T3-L1 adipocytes with adenovirus encoding PIKfyve WT (22) (Fig. 2B). This was also evident in the HEK293 stable cell line, where we documented higher PtdIns-5-P levels by mass assay (Fig. 2B) and an increase of the accumulated $^{[32P]}$PtdIns-3,5-P2 by HPLC analysis of in vivo labeled cells induced to express PIKfyve WT (Ref. 22 and data not shown). This dual specificity implies that distinct intracellular signals may differentially regulate PIKfyve lipid substrate preferences. Although direct experimental evidence is currently unavailable, the assumption that such differential regulation may operate in the cellular context is supported by our recent observation documenting altered lipid substrate preferences for either PtdIns-3-P or PtdIns upon substitution of Lys-2000 or Lys-1999 in PIKfyve, respectively (35).

A key observation in the present study is the demonstration that PtdIns-5-P synthesis is profoundly decreased upon hypo-osmotic shock in 3T3-L1 fibroblasts and adipocytes. Because these cells showed no hyperosmotically dependent increase in PtdIns-5-P, but a substantially augmented PtdIns-4,5-P2 production, we suspect a concomitant activation of type II PIPK that should be tested in future studies. Whereas reports related to osmotically regulated PtdIns-5-P synthesis in mammalian cells are, to the best of our knowledge, unavailable, a recent study (19) in Chlamydomonas cells has demonstrated a rapid and sustained increase in PtdIns-5-P upon treatment with 300 mM NaCl for a period of 15 min. Intriguingly, in the same study, the PtdIns-4,5-P2 levels were back to normal at the 15-min time point (19), suggesting a less efficient type II PIPK-directed conversion of PtdIns-5-P in this cell system. Together, these data are consistent with the concept that acute changes in PtdIns-5-P levels are involved in the cellular osmoregulatory pathway, and this mechanism may be evolutionarily conserved. It is tempting to speculate that PtdIns-5-P, like other PIs, has a direct signaling potential and is able to selectively recruit specific yet-to-be identified protein intermediates with relevance to osmoprotective cell responses. Noteworthy, osmotic shock of 3T3-L1 adipocytes is shown to stimulate the glucose transport activity, translocation of GLUT4, and membrane ruffling. These cellular effects resemble those elicited by insulin, and studies indicate that these two stimuli share both common and unique signaling pathway mechanisms (36–38). It would be interesting in future studies to examine a plausible regulatory role of PtdIns-5-P in the molecular mechanism of osmotic shock- and insulin-induced responses in this cell type. Moreover, dominant-negative kinase-deficient PIKfyve K1831E has been shown recently to inhibit insulin-regulated GLUT4 translocation in 3T3-L1 adipocytes (39).

Our data demonstrating PIKfyve-dependent intracellular PtdIns-5-P production would imply a role for PIKfyve in osmotically regulating PtdIns-5-P levels. Although this assumption may be correct, the complex enzymology of PIs, including that of PtdIns-5-P, precludes us from making such a conclusion. Moreover, there is the unfavorable fact that we were unable to detect sustained osmotically dependent regulation in the in vitro PIKfyve lipid kinase activity. Theoretically, levels of PtdIns-5-P could decrease due to osmotically dependent inhibition of PIKfyve, PtdIns-4,5-P2 4-phosphatase, and PtdIns-3,5-P2 3-phosphatase activities or activation of type II PIPK and PtdIns-5-P phosphatase. However, no such phosphatases have been clearly described thus far (6–8). Also, it should be emphasized that hypo-osmotic shock in 3T3-L1 cells caused a substantial drop in accumulated $^{[32P]}$PtdIns-3,5-P2 and PtdIns-4,5-P2 amounts corresponding to 2.8- and 1.6-fold, respectively. These results and considerations eliminate the action of the first two hypothetical candidate phosphatases, making PIKfyve a more likely candidate for osmoregulation. Thus, the simultaneous inhibition of PtdIns-5-P and PtdIns-3,5-P2 production both associated with the dual lipid kinase activity of PIKfyve is consistent with the idea for a hypo-osmotic down-regulation of PIKfyve activity in vivo, although it remains to be experimentally proven in future studies.

In conclusion, we demonstrate here that PIKfyve lipid kinase activity is responsible for biosynthesis of PtdIns-5-P, a lipid whose natural occurrence was identified several years ago but whose kinase remained elusive. The turnover of PtdIns-5-P likely involves further conversion to PtdIns-4,5-P2 by type II PIPK, supporting the previously suggested alternative pathway of PtdIns-4,5-P2 biosynthesis. It is also possible that PtdIns-5-P is a precursor for an alternative production of PtdIns-3,5-P2 that would require the specificity of a separate PI 3-kinase. The fact that hypo-osmotic shock of 3T3-L1 cells induces a robust decrease of PtdIns-5-P levels indicates that PtdIns-5-P may be a regulatory intermediate in osmoprotective response pathways in mammalian cells.

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Phosphatidylinositol 5-Phosphate Biosynthesis Is Linked to PIKfyve and Is Involved in Osmotic Response Pathway in Mammalian Cells
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