The Identification of Phosphatidylinositol 3,5-bisphosphate in T-lymphocytes and Its Regulation by Interleukin-2*

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In recent times 3-phosphoinositides have emerged as important regulators of cell metabolism, survival, and proliferation. During the last year, the phospholipid phosphatidylinositol 3,5-bisphosphate (PtdIns3,5P2) was identified in yeast, fibroblasts, SV40-transformed kidney (COS-7) cells, and platelets. The discovery of this novel phospholipid has increased the complexity of the metabolism relating to the generation of biologically active inositol-containing lipids. We describe here the metabolism relating to the generation of biologically novel phospholipid has increased the complexity of the kidney (COS-7) cells, and platelets. The discovery of this was identified in yeast, fibroblasts, SV40-transformed lymphocyte cell line using two in vivo radiolabeling protocols. Treatment of the cells with UV radiation led to an increase in the cellular content of PtdIns3,5P2. In contrast, preincubation of the cells with wortmannin or treatment with hypertonic medium (high concentration sorbitol) led to the opposite effect. Herein we demonstrate that interleukin-2 (IL-2), the growth factor required for CTL-L2 cell proliferation, was able to increase the level of PtdIns3,5P2 with similar kinetics to that of the formation of phosphatidylinositol 3,4-bisphosphate (PtdIns3,4P2). An increase in this novel 3-phosphorylated lipid in response to IL-2 seems to be a general property of this cytokine because a similar result was obtained when the pre-B cell line BaF3 expressing the high affinity IL-2 receptor was used. Using a constitutively active regulatory subunit of type I phosphatidylinositol 3-kinase and cells expressing a deletion of the serine-rich domain of the IL-2 receptor β chain, which is required for IL-2-stimulated type I phosphatidylinositol 3-kinase activation, we demonstrate that IL-2-induced generation of PtdIns3,5P2 is related to the activation of this enzyme. The results show for the first time the identification of PtdIns3,5P2 in both T- and B-lymphocytes and indicate its positive regulation by the mitogen IL-2.

Phosphoinositides represent a small (less than 5%) proportion of the total cell phospholipids, yet they play crucial roles in the regulation of cell metabolism through their involvement in intracellular signaling mechanisms. In the last decade there has been a large increase in the number of reports describing the phosphorylation on the 3'-hydroxyl of inositol giving rise to a small family of 3-phosphorylated phosphoinositides consisting of phosphatidylinositol 3-phosphate (PtdIns3P),1 PtdIns3,4P2, and phosphatidylinositol 3,4,5-trisphosphate (PtdIns3,4,5P3). These 3-phosphorylated phosphoinositides are not substrates for activated phospholipase C isoenzymes, but they have biological activity per se as they are able to activate downstream effectors such as PKB/Akt and modulate several metabolic processes (1–3). Currently there are three families of mammalian PI3-K enzymes (type I, II, and III), the former two consisting of multiple isoforms (4). The substrate specificities of the PI3-Ks show some differences. Type I PI3-Ks preferentially phosphorylate PtdIns4,5P2 (over PtdIns and phosphatidylinositol 4-phosphate (PtdIns4P) in vivo), whereas the type II PI3-Ks prefer PtdIns and PtdIns4P as substrates, and PtdIns4,5P2 is poorly used (6, 7). Type III PI3-K is only able to phosphorylate PtdIns (8). Additionally, other PtdIns kinases involved in the regulation of the levels of 3-phosphorylated phosphoinositides have been characterized; these include isoforms of the PtdIns4P 5-kinase family which are able to synthesize the 3-phosphoinositides PtdIns3,4P2 and PtdIns3,4,5P3 (9) and the unusual phospholipids PtdIns3,5P2 and phosphatidylinositol 5-phosphate (10). One of the latest developments in the phosphoinositide field has been the identification in vivo of the novel phospholipid PtdIns3,5P2 in yeast, fibroblasts, COS-7 cells, and platelets (11–13). PtdIns3,5P2 has been proposed to arise from PtdIns3P via a PtdIns3P 5-kinase (11, 12), which, in the case of mammalian cells, is a wortmannin-sensitive process (11). Studies with yeast and COS-7 cells have indicated that PtdIns3,5P2 might be an intracellular signaling molecule involved in controlling responses to stress (12).

IL-2 is the growth factor responsible for the proliferation of T-cells. Its signal transduction mechanisms have been studied in depth revealing that multiple signal transduction cascades are initiated after just seconds of receptor ligation (14). IL-2 does not cause the activation of the classical PtdIns cycle (15–17), but instead is a strong activator of the PI3-K pathway (18–20), which has been shown to be crucial for cell prolifera-

1 The abbreviations used are: PtdIns3P, phosphatidylinositol 3-phosphate; PtdIns3,5P2, phosphatidylinositol 3,5-bisphosphate; PtdIns3,4P2, phosphatidylinositol 3,4-bisphosphate; PtdIns4,5P2, phosphatidylinositol 4,5-bisphosphate; PtdIns4,5P3, phosphatidylinositol 4,5,6-trisphosphate; PtdIns5P, phosphatidylinositol 5-phosphate; PtdIns6P, phosphatidylinositol 6-phosphate; PtdIns7P, phosphatidylinositol 7-phosphate; GroPIns, glycerophosphoinositol; GroPIns3P, glycerophosphoinositol 3-phosphate; GroPIns4P, glycerophosphoinositol 4-phosphate; GroPIns4,5P2, glycerophosphoinositol 4,5-bisphosphate; GroPIns4,5,6P3, glycerophosphoinositol 4,5,6-trisphosphate; GroPIns5P, glycerophosphoinositol 5-phosphate; GroPIns6P, glycerophosphoinositol 6-phosphate; GroPIns7P, glycerophosphoinositol 7-phosphate; GroPIns8P, glycerophosphoinositol 8-phosphate; GroPIns9P, glycerophosphoinositol 9-phosphate; GroPIns10P, glycerophosphoinositol 10-phosphate; GroPIns11P, glycerophosphoinositol 11-phosphate; GroPIns12P, glycerophosphoinositol 12-phosphate; GroPIns13P, glycerophosphoinositol 13-phosphate; GroPIns14P, glycerophosphoinositol 14-phosphate; GroPIns15P, glycerophosphoinositol 15-phosphate; GroPIns16P, glycerophosphoinositol 16-phosphate; GroPIns17P, glycerophosphoinositol 17-phosphate; GroPIns18P, glycerophosphoinositol 18-phosphate; GroPIns19P, glycerophosphoinositol 19-phosphate; GroPIns20P, glycerophosphoinositol 20-phosphate; GroPIns21P, glycerophosphoinositol 21-phosphate; GroPIns22P, glycerophosphoinositol 22-phosphate; GroPIns23P, glycerophosphoinositol 23-phosphate; GroPIns24P, glycerophosphoinositol 24-phosphate; GroPIns25P, glycerophosphoinositol 25-phosphate; GroPIns26P, glycerophosphoinositol 26-phosphate; GroPIns27P, glycerophosphoinositol 27-phosphate; GroPIns28P, glycerophosphoinositol 28-phosphate; GroPIns29P, glycerophosphoinositol 29-phosphate; GroPIns30P, glycerophosphoinositol 30-phosphate; GroPIns31P, glycerophosphoinositol 31-phosphate; GroPIns32P, glycerophosphoinositol 32-phosphate; GroPIns33P, glycerophosphoinositol 33-phosphate; GroPIns34P, glycerophosphoinositol 34-phosphate; GroPIns35P, glycerophosphoinositol 35-phosphate; GroPIns36P, glycerophosphoinositol 36-phosphate; GroPIns37P, glycerophosphoinositol 37-phosphate; GroPIns38P, glycerophosphoinositol 38-phosphate; GroPIns39P, glycerophosphoinositol 39-phosphate; GroPIns40P, glycerophosphoinositol 40-phosphate; GroPIns41P, glycerophosphoinositol 41-phosphate; GroPIns42P, glycerophosphoinositol 42-phosphate; GroPIns43P, glycerophosphoinositol 43-phosphate; GroPIns44P, glycerophosphoinositol 44-phosphate; GroPIns45P, glycerophosphoinositol 45-phosphate; GroPIns46P, glycerophosphoinositol 46-phosphate; GroPIns47P, glycerophosphoinositol 47-phosphate; GroPIns48P, glycerophosphoinositol 48-phosphate; GroPIns49P, glycerophosphoinositol 49-phosphate; GroPIns50P, glycerophosphoinositol 50-phosphate; GroPIns51P, glycerophosphoinositol 51-phosphate; GroPIns52P, glycerophosphoinosito
tion (21) and activation of the transcription factor E2F via PKB/Akt (22). IL-2 stimulation of T-cells induces the rapid elevation of PtdIns3,4P2 and PtdIns3,4,5P3, but to this date, no analysis of PtdIns3,5P2 either in resting or proliferating lymphocytes has been reported. The present study describes the presence of PtdIns3,5P2 in both T- and B-lymphocytes. We also report here for the first time that mitogenic stimulation by IL-2 increases the level of this novel PtdInsP2 isofrom.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human recombinant IL-2 was a generous gift from Hoffmann-LaRoche, Inc. (Nutley, NJ). [3H]Orthophosphate, [γ-32P]ATP, myo-2-[3H]PtdIns, myo-2-[3H]PtdIns4P, myo-2-[3H]PtdIns4,5P2, and myo-2-[3H]inositol were purchased from Amersham Pharmacia Biotech. myo-2-[3H]inositol-1,4-bisphosphate and myo-2-[3H]inositol-1,4,5-trisphosphate were obtained from NEN Life Science Products. Fetal calf serum and all cell culture media/supplements were bought from Life Technologies, Inc. Bovine serum albumin (fraction V), 1000 Da molecular-cutoff fetal calf serum, ammonium hydrogen sulfate, unlabeled myo-inositol, PtdIns, PtdIns4P, PtdIns4,5P2, and Wortmannin were from Sigma. Anti-p85 antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). All organic solvents were supplied from Merck (Darmstadt, Germany).

**Cell Culture, Radiolabeling, and Treatments**—CTLL-2 cells were maintained and grown for experiments according to previously published work (23, 24). When necessary, the CTLL-2 cells were transfected according to the protocol described by Jimenez et al. (24). Murine IL-3-dependent BaF/3 cells expressing the full-length human IL-2 receptor β chain (Baf/β) or a deletion mutant of the IL-2 receptor β chain lacking the serine-rich domain (amino acids 267-322) (Baf/SD1) were generated as described previously (21). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin buffered to a pH of 7.2 with 10 mM HEPES, with either 50 mM sodium hydrogen sulfate, or 10 mM HCl-conditioned medium as a source of IL-3, respectively. When cells reached a density of approximately 10^6/ml, they were washed twice in phosphate-free RPMI 1640 medium supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin buffered to a pH of 7.2 with 10 mM HEPES before re-incubating during a starving period of 6 h in the presence of 0.1% bovine serum albumin. For in vivo radiolabeling and radiolysis measurements, [2-3H]inositol was included in the medium during days 2–6 at a concentration of 10 μCi/ml. At the end of cell treatments, the cells were immediately frozen on dry ice. Approximately 15 × 10⁴ cells were used for each incubation/extraction, yielding a total incorporation of [32P] radioactivity into phospholipids of approximately 4.5 × 10⁶ cpm and 5 × 10⁵ cpm in the case of [3H].

**Analysis of Polyphosphoinositides by HPLC**—**In vivo** production of 3-phosphorylated lipids was determined essentially as described previously (39). Briefly, phospholipids were extracted using CHCl₃/MeOH/HCl in the presence of 5 mM EDTA, 5 mM tetraubutylammonium hydrogen sulfate, and 10 μg of a mixture of unlabeled PtdIns, PtdIns4P, and PtdIns4,5P2 to act as cold carriers. The isolated lower organic phases were washed once with freshly prepared artificial acidic aqueous methanolic top phase containing 5 mM EDTA and 5 mM tetraubutylammonium hydrogen sulfate. Washed organic phases were transferred into a screw-capped glass vial and dried under a gentle stream of nitrogen gas. 500 μl of freshly prepared methylene reagent (1-butanol, methanol, 25% aqueous methylamine, 11.5/45.7/42.8, v/v/v) containing 1 mM EDTA was added to the glass vials before heating at 53 °C for 1 h. After cooling to room temperature, the contents of the vials were transferred to an Eppendorf tube and vacuum-dried at room temperature. After resuspending the mixture in 500 μl of H₂O, fatty acids and any unacylated lipids were removed by washing twice with 500 μl of the freshly prepared solvent mixture consisting of 1-butanol, petroleum ether, ethyl formate (20:41, v/v/v). More than 95% of the radioactivity routinely partitioned into the aqueous phase, indicating almost complete phospholipid deacylation. The aqueous phase was vacuum-dried before storage at ~70 °C. Separation of all the deacylated phospholipids was performed by HPLC employing a Partisphere SAX column (4.6 × 235 mm, 5 μm, Whatman) with a gradient of 0–1 M ammonium phosphate, pH 7.2, over 120 min. The gradient consisted of 0–10 min 100% for pump A, 10–70 min linear rise to 25% for pump B, 70–120 min steep linear rise to 100% for pump B. The pump and column washout was from 120 to 130 min with 100% for pump A (H₂O). Radiolabeled deacylated phospholipids were detected by on-line radiochemical monitoring (Beckman Instruments, Inc., Fullerton, CA and EG & G Berthold, Bad Wildbad, Germany). Peak-associated radioactivity was expressed as % of total radioactivity detected to eliminate any inter-sample variation. 3-Phosphorylated phosphoinositide HPLC standards ([32P]GroPIns3P, [3H]GroPIns3,4P2, and [3H]GroPIns3,4,5P2) were prepared by the action of PI3-K (immunoprecipitated from 10⁸ CTLL-2 cells using an anti-p85 antibody) on the substrates PtdIns, PtdIns4P, and PtdIns4,5P2 in the presence of [γ-32P]ATP followed by their deacylation as described previously (24). In addition, [3H]GroPIns2, [3H]GroPIns4P, and [3H]GroPIns4,5P2 were prepared by deacylation of commercially available myo-2-[3H]PtdIns, myo-2-[3H]PtdIns4P, and myo-2-[3H]PtdIns4,5P2. Authentic [3H]GroPIns3,5P2 and [14C]GroPIns3,5P2 standards were provided by Dr. Stephen Deve and Prof. R. H. Michell, University of Birmingham (Birmingham, UK).

**RESULTS**

Using SAX-HPLC methodology, we were able to achieve base-line separation of the three radiolabeled deacylated isomers of PtdInsP2 ([3H]GroPIns3,5P2, [3H]GroPIns3,4P2, and [3H]GroPIns3,4,5P2) known at this time. This separation of the standards is shown in Fig. 1. Metabolic radiolabeling of CTLL-2 cells with [3H]Orthophosphate introduced radioactivity into GroPIns3,4P2 and the GroPIns4,5P2 isomer. In addition, radioactivity was also found in a third peak, which eluted approximately 2 min before that of the GroPIns4,5P2 isomer (Fig. 1). This peak coincided with the [3H]GroPIns3,5P2 standard and was well separated from the internal standards myo-2-[3H]inositol 1,4-bisphosphate, myo-2-[3H]inositol 4,5-bisphosphate and myo-2-[3H]inositol 1,4,5-trisphosphate which were also injected. This observation was completely reproducible and suggested that PtdIns3,5P2 was present in CTLL-2 cells. To confirm the identification of GroPIns3,5P2 in the
HPLC profile, we used long term myo-[3H]inositol metabolic labeling of CTLL-2 cells to show the presence of myo-inositol in the peak putatively identified as PtdIns3,5P$_2$. Indeed, 3H radioactivity was found in a peak co-eluting with an authentic [$^{14}$C]GroPIns3,5P$_2$ standard and before the [$^{32}$P]GroPIns3,4P$_2$ standard (data not shown). The structure of GroPIns3,5P$_2$ was confirmed by its deglyceration to Ins(1,3,5)P$_3$ and its separation from the standards Ins(1,3,4)P$_3$ and Ins(1,4,5)P$_3$ by isoocratic SAX-HPLC using 510 mM NaH$_2$PO$_4$ at 1 ml min (data not shown). Thus, on the basis of these preliminary experiments, we positively identified the presence of PtdIns3,5P$_2$ in CTLL-2 cells.

It has been reported that treatment of eukaryotic and prokaryotic cells with agents that induce stress result in the modulation of the levels of PtdIns3,5P$_2$ (12). To study the regulation of PtdIns3,5P$_2$ in CTLL-2 cells, we treated the cells with various defined agents to determine their effects on the level of PtdIns3,5P$_2$. PtdIns3P, PtdIns3,4P$_2$, and PtdIns3,4,5P$_3$. Cells that were treated with UV radiation for 20 min increased their PtdIns3,5P$_2$ by approximately 75% above control (Fig. 2). Furthermore, UV radiation caused an approximately 2-fold increase in the radioactivity associated with PtdIns3,5P$_2$, a small (15%) decrease in the level of PtdIns3,4P$_2$, and a large decrease (80%) in PtdIns3,4,5P$_3$ compared with control-treated cells (Fig. 3). In response to hypertonic shock (high concentration sorbitol), a decrease (to approximately 65% of control) in the radiolabeling associated with PtdIns3,5P$_2$ was observed (Fig. 2), which was in agreement with that seen in COS-7 cells (12). Fig. 3 also indicates that the action of sorbitol on the levels of PtdIns3,5P$_2$, PtdIns3,4P$_2$, and PtdIns3,4,5P$_3$ was slight, as only slight (10–30%) decreases in their levels were observed. In addition to agents that induce stress, the level of PtdIns3,5P$_2$ has also been shown to be sensitive to the PI3-K inhibitor wortmannin (11). To further characterize our cell system and to determine whether PtdIns3,5P$_2$ in CTLL-2 cells showed similar wortmannin sensitivity, we treated the cells with the PI3-K inhibitor. The radioactivity associated with PtdIns3,5P$_2$ fell to approximately 25% of control (Fig. 2). In addition, the levels of PtdIns3P, PtdIns3,4P$_2$, and PtdIns3,4,5P$_3$ were affected by treatment with wortmannin. The former two phospholipids...
suffered moderate decreases (between 20 and 50%), whereas PtdIns3,4,5P3 was reduced by more than 90% (Fig. 3). In the light of the discovery of PtdIns3,5P2 (11, 12), it has now become important to completely separate all three isomers (PtdIns3,5P2, PtdIns3,4P2, and PtdIns4,5P2) of PtdInsP2 to determine their cellular levels before and after cell stimulation. For that reason we decided to investigate whether IL-2 affected the level of PtdIns3,5P2 in CTLL-2 cells by using our SAX-HPLC system capable of high resolution PtdInsP2 isomer separation. Fig. 4 shows that IL-2 stimulated an early and transient increase in the PtdIns3,4,5P3 content of the cells (approximately 75% above control). This finding is in contrast to the slow rise in the amount of PtdIns3,4P2 (approximately a 2-fold increase above control at 20 min). As is the case for other mitogens, IL-2 caused no consistent perturbation of the amount of PtdIns3P in CTLL-2 cells at all the time points examined (data not shown). Our results agree with those reported by Remillard and co-workers (18). In response to IL-2, there was a gradual accumulation of PtdIns3,5P2 (approximately 75% above control at 20 min). This increase in PtdIns3,5P2 displayed kinetics similar to those in the accumulation of PtdIns3,4P2. The increase in PtdIns3,4,P2 at 20 min was approximately 50% higher than for PtdIns3,5P2.

To demonstrate that the mitogenic activity of IL-2 correlated with the generation of PtdIns3,5P2, we determined the accumulation of this novel lipid in Baf/3 cells expressing either the wild-type human β chain of the IL-2 receptor or a deletion of the serine-rich domain within the human β chain of the IL-2 receptor, which abolishes IL-2-stimulated PI3-K activity and cell proliferation. In Bafα/β cells, IL-2 caused an approximately 2-fold increase in the radioactivity associated with both PtdIns3,5P2 and PtdIns3,4P2 after 20 min of incubation, which was completely inhibited by wortmannin (Fig. 5). These IL-2-stimulated increases in PtdIns3,5P2 and PtdIns3,4P2 were not observed in Bafα/βSD1 cells (Fig. 5). Both types of cells responded to UV radiation in a manner similar to that seen in CTLL-2 cells by increasing the radioactivity found in the PtdIns3,5P2 fraction (approximately 2-fold) and decreasing by approximately 30% that found in PtdIns3,4P2 (Fig. 5). So far, the results suggested that IL-2-activated type I PI3-K activity was a prerequisite for an increase in the cellular PtdIns3,5P2 content. To consolidate these findings, we chose to take advantage of the recently described constitutively active regulatory subunit of type I PI3-K p65 (24). In vivo metabolic radiolabeling and HPLC analysis of p65-transfected cells indicated virtually no increases in PtdIns3P compared with wild-type cells, despite the increase in the levels of both PtdIns3,4P2 and PtdIns3,5P2 by approximately 50–60% (Fig. 6), which was in agreement with previous results (24). When the level of PtdIns3,5P2 was analyzed in CTLL-2 cells expressing p65, a
dramatic increase in the level of this lipid, more than 3-fold compared with empty vector-transfected cells, was observed (Fig. 7). When both cell types were stimulated with IL-2 for 20 min, the PtdIns3,5P2 content of empty vector-transfected cells and p65 vector-transfected cells was increased by approximately 80 and 110%, respectively (Fig. 7).

**DISCUSSION**

We have demonstrated for the first time the existence of an agonist (IL-2)-stimulated pathway leading to the accumulation of PtdIns3,5P2 in T- and B-lymphocytes. In addition to lymphocytes, PtdIns3,5P2 has been identified (11–13) and speculated to exist (25–27) in both yeast and other mammalian cells.

The mechanism for the formation of PtdIns3,5P2 appears to be through the enzymatic conversion of PtdIns3P by a 5-kinase. Evidence for this hypothesis has come from *in vivo* radiolabeling procedures indicating that the last phosphate group attached to the PtdIns3,5P2 is the group that has the highest specific activity (11, 12). Furthermore, this activity is, at least in mouse fibroblasts, sensitive to wortmannin (11). The responsible enzyme, Fab1p, has recently been described in yeast (28, 29). To date no information is available concerning its mammalian counterpart. Treatment of cells with UV radiation is considered to be a stress signal that immediately causes the activation of various enzymes including the c-Jun amino-terminal protein kinase (JNK) cascade (30). Because UV irradiation of cells is anti-mitogenic, it is consistent with the observation in our cells of a decrease in phospholipids considered to be mitogenic (PtdIns3,4P2 and PtdIns3,4,5P3). Our results indicated that the enzyme activated in response to UV radiation was a PI3-K specific for the phosphorylation of PtdIns, most likely a type III PI3-K. The rise in PtdIns3P was far greater than the rise in PtdIns3,5P2, which suggests that only a small proportion of the PtdIns3P is used for PtdIns3,5P2 synthesis, leaving the majority for other roles that could include regulation of endocytosis, membrane trafficking, and protein sorting (31, 32). Another possible role of the PtdIns3P generated in response to UV radiation could be that of the activation of the JNK cascade. In this regard, JNK activation has been shown to be mediated by a PI3-K exhibiting wortmannin/LY294002 sensitivity (33).

The proliferation of T-lymphocytes is a process primarily

**Fig. 6.** Stable p65-transfection increases the PtdIns3,4P2 and PtdIns3,4,5P3 content of CTLL-2 cells without affecting the level of PtdIns3P. Stable CTLL-2 transfected (both empty vector and p65 vector) cell lines were arrested and radiolabeled with [32P]orthophosphate. Phospholipids were extracted, deacylated, and analyzed by SAX-HPLC. Representative SAX-HPLC profiles corresponding to the regions containing PtdIns3P (from 38 to 42 min), PtdIns3,4P2 (from 81 to 84 min), and PtdIns3,4,5P3 (from 103 to 106 min) are shown in panels A, B, and C, respectively. In panel D the effect of p65 vector transfection on the levels of PtdIns3P, PtdIns3,4P2, and PtdIns3,4,5P3, with respect to empty vector-transfected cells are indicated. The results are mean ± S.D. (n = 3).

**Fig. 7.** Stable p65 transfection increases the PtdIns3,5P2 content of CTLL-2 cells. Stable CTLL-2-transfected (both empty vector and p65 vector) cell lines were arrested and radiolabeled with [32P]orthophosphate. After treatment with either control medium (20 min) or IL-2 (500 units/ml, 20 min), phospholipids were extracted, deacylated, and analyzed by SAX-HPLC. Representative SAX-HPLC profiles corresponding to the region containing PtdIns3,5P2 (from 78 to 81 min) after distinct cell treatments (see symbol key) are shown in panel A. In panel B, the percentage increases in the level of PtdIns3,5P2 with respect to empty vector transfection and control cell treatments are indicated. The results are mean ± S.D. (n = 2).
controlled by IL-2. It activates various signal transduction pathways including the ras-MAPK (mitogen-activated protein kinase), PI3-K/Akt, and Jak/STAT cascades (14). IL-2 is a strong stimulator of the PI3-K pathway, and it is essential for IL-2-driven cell proliferation. IL-2-stimulated PI3-K has been relatively well characterized and has involved the use of in vivo metabolic radiolabeling experiments to demonstrate increases in PtdIns3,4P2 and PtdIns3,4,5P3 (18–20). However, to date no analysis of PtdIns3,5P2 generation in lymphocytes in response to IL-2 has been performed. IL-2 stimulation of CTTL-2 cells also increases PtdIns3,5P2 levels, although the mechanism responsible for the elevation of this novel lipid is not completely clear. Results from our experiments using Bafα/β cells and Bafα/βSD1 cells have helped to clarify this point. Within the β chain of the IL-2 receptor is a serine-rich cytoplasmic domain that is responsible for the association of Jak1 (34). Deletion of this domain abolishes PI3-K activation following IL-2 binding (21, 35), probably because of the role of Jak1 in p85 association (36). In Bafα/βSD1 cells, no such domain is present, and no increase in the PtdIns3,5P2 content of the cells was found in response to IL-2 stimulation. This finding suggests that type I PI3-K activity is required for IL-2-stimulated PtdIns3,5P2 generation. As a consequence of irradiation with UV light, both cells were able to elevate the cellular level of PtdIns3,5P2 to approximately the same level, thereby demonstrating that their response to a non-receptor-mediated event was identical and fully independent of the type of IL-2 receptor β chain that they expressed. We have previously demonstrated that CTTL-2 cells stably transfected with a mutant form of the regulatory subunit of PI3-K, p65, induces constitutive activation of PI3-K (24). We have used this model herein to determine its effects on the accumulation of PtdIns3,5P2. In these cells the PtdIns3,5P2 content was elevated with respect to empty vector-transfected cells. In addition, IL-2 stimulation of these cells induces an approximately two-fold elevation of the level of PtdIns3,5P2, as it does in wild-type CTTL-2 cells. It can be concluded from these experiments that in CTTL-2 cells expressing a constitutively active allele of PI3-K, there is a constitutive elevation of the PtdIns3,5P2 level. This observation coincides with recent work by Klippel and co-workers (27) who have demonstrated that an inducible constitutively activated PI3-K was able to increase the levels of PtdIns3,4P2 and PtdIns3,4,5P3 and that of a peak in the HPLC profile putatively identified as PtdIns3,5P2. Interestingly, IL-2 stimulation of CTTL-2 and Bafα/β cells as well as control-treated p65-transfected CTTL-2 cells results in the generation of PtdIns3,5P2 without increases in PtdIns3P3. Although the increase in PtdIns3,5P2 provoked by IL-2 was approximately the same as seen in response to UV irradiation, the apparent lack of PtdIns3P3 production suggested that the pathway for the formation of PtdIns3,5P2 did not totally overlap that initiated by UV radiation, despite the fact that IL-2 stimulates JNK activation (37). Generation of PtdIns3P3 is usually attributable to type III PI3-K activity, which has been described as having constitutive activity in both resting and actively growing cells (4). It has been generally accepted that PtdIns is not a good substrate for type I PI3-K in vivo. These observations are based on the fact that no accumulation of PtdIns3P3 is detected following mitogen stimulation. However, when p65 is transiently expressed in COS-7 cells, its PI3-K activity is extremely high, which forces the in vivo elevation of PtdIns3P3, PtdIns3,4P2, and PtdIns3,4,5P3 levels.3 In this situation the degradation of PtdIns3P3 is delayed as compared with p65 stably transfected cells. This observation would suggest that the PtdIns3P3 generated in vivo following type I PI3-K activation, either by IL-2 receptor ligation or p65 cell transfection, is removed rapidly by one or more of the following enzymes: a PtdIns3P3 3-phosphatase, a PtdIns3P4 4-kinase, or a PtdIns3P5 5-kinase. It could be envisaged that in response to IL-2, and for that matter other ligands, no net increases in PtdIns3P3 are observed because of its rapid removal via one or more of the three enzymes mentioned above.

PtdIns3,5P2 represents a new member of the family of 3-phosphoinositides, which are known to be essential for the regulation of numerous cellular activities. However, to date PtdIns3P3 and PtdIns3,5P2 have not been assigned definite roles. Observations of the involvement of phosphoinositides during protein transduction and membrane reorganization or protein trafficking events are the possibilities so far proposed (31, 32, 38). PtdIns3,5P2 may be a strong candidate for a true second messenger. To substantiate this claim, work focusing on its generation through a PtdIns3P5 5-kinase, its subcellular site of synthesis, its possible relocalization, and its interaction with proteins, which may or may not contain pleckstrin homology domains (which bind polyphosphoinositides), will lead the way to understanding why cells have a requirement for yet another isomer of PtdInsP3, particularly in the case of mitogenic signals such as that delivered by IL-2 in T-lymphocytes.

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Phosphatidylinositol 3,5-bisphosphate in T-lymphocytes

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