Acquisition of Unprecedented Phosphatidylinositol 3,5-Bisphosphate Rise in Hyperosmotically Stressed 3T3-L1 Adipocytes, Mediated by ArPIKfyve-PIKfyve Pathway*

Diego Sbrissa and Assia Shisheva‡

From the Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201

Unlike yeast, where hyperosmotic stress induces a dramatic increase in phosphatidylinositol 3,5-bisphosphate (PtdIns 3,5-P$_2$) synthesis, in mammalian cells, although activating a complex array of signaling events, hyperosmotic stress fails to up-regulate PtdIns 3,5-P$_2$, indicating the PtdIns 3,5-P$_2$ pathway is not involved in mammalian osmo-protective responses. Here we report an unexpected and marked PtdIns 3,5-P$_2$ increase in response to hyperosmotic stress in differentiated 3T3-L1 adipocytes. Because this effect was not observed in the precursor preadipocytes, a specific role during acquisition of the adipocyte phenotype and transition into insulin-responsive cells could be suggested. However, acute insulin action did not result in a measurable PtdIns 3,5-P$_2$ rise, indicating the PtdIns 3,5-P$_2$ pathway is acutely operating in differentiated 3T3-L1 adipocytes. Hyperosmolarity activates different components of several kinase cascades, including p38 mitogen-activated protein kinases, and tyrosine kinases, but these appear to be separate from the activated PtdIns 3,5-P$_2$ pathway. Because PtdIns 3,5-P$_2$ is primarily produced by PIKfyve-catalyzed synthesis and requires the upstream activator hVac14 (called herein ArPIKfyve) that physically associates with and activates PIKfyve, we examined the contribution of ArPIKfyve-PIKfyve for the hyperosmotic stress-induced rise in PtdIns 3,5-P$_2$. Small interfering RNA-directed gene silencing to selectively deplete ArPIKfyve or PIKfyve in 3T3-L1 adipocytes determined the ArPIKfyve-PIKfyve axis fully accountable for the hyperosmotically activated PtdIns 3,5-P$_2$. Together these results reveal a previously uncharacterized PtdIns 3,5-P$_2$ pathway activated selectively in hyperosmotically stressed 3T3-L1 adipocytes and suggest a plausible role for PtdIns 3,5-P$_2$ in the osmo-protective response mechanism in this cell type.

Dynamic changes in the phosphorylation status of phosphatidylinositol (PtdIns)$^1$ in eukaryotic cells regulate diverse cellular processes (for recent reviews, see Refs. 1–8). In most cases the membrane-localized PI signals serve to recruit and activate enzymes or intermediate proteins through interaction with distinct lipid-binding protein modules displayed by the effector molecules. PtdIns 3,5-P$_2$, one of the seven Pls, is widespread in eukaryotes but is present only in minute quantity, comprising as little as 0.8% of total Pls (9, 10). Its identification goes back ~15 years, when Auger et al. (11) detected a compound with a migration pattern of PtdIns 3,5-P$_2$ in metabolically labeled smooth muscle cells. Steady-state levels were detected thereafter in different mammalian cells such as fibroblasts, COS, Chinese hamster ovary, HEK293, platelets, and CTLL-2 T lymphocytes as well as in yeast and plants (9, 12–16). The enzymes responsible for PtdIns 3,5-P$_2$ synthesis were cloned and found to comprise an evolutionarily ancient gene family represented by a single copy gene in most if not all species with sequenced genomes, with Fab1 in Saccharomyces cerevisiae and PIKfyve in mammals the most well studied (for reviews, see Refs. 3, 8, and 17). Another family of evolutionarily conserved proteins has been recently identified to serve as up-stream activators of Fab1 (Vac14; Ref. 18) or PIKfyve activities (hVac14; Ref. 19; referred to herein as ArPIKfyve, Associated regulator of PIKfyve) to control PtdIns 3,5-P$_2$ synthesis. Both specific and more promiscuous PtdIns 3,5-P$_2$-interacting protein modules have been determined in several candidate PtdIns 3,5-P$_2$ downstream effectors, including the PX domain in CISK and sorting nexin 1 (20, 21), the PH domain in centaurin $\beta_2$ (22), the GRAM domain in myotubularin (23, 24), and the ENTH (epsin N-terminal homology) domain in Emt3P (25). Specific binding has also been observed with proteins (Vps24, Syp1p, and WIP14; Refs. 26–28) that lack any of the characterized lipid binding domains (2–8), indicating the PtdIns 3,5-P$_2$-protein interaction may proceed under a mechanism involving yet-to-be identified PI binding pockets. Whereas the existence of multiple effector molecules may suggest multiple cellular functions for PtdIns 3,5-P$_2$, experimental evidence currently implicates the basal PtdIns 3,5-P$_2$ synthesis in the normal late-endosomes/multivesicular body organization and dynamics in both yeast and mammalian cells (3, 9, 29).

A hallmark of PI regulation in the budding yeast S. cerevisiae is the drastic rise of PtdIns 3,5-P$_2$ up to 20-fold above the steady-state levels, in response to hyperosmotic stress (13, 30, 31). Hyperosmotic stress increases of PtdIns 3,5-P$_2$ levels have also been observed in higher plants (16, 32). Intriguingly, hyperosmotic stress does not elevate PtdIns 3,5-P$_2$ in several mammalian cell types studied thus far, including COS-7 cells, CTLL-2 T lymphocytes, and likely others (13, 15, 30). It has been suggested, therefore, that the hyperosmotically activated PtdIns 3,5-P$_2$ pathway signals a protective response to rapid changes in osmolarity of the yeast or plant environment (for reviews, see Refs. 18 and 33). In mammalian cells, this mech-
anism likely degenerated as a result of the relatively constant extracellular osmotic potential. In this study we have revisited the role of PtdIns 3,5-P_2 in the osmo-regulatory response pathway in mammalian cells. Consistent with previous studies (13, 15, 30), we have observed from no change to a moderate decrease of PtdIns 3,5-P_2 levels in HEK293 cells or 3T3-L1 preadipocytes. Unexpectedly, in differentiated 3T3-L1 adipocytes hyperosmolarity profoundly increased PtdIns 3,5-P_2 levels in ArPIKfyve-PIKfyve-dependent fashion, suggesting a role for a previously uncharacterized PtdIns 3,5-P_2 pathway in the osmo-regulatory response in this cell type.

EXPERIMENTAL PROCEDURES

Cell Cultures, Cell Treatment, and Antibodies—3T3-L1 and HEK293 cells were maintained as detailed elsewhere (9, 34). Mouse 3T3-L1 fibroblasts were differentiated into adipocytes by a standard differentiation protocol detailed elsewhere (34). 3T3-L1 adipocytes were used between days 8 and 12 of the differentiation program, whereas 3T3-L1 fibroblasts or HEK293 cells were used on day 3 post-seeding when they reached confluence. Cells were labeled (see below) and then treated for the indicated time periods with sorbitol (0.6 M) or insulin (100 nM) in Dulbecco’s modified Eagle’s medium as described previously (35). Where indicated, labeled 3T3-L1 adipocytes were pretreated with genistein (300 mM; 20 min). Polyclonal anti-phospho-p38 MAP kinase antibodies were from Cell Signaling (Beverly, MA).

([32P]Orthophosphate Cell Labeling, Lipid Extraction, and HPLC—Cells (60-mm plates) were phosphate-serum-deprived for 1 h and then labeled with [32P]orthophosphate in phosphate-serum-free Dulbecco’s modified Eagle’s medium for 2.5 h at 37 °C as described previously (35). In siRNA experiments, after electroporation, 3T3-L1 adipocytes were seeded on 6-well plates and labeled (2.5 h) with [32P]orthophosphate 72 h post-electroporation subsequent to overnight starvation in serum-phosphate-free media supplemented with 0.5% bovine serum albumin. Lipids were extracted, deacylated, and analyzed on an HPLC column (Whatman 5-μm Partisphere SAX) as detailed elsewhere (9, 35). Column elution was monitored for H^3 radioactivity and area under the curve for the summed radioactivity from the labeled GroPIns 3-P, GroPIns 4-P, GroPIns 4,5-P_2, GroPIns 3,4-P_2, and GroPIns 3,5-P_2 peaks ("total radioactivity").

myo-[2-3H]inositol Labeling of 3T3-L1 Adipocytes—Fully differentiated 3T3-L1 adipocytes (60-mm plates) were maintained for 24 h in glucose- and inositol-free Dulbecco’s modified Eagle’s medium containing 0.5% bovine serum albumin, 5% dialyzed fetal bovine serum, 2 mM pyruvate, 25 mM HEPES, pH 7.4, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were then labeled for 40 h with 25 μCi/ml myo-[2-3H]inositol (PerkinElmer Life Sciences) in the above medium except for fetal bovine serum and antibiotics. Cells were then labeled and deacylated as described above. [3H]Labeled lipids were co-injected on the HPLC column (Whatman 5-μm Partisphere SAX) with the above-specified [32P]GroPIns as internal standards. [3H]GroPIns 4-P, [3H]GroPIns 4,5-P_2, and [3H]GroPIns 3-P were used as external HPLC standards. Fractions were collected every 0.25 min, and their H^3 and P^32 radioactivity was analyzed after the addition of scintillation mixture (ScintiVerse). Results were corrected for background radioactivity from the labeled GroPIns. Immunoblotting—Immunoblotting was performed subsequent to protein separation by SDS-PAGE and electrophoretic transfer onto nitrocellulose membranes as described previously (9, 34–36). Protein levels were quantified from the intensity of the bands with a laser scanner (Microtek) and UN-SCAN-IT software (Silk Scientific). Several films of different exposure times were quantified to assure the signals were within the linear range.

RESULTS

Unique Increases of PtdIns 3,5-P_2 in Differentiated 3T3-L1 Adipocytes by Hyperosmotic Stress—While examining the phosphoinositide profiles in fully differentiated mouse 3T3-L1 adipocytes and a possible acute regulation by extracellular stimuli, we have observed a dramatic increase in PtdIns 3,5-P_2 levels, reaching up to 10-fold, in response to acute hyperosmotic stress (Fig. 1). This result was quite surprising since previous studies in yeast and other mammalian cell types have documented that only the former, but not the latter, respond to hyperosmotic stress by a robust rise in PtdIns 3,5-P_2 levels (13, 15, 30). This unexpected elevation of PtdIns 3,5-P_2 accumulation was manifested in both [32P]orthophosphate-labeled (Fig. 1A) and myo-[3H]inositol-labeled 3T3-L1 adipocytes (Fig. 1C). As in yeast (13), the PtdIns 3,5-P_2 rise in 3T3-L1 adipocytes in response to hyperosmotic stress was transient, with a peak at about 10 min and a return toward the basal levels at ~60 min (Fig. 1C). Quantitation from five independent HPLC inositol-head-group analyses in [32P]orthophosphate-labeled 3T3-L1 adipocytes treated with sorbitol (10 min) estimated an average of a 3-fold increase in [32P]PtdIns 3,5-P_2 accumulation (Table I).

Fully differentiated 3T3-L1 adipocytes display not only quite different morphology from their fibroblastic precursors but also develop adipocyte-specific signaling pathways not observed in the precursor cell line. To assess the possibility that the elevated PtdIns 3,5-P_2 is part of a novel adipocyte-specific hyperosmotic regulatory mechanism, we examined the effect of hyperosmolarity on radiolabeled PtdIns 3,5-P_2 accumulation in 3T3-L1 preadipocytes metabolically labeled with [32P]orthophosphate just before initiation of the differentiation program. Remarkably, the precursor cell line not only failed to show a rise in the accumulated [32P]PtdIns 3,5-P_2 in response to hyperosmotic stress but even showed a 2-fold decline in [32P]PtdIns 3,5-P_2 (Table I). Whereas PtdIns 3,5-P_2 levels were changed in opposite directions, hyperosmolarity similarly increased the levels of PtdIns 3-P in both 3T3-L1 adipocytes and 3T3-L1 preadipocytes (Table I, Fig. 1). This result indicates that the increased [32P]PtdIns 3,5-P_2 accumulation results from an activation of a distinct pathway that operates specifically in hyperosmotically stressed differentiated 3T3-L1 adipocytes and does not simply mirror the concomitant PI 3-kinase activation and the rise of PtdIns 3-P, the substrate for PI 3-kinase. As such, the substrate for PI 3-kinase in differentiating 3T3-L1 adipocytes (13). PtdIns 3-P has been previously shown to increase in hyperosmotically stressed Swiss 3T3 mouse fibroblasts; however, PtdIns 3,5-P_2 was not examined in this study (38).

To relate the differential changes in PtdIns 3,5-P_2 accumulation seen in hyperosmotically stimulated 3T3-L1 adipocytes and preadipocytes to a different cell type, we subjected metabolically labeled HEK293 cells to a similar analysis. As illustrated in Table I, hyperosmotic stress in this cell line was associated with a tendency for a decrease in accumulated amounts of [32P]PtdIns 3,5-P_2, whereas levels of [32P]PtdIns 3-P remained practically unaltered. Noteworthy, whereas both the [32P]PtdIns 4-P and [32P]PtdIns 4,5-P_2 accumulation slightly changed by hyperosmolarity (1–10%; data not shown), the direction and magnitude of these alterations were roughly comparable among the 3T3-L1 adipocytes, 3T3-L1 preadipocytes, and HEK293 cells. Together these data indicate that the
FIG. 1. Hyperosmotic stress in 3T3-L1 adipocytes transiently elevates PtdIns 3,5-P$_2$. Serum-starved 3T3-L1 adipocytes (60-mm plates) on days 8–12 of the differentiation program were labeled with [32P]orthophosphate (A and B) or myo-[2-3H]inositol (C) as detailed under “Experimental Procedures.” Cells were then treated or not treated with 0.6 M sorbitol for 10 min (A and C) or for indicated time periods (B), and 32P-labeled lipids were extracted, deacylated, and co-injected with [3H]GroPIns 4-P, [3H]GroPIns 3-P, and [3H]GroPIns 4,5-P$_2$ as internal HPLC standards (elution times are indicated). The elution times of [32P]GroPIns 3,5-P$_2$ and [32P]GroPIns 3,4-P$_2$ standards (arrows, the latter is not seen in here) were determined from parallel HPLC runs. Radioactivity was monitored automatically with an online flow scintillation analyzer. Shown are HPLC elution profiles of a labeling experiment out of six experiments with similar results (A) and quantitation of the HPLC elution profiles from two independent time kinetics experiments of sorbitol treatment (B). For the quantitation, the PtdIns 3,5-P$_2$ was first calculated as percentage of the total radioactivity as described under “Experimental Procedures” and then expressed relatively to the sorbitol-untreated controls for the corresponding time period (B). C, extracted and deacylated 3H-labeled lipids were co-injected with [32P]GroPIns 3-P, [32P]GroPIns 3,5-P$_2$, and [32P]GroPIns 3,4-P$_2$ or [3H]GroPIns 4-P, [3H]GroPIns 3-P, and [3H]GroPIns 4,5-P$_2$ as internal or external standards, respectively (indicated). Fractions were collected every 0.25 min from 65 to 85 min (GroPInsP elution) and from 105 to 125 min (GroPInsP$_2$ elution). Fraction radioactivity was monitored for 3H and 32P radioactivity by liquid scintillation counting. Shown are HPLC elution profiles of a labeling experiment out of two experiments with similar results.
transition into the adipocyte phenotype is associated with the acquisition of a unique rise in PtdIns 3,5-P2 accumulation in response to acute hyperosmotic stress.

Similar Activation of p38 MAP Kinase in Different Mammalian Cell Types by Hyperosmotic Stress—Hyperosmotic stress is a potent inducer of p38 MAP kinase (39, 40). We, therefore, sought to link the differential hyperosmotic effect on the PtdIns 3,5-P2 levels observed among the cell types studied herein with a potential differential activation of p38 MAP kinase. However, we have observed a similar extent of the hyperosmotic-induced p38 MAP kinase activation in the three cell types as revealed by Western blotting with anti-phospho-p38-specific antibodies (Fig. 2). This result is consistent with the notion that the p38 pathway most likely does not govern the 3T3-L1 adipocyte-specific rise in PtdIns 3,5-P2 in response to hyperosmolality. This conclusion is in agreement with genetic studies in yeast (13), which failed to relate hyperosmotically induced PtdIns 3,5-P2 to activation of Hog1, the yeast ortholog of mammalian p38 MAP kinase.

ArPIKfyve-PIKfyve Axis in Hyperosmotically Increased PtdIns 3,5-P2 in 3T3-L1 Adipocytes—We have previously identified that steady-state levels of PtdIns 3,5-P2 produced by PIKfyve-catalyzed synthesis from PtdIns 3-P require an upstream activator, ArPIKfyve, that physically associates with and activates PIKfyve (9, 19). To examine whether the ArPIKfyve-PIKfyve axis is part of the molecular mechanism underlying the hyperosmotic response in 3T3-L1 adipocytes, we used an siRNA-directed gene silencing approach to selectively eliminate endogenous PIKfyve or ArPIKfyve proteins. Western blot analysis in 3T3-L1 adipocytes electroporated with either mouse PIKfyve or ArPIKfyve siRNA duplexes confirmed selective and substantial knockdown of the respective protein and no effect on the reciprocal one (Fig. 3 and not shown). Quantitation of immunoblots derived from three independent cell electroporation experiments revealed that endogenous PIKfyve or ArPIKfyve were depleted by 45–50 or 70–75%, respectively, without significant changes in other protein bands (Fig. 3). Under conditions of this selective attenuation of PIKfyve or ArPIKfyve protein expression, the HPLC inositol-head-group analysis documented a mild reduction of [32P]PtdIns 3,5-P2 steady-state levels, equal to 77 and 86%, respectively, of the control levels measured in cells treated with cyclophilin A siRNA duplexes (Fig. 4). Importantly, PIKfyve protein depletion by ∼50% completely eliminated the hyperosmotically dependent rise in PtdIns 3,5-P2 levels (Fig. 4). By contrast, the hyperosmotic stress-induced elevation of PtdIns 3-P persisted and even exceeded the control levels seen in stressed cells treated with cyclophilin A siRNAs (Fig. 4), apparently due to increased [32P]PtdIns 3-P accumulation in the absence of PIKfyve-directed conversion to PtdIns 3,5-P2. Abrogation of a hyperosmotic stress-rise in [32P]PtdIns 3,5-P2 accumulation was also seen upon selective attenuation of ArPIKfyve protein expression. This decrease, however, was to a lesser extent compared with that observed upon PIKfyve loss if normalized for the degree of the protein depletion (Fig. 4). Together, these results indicate that PIKfyve activity is responsible for the normal increase of PtdIns 3,5-P2 in response to hyperosmotic stress in 3T3-L1 adipocytes and that it requires an activator, ArPIKfyve, for a full effect.

**TABLE I**

| Treatment   | PtdIns 3,5-P2 | PtdIns 3-P |
|-------------|--------------|------------|
| 3T3-L1 adipocytes | % Total | % Total |
| Control     | 0.14 ± 0.05  | 1.11 ± 0.14|
| Sorbitol    | 0.42 ± 0.04  | 2.53 ± 0.37|

**FIG. 2.** p38 MAP kinase is activated to a similar extent in 3T3-L1 adipocytes, 3T3-L1 preadipocytes, and HEK293 cells. Indicated cell lines were serum-starved for 3 h and then treated with 0.6 M sorbitol (10 min) or left untreated. Lysates (60 μg of protein) were analyzed by SDS-PAGE and immunoblotting with anti-phospho-p38 MAP kinase antibodies. Shown are chemiluminescence detections of representative immunoblots out of two to three independent experiments in each cell line with similar results. The quantitation shows >50-fold increases of phospho-p38 MAP kinase for each cell line.

**FIG. 3.** Knockdown of endogenous PIKfyve and ArPIKfyve proteins by gene silencing in 3T3-L1 adipocytes, siRNA duplexes (0.6 nM) targeting specific regions of mouse PIKfyve, ArPIKfyve, or cyclophilin B (control) were used to transfect 3T3-L1 adipocytes by electroporation as indicated. Reseeded cells were then lysed 72 h after transfection. Equal amounts (100 μg of protein) were resolved by SDS-PAGE and analyzed by immunoblotting (WB) with the indicated antibodies. Shown are chemiluminescence detections of representative immunoblots out of three independent cell electroporation experiments with similar results.
For quantitation, the $[32P]$PtdIns 3,5-P$_2$ or PtdIns 3-P radioactive peaks untreated. $32P$-labeled lipids were extracted, deacylated, and co-injected with $[3H]$GroPIns 5-P, $[3H]$GroPIns 4-P, $[3H]$GroPIns 3-P, and $[3H]$GroPIns 4,5-P$_2$ as internal HPLC standards. $[32P]$GroPIns 3,5-P$_2$, and $[32P]$GroPIns 3,4-P$_2$ external standards were injected in parallel HPLC runs. Fractions, collected every 0.25 min, were monitored for $^3$H and $^32P$ radioactivity by liquid scintillation counting. $^32P$ radioactivity was plotted, and the counts within the elution times corresponding to the $[32P]$GroPIns peaks determined by the above standards were summed (total radioactivity). Shown is quantitation of HPLC elution profiles with respect to insulin-stimulated $[32P]$PtdIns 3,5-P$_2$ accumulation expressed as a percentage of the total $[32P]$PtdIns 3,5-P$_2$. Quantitation is from two to three independent experiments, with similar results for each time interval; mean ± S.E.

responsive cells, poses the question as to whether acute insulin action in 3T3-L1 adipocytes activates the PtdIns 3,5-P$_2$ pathway. Therefore, we determined the $[32P]$PtdIns 3,5-P$_2$ accumulation at various time periods (1–30 min) of insulin stimulation of fully differentiated 3T3-L1 adipocytes metabolically labeled with $[32P]$orthophosphate. HPLC inositol-head-group analysis of extracted lipids revealed that at no time interval was there a significant increase in the $[32P]$PtdIns 3,5-P$_2$-accumulated levels (Fig. 5). This result indicates that hyperosmotic stress, but not insulin, is able to selectively activate the PtdIns 3,5-P$_2$ pathway in 3T3-L1 adipocytes that could be detected by means of the total $[32P]$PtdIns 3,5-P$_2$ accumulation. The approach used could not rule out insulin-induced changes in localized PtdIns 3,5-P$_2$ production at distinct membrane microdomains. It has been known for many years that hyperosmolarity has a potent insulin-mimetic effect and affects glucose metabolism in fat and muscle (for recent reviews, see Refs. 8 and 43). In 3T3-L1 adipocytes the hyperosmotically activated glucose transport is due to translocation of the insulin-responsive GLUT4 glucose transporter to the cell surface. Although still elusive, the proximal signaling involved in the GLUT4 vesicles response to hyperosmolarity appears to be distinct from that induced by insulin. It includes tyrosine phosphorylation of a subset of proteins, distinct from the insulin receptor itself. Consistent with the dependence on tyrosine phosphorylation, hyperosmotically induced GLUT4 translocation and glucose transport have been found to be completely blocked if 3T3-L1 adipocytes were pretreated with genistein, a potent inhibitor of tyrosine kinases (Refs. 44 and 45 and this study; data not shown). To examine whether the hyperosmotically activated PtdIns 3,5-P$_2$ pathway in 3T3-L1 adipocytes is dependent on tyrosine kinases and related to GLUT4 vesicle dynamics, metabolically labeled cells were treated with genistein before the hyperosmotic stress. Intriguingly, HPLC profiles of deacylated lipids revealed that genistein did not prevent the dramatic accumulation of $[32P]$PtdIns 3,5-P$_2$ in response to hyperosmotic stress (Fig. 6). In contrast, the $[32P]$PtdIns 3-P rise was abolished (Fig. 6). This result is consistent with the notion that the osmotically activated PtdIns 3,5-P$_2$ (but not PtdIns 3-P) pathway operates independently of tyrosine phosphorylation cascades. The lack of inhibition also implies that the hyperosmotic rise in PtdIns 3,5-P$_2$ is either unrelated to the sorbitol-induced activation of glucose metabolism in 3T3-L1 adipocytes or, if involved, it precedes the genistein-sensitive step.
PtdIns 3,5-P₂ Rise in Hyperosmotically Stressed Adipocytes

The identification of PtdIns 3,5-P₂ as a ubiquitous but minor inositol phospholipid in cells of the yeast, plant, and animal kingdom (9–19) together with the discovery of multiple PtdIns 3,5-P₂ effector molecules (20–28) suggests PtdIns 3,5-P₂ is a signaling molecule in its own right. However, its intracellular dynamics and regulation in the context of mammalian cells remained largely enigmatic. To date, mitogenic signals such as interleukin-2 or UV light in CTLL-2 T lymphocytes, platelets, and EGF stimulation in COS cells have been reported to dramatically rise by hyperosmolarity, the mammalian cells examined thus far appear unresponsive (3, 13, 15, 30, 31). We demonstrate here a dramatic hyperosmotic-dependent elevation of PtdIns 3,5-P₂ that is uniquely manifested in differentiated 3T3-L1 adipocytes but not in the precursor 3T3-L1 fibroblastic line or HEK293 cells (Fig. 1, Table I). This result is consistent with the notion that acquisition of an adipocyte phenotype is associated with the development of a novel PtdIns 3,5-P₂ pathway that is activated to perceive and transduce osmotic stress.

One important mechanistic observation in our study is that the osmotically regulated increase in PtdIns 3,5-P₂ levels in 3T3-L1 adipocytes is fully dependent on the PIKfyve enzymatic activity and the PIKfyve activator, hVac14, that we recently identified (19) and refer to herein as ArPIKfyve. Thus, only 2-fold depletion of PIKfyve protein expression levels (Fig. 3) yielded a complete abrogation of the hyperosmotic increase in PtdIns 3,5-P₂, whereas the PtdIns 3,5-P₂ basal levels were decreased only slightly (Fig. 4). Likewise, about a 70% loss of ArPIKfyve protein, the up-stream associated regulator of PIKfyve activity, completely suppressed the hyperosmotic stress-induced PtdIns 3,5-P₂ rise (Figs. 3 and 4). Thus, the hyperosmotic stress response of elevated PtdIns 3,5-P₂ in 3T3-L1 adipocytes, like in S. cerevisiae (18), requires both PIKfyve and ArPIKfyve.

If ARPIKfyve and PIKfyve are evolutionarily conserved and widespread proteins, why is the hyperosmotic elevation of PtdIns 3,5-P₂ only a prerogative of 3T3-L1 adipocytes among all mammalian cell types examined thus far? One explanation may lie in the substantially higher levels of PIKfyve protein in this cell type versus the 3T3-L1 precursors or other cell types, which have been tested for PIKfyve protein expression (36, 46). Thus, as became apparent in this study, attenuation of endogenous PIKfyve protein expression to only ~50% fully abrogated the hyperosmotically induced PtdIns 3,5-P₂ elevation in 3T3-L1 adipocytes (Figs. 3 and 4). Alternatively, additional yet-to-be-identified molecular mechanisms that involve specific associations of ArPIKfyve with PIKfyve or with other proteins as well as intracellular compartmentalization of the protein complexes may operate uniquely in this cell type. This notion is supported by several lines of experimental evidence both in 3T3-L1 adipocytes and in yeast. Thus, no in vitro measurable activation of the PIKfyve lipid kinase activity in hyperosmotically stressed 3T3-L1 adipocytes has been observed (35). Next, PIKfyve expression from a yeast promoter in a S. cerevisiae strain harboring deletion of the PIKfyve-orthologous gene fab1 produced adequate basal levels of PtdIns 3,5-P₂ but did not support the hyperosmotically elevated levels (30). Likewise, Schizosaccharomyces pombe Fab1 complementation in the same S. cerevisiae strain provoked only a negligible stimulation in PtdIns 3,5-P₂ in response to hyperosmotic stress (30). Thus, whereas the ARPIKfyve-PIKfyve axis or its counterpart in yeast is absolutely necessary, additional regulatory elements and mechanisms are required to explain the detailed mechanism whereby the hyperosmotic stress-induced elevation of PtdIns 3,5-P₂ is an attribute of only 3T3-L1 adipocytes.

It is not clear at present what is the physiological significance of elevated PtdIns 3,5-P₂ for the hyperosmotically stressed 3T3-L1 adipocytes. One plausible explanation comes from studies in yeast where hyperosmotically elevated PtdIns 3,5-P₂, by increasing the vacuole surface area to volume ratio, is thought to protect cells against rapid dehydration and lysis (18). We therefore speculate that, much as in yeast cells, the PtdIns 3,5-P₂ rise in 3T3-L1 adipocytes is one of the mechanisms that promotes an osmo-protective response against cell volume loss. The cell specificity of this mechanism is likely determined by the distinct 3T3-L1 adipocyte morphology. Unlike 3T3-L1 fibroblasts and other mammalian cells that do not hyperosmotically elevate PtdIns 3,5-P₂, adipocytes are fat-laden cells whose cytoplasm constitutes only a negligible amount of the cell volume (47). Thus, relatively disadvantaged on the basis of cytoplasmic volume compared with fibroblastic or other cell types, the 3T3-L1 adipocytes may have acquired a specific although evolutionarily ancient mechanism of activating the PtdIns 3,5-P₂ pathway to regulate cell volume loss. Whether this mechanism plays a role in patients with severe diabetic conditions such as hyperglycemic and hyperosmotic coma and the associated dehydration are important questions to be addressed in future studies. In any case, the present results demonstrate a novel hyperosmotically activated PtdIns 3,5-P₂ pathway selectively operating in differentiated 3T3-L1 adipocytes among all mammalian cell types tested thus far.

DISCUSSION

The identification of PtdIns 3,5-P₂ as a ubiquitous but minor inositol phospholipid in cells of the yeast, plant, and animal kingdom (9–19) together with the discovery of multiple PtdIns 3,5-P₂ effector molecules (20–28) suggests PtdIns 3,5-P₂ is a signaling molecule in its own right. However, its intracellular dynamics and regulation in the context of mammalian cells remained largely enigmatic. To date, mitogenic signals such as interleukin-2 or UV light in CTLL-2 T lymphocytes, the protein kinase C-activating agent phorbol 12-myristate 13-acetate in interleukin-2 or UV light in CTLL-2 T lymphocytes, platelets, and EGF stimulation in COS cells have been reported to dramatically rise by hyperosmolarity, the mammalian cells examined thus far appear unresponsive (3, 13, 15, 30, 31). We demonstrate here a dramatic hyperosmotic-dependent elevation of PtdIns 3,5-P₂ that is uniquely manifested in differentiated 3T3-L1 adipocytes but not in the precursor 3T3-L1 fibroblastic line or HEK293 cells (Fig. 1, Table I). This result is consistent with the notion that acquisition of an adipocyte phenotype is associated with the development of a novel PtdIns 3,5-P₂ pathway that is activated to perceive and transduce osmotic stress.

One important mechanistic observation in our study is that the osmotically regulated increase in PtdIns 3,5-P₂ levels in 3T3-L1 adipocytes is fully dependent on the PIKfyve enzymatic activity and the PIKfyve activator, hVac14, that we recently identified (19) and refer to herein as ArPIKfyve. Thus, only 2-fold depletion of PIKfyve protein expression levels (Fig. 3) yielded a complete abrogation of the hyperosmotic increase in PtdIns 3,5-P₂, whereas the PtdIns 3,5-P₂ basal levels were decreased only slightly (Fig. 4). Likewise, about a 70% loss of ArPIKfyve protein, the up-stream associated regulator of PIKfyve activity, completely suppressed the hyperosmotic stress-induced PtdIns 3,5-P₂ rise (Figs. 3 and 4). Thus, the hyperosmotic stress response of elevated PtdIns 3,5-P₂ in 3T3-L1 adipocytes, like in S. cerevisiae (18), requires both PIKfyve and ArPIKfyve.

If ARPIKfyve and PIKfyve are evolutionarily conserved and widespread proteins, why is the hyperosmotic elevation of PtdIns 3,5-P₂ only a prerogative of 3T3-L1 adipocytes among all mammalian cell types examined thus far? One explanation may lie in the substantially higher levels of PIKfyve protein in this cell type versus the 3T3-L1 precursors or other cell types, which have been tested for PIKfyve protein expression (36, 46). Thus, as became apparent in this study, attenuation of endogenous PIKfyve protein expression to only ~50% fully abrogated the hyperosmotically induced PtdIns 3,5-P₂ elevation in 3T3-L1 adipocytes (Figs. 3 and 4). Alternatively, additional yet-to-be-identified molecular mechanisms that involve specific associations of ArPIKfyve with PIKfyve or with other proteins as well as intracellular compartmentalization of the protein complexes may operate uniquely in this cell type. This notion is supported by several lines of experimental evidence both in 3T3-L1 adipocytes and in yeast. Thus, no in vitro measurable activation of the PIKfyve lipid kinase activity in hyperosmotically stressed 3T3-L1 adipocytes has been observed (35). Next, PIKfyve expression from a yeast promoter in a S. cerevisiae strain harboring deletion of the PIKfyve-orthologous gene fab1 produced adequate basal levels of PtdIns 3,5-P₂ but did not support the hyperosmotically elevated levels (30). Likewise, Schizosaccharomyces pombe Fab1 complementation in the same S. cerevisiae strain provoked only a negligible stimulation in PtdIns 3,5-P₂ in response to hyperosmotic stress (30). Thus, whereas the ARPIKfyve-PIKfyve axis or its counterpart in yeast is absolutely necessary, additional regulatory elements and mechanisms are required to explain the detailed mechanism whereby the hyperosmotic stress-induced elevation of PtdIns 3,5-P₂ is an attribute of only 3T3-L1 adipocytes.

It is not clear at present what is the physiological significance of elevated PtdIns 3,5-P₂ for the hyperosmotically stressed 3T3-L1 adipocytes. One plausible explanation comes from studies in yeast where hyperosmotically elevated PtdIns 3,5-P₂, by increasing the vacuole surface area to volume ratio, is thought to protect cells against rapid dehydration and lysis (18). We therefore speculate that, much as in yeast cells, the PtdIns 3,5-P₂ rise in 3T3-L1 adipocytes is one of the mechanisms that promotes an osmo-protective response against cell volume loss. The cell specificity of this mechanism is likely determined by the distinct 3T3-L1 adipocyte morphology. Unlike 3T3-L1 fibroblasts and other mammalian cells that do not hyperosmotically elevate PtdIns 3,5-P₂, adipocytes are fat-laden cells whose cytoplasm constitutes only a negligible amount of the cell volume (47). Thus, relatively disadvantaged on the basis of cytoplasmic volume compared with fibroblastic or other cell types, the 3T3-L1 adipocytes may have acquired a specific although evolutionarily ancient mechanism of activating the PtdIns 3,5-P₂ pathway to regulate cell volume loss. Whether this mechanism plays a role in patients with severe diabetic conditions such as hyperglycemic and hyperosmotic coma and the associated dehydration are important questions to be addressed in future studies. In any case, the present results demonstrate a novel hyperosmotically activated PtdIns 3,5-P₂ pathway selectively operating in differentiated 3T3-L1 adipocytes among all mammalian cell types tested thus far.
Acknowledgments—We thank Linda McCraw for the excellent secretarial assistance. We thank Chris Tierney and Rohaizah James, Dharmacorn, for the helpful discussions of the siRNA approach.

REFERENCES
1. Vanhaesbroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J., and Waterfield, M. D. (2001) Annu. Rev. Biochem. 70, 535–622
2. Corvera, S. (2001) Traffic 2, 859–866
3. Odorizzi, G., Babst, M., and Emr, S. D. (2000) Trends Biochem. Sci. 25, 229–234
4. Simonsen, A., Wurmser, A. E., Emr, S. D., and Stennard, H. (2001) Curr. Opin. Cell Biol. 13, 485–492
5. Hurley, J. H., and Meyer, T. (2001) Curr. Opin. Cell Biol. 13, 146–152
6. Wishart, M. J., Taylor, G. S., and Doxn, J. E. (2001) Cell 105, 817–820
7. Czech, M. P. (2001) Ann. Rev. Physiol. 65, 791–815
8. Shisheva, A. (2003) Front. Biosci. s845–s867
9. Ikonomov, O. C., Shrissa, D., and Shisheva, A. (2001) J. Biol. Chem. 276, 26141–26147
10. Rudge, S. A., Anderson, D. M., and Emr, S. D. (2004) Mol. Biol. 15, 24–36
11. Auger, K. R., Serunian, L. A., Sotloff, S. P., Libby, P., and Cantley, L. C. (1997) Biochem. J. 323, 597–601
12. McEwen, R. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J., and Michell, T. H. (1997) Nature 390, 158–159
13. Baglin, H., Downes, C. P., and Rittenhouse, S. E. (1998) J. Biol. Chem. 273, 11630–11637
14. Jones, D. R., Gonzalez-Garcia, A., Diaz, E., Martinez-A, C., Carrera, A. C., and Merida, I. (1999) J. Biol. Chem. 274, 18407–18413
15. Meijer, H. J. G., Divecha, N., van den Ende, H., Musgrave, A., and Munnik, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 100, 3024–3028
16. Weisman, L. S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 12177–12182
17. Tsujita, K., Itoh, T., Ijuin, T., Yamamoto, A., Shisheva, A., Laporte, J., and Takenawa, T. (2004) J. Biol. Chem. 279, 13817–13824
18. Izumi, S., Iwakura, Y., Bellet, M. F., and Nourissin, D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10767–10772
19. Whiteley, P., Reaves, B. J., Hashimoto, M., Riley, A. M., Potter, B. V. L., and Holman, G. D. (2003) J. Biol. Chem. 278, 33786–33795
20. Dow, S. K., Piper, R. C., McEwen, R. K., Ya, J. W., King, M. C., Hughes, D. C., Thuring, J., Holmes, A. B., Cooke, F. T., Michell, R. H., Parker, P. J., and Lemmon, M. A. (2004) EMBO J. 23, 1922–1933
21. Jeffries, T. R., Doe, S. K., Michell, R. H., and Parker, P. J. (2004) Mol. Biol. Cell 15, 2652–2663
22. Ikonomov, O. C., Shrissa, D., Fari, M., Carpenter, J.-L., and Shisheva, A. (2003) Mol. Biol. Cell 14, 4581–4591
23. McEwen, R. K., Doe, S. K., Cooke, F. T., Painter, G. F., Holmes, A. B., Shisheva, A., Ohya, Y., Parker, P. J., and Michell, R. H. (1999) J. Biol. Chem. 274, 33905–33912
24. Visani, D., J. A., Nau, J. J., Dauer, E. J., Brinkman, M., Wurmser, A. E., Gary, J. D., Emr, S. D., and Weisman, L. S. (2002) J. Cell Biol. 156, 1015–1028
25. Zong, L., and Munnik, T. (2004) Plant Physiol. 134, 813–823
26. Meijer, H. J. G., and Munnik, T. (2003) Annu. Rev. Plant Biol. 4, 35–60
27. Shisheva, A., Shrissa, D., and Ikonomov, O. (1999) Mol. Cell. Biol. 19, 623–634
28. Broom, D., Ikonomov, O. C., Deeb, R., and Shisheva, A. (2002) J. Biol. Chem. 277, 47289–47294
29. Shisheva, A., and Shinoda, N. (1999) J. Biol. Chem. 274, 21589–21597
30. Zhang, Z. Y., Zhou, Q. L., Coleman, K. A., Chouinard, M., Bese, Q., and Czech, M. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7569–7574
31. Van Der Kaay, J., Beck, M., Gray, A., and Downes, C. P. (1999) J. Biol. Chem. 274, 35963–35968
32. Nance, M., Flavell, R. A., and Davis, R. A. (2000) Free Radic. Biol. Med. 28, 1328–1337
33. Kyriakis, J. M., and Avruch, J. (2001) Physiol. Rev. 81, 807–869
34. Rosen, E. D., and Spiegelman, B. M. (2000) Annu. Rev. Cell Dev. Biol. 16, 145–171
35. Accili, D., and Taylor S.T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4708–4712
36. Guan, P., Le Marchand-Brustel, Y., and Tanti, J. F. (2003) Diabetes Metab. 29, 565–576
37. Chen, D., Elmdendorf, J. S., Olsen, A. L., Li, X., Earp, H. S., and Pessin, J. E. (1997) J. Biol. Chem. 272, 27401–27410
38. Jin, X., Kear, K., Teasdale, R. D., Shimizu, T., Sharma, P. M., and Olefsky, J. M. (2000) J. Biol. Chem. 275, 26870–26876
39. Shisheva, A., DeMarco, C., Ikonomov, O. C., and Shrissa, D. (2002) in Insulin Signaling: From Cultured Cells to Animal Models (Grunberger, G., and Zick, Y., eds) pp. 189–205, Taylor & Francis Ltd., London
