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Modification of protein sub-nuclear localization by synthetic phosphoinositides: Evidence for nuclear phosphoinositide signaling mechanisms

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Introduction

Phosphoinositides (PtdInsPs) regulate diverse cellular functions that influence survival, growth, and proliferation, and dysregulated PtdInsP homeostasis is implicated in many disease processes (Cantley, 2002; Maehama et al., 2001; Payrastré et al., 2001). For example, in tumor tissues, the PtdInsP phosphatase gene, PTEN (phosphatase and tensin homologue deleted on chromosome 10; EC 3.1.3.67) is frequently mutated, whereas expression of the PtdInsP kinase, phosphatidylinositol 3-kinase (PI3K; EC 2.7.1.153) is often upregulated (Cantley, 2002). Mouse models have also implicated PI3K in the pathogenesis of diabetes and immunodeficiencies, and mutations in the myotubularin family of PtdInsP phosphatases have been linked to myopathies and neuropathies (Fruman and Cantley, 2002; Mauvais-Jarvis et al., 2002; Taylor et al., 2000). These and many other PtdInsP kinases and phosphatases generate seven different PtdInsP species, which are thought to mediate

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signaling pathways by modulating the sub-cellular localization and/or activity of PtdInsP-binding proteins (DiNitto et al., 2003; Toliás and Cantley, 1999; Vanhaesebroeck and Alessi, 2000).

The majority of study on PtdInsP regulatory mechanisms has centered on cytoplasmic processes. Nevertheless, there is a great deal of evidence that the functions of PtdInsPs in the nucleus are as diverse as their roles in the cytoplasm (Cocco, et al., 2000; Irvine, 2003; Jones and Divecha, 2004; Martelli, et al., 1992, 2002, 2003, 2004a, b; Ye et al., 2000). For instance, a number of clinically important PtdInsP kinases and phosphatases, including PI3K, PTEN and *src* homology 2 domain-containing inositol phosphatase 2 (SHIP2; EC number not identified), translocate into the nucleus, often upon activation (Deleris et al., 2003; Lachyankar et al., 2000; Neri et al., 2002). Specifically, PI3K translocates into the nucleus during its activation and the nuclear GTPase PIKE (EC number not identified) enhances its activity (Rong et al., 2003; Ye et al., 2002, 2000). In addition, SHIP2 and PTEN have been observed to partly reside in the nucleus and, for PTEN, this localization is specifically lost in some cancer tissue (Deleris et al., 2003; Ginn-Pease and Eng, 2003; Neri et al., 2002; Tachibana et al., 2002; Tanaka et al., 1999; Whiteman et al., 2002; Ye et al., 2000). Many other phosphoinositide kinases and phosphatases are reported to localize within the nucleus and moreover, a number of PtdInsP species, including PtdIns(3)P, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, have been directly observed to be intranuclear (Boronenkov et al., 1998; Chen et al., 2002; Deleris et al., 2003; Garcia-Bustos et al., 1994; Gillooly et al., 2000; Neri et al., 2002; Tabellini et al., 2003; Watt et al., 2002; Yokogawa et al., 2000). The presence of these molecules and their modifying enzymes in the nucleus argues that many nuclear events are regulated by, at present, undiscovered PtdInsP-signaling mechanisms.

Indeed, functional evidence has emerged that PtdInsPs modulate a number of nuclear processes (Martelli et al., 2004a). For example, PtdIns(4,5)P₂ induces association of the SWI/SNF-like BAF complex with chromatin in T cells during antigen stimulation and likely has a role in pre-mRNA splicing (Boronenkov et al., 1998; Osborne et al., 2001; Zhao et al., 1998). Finally, we have recently identified the plant homeodomain (PHD) finger, a protein domain commonly found on nuclear-localized, chromatin-regulatory proteins, as a nuclear PtdInsP receptor (Gozani et al., 2003). We have shown that interactions between the PHD finger of the candidate tumor suppressor ING2 and PtdIns(5)P regulate the ability of ING2 to associate with chromatin and induce p53-mediated apoptosis in response to genotoxic stimuli (Gozani et al., 2003). Thus, it is becoming abundantly clear that understanding the role of PtdInsPs in the nucleus is essential for understanding how altered PtdInsP homeostasis leads to disease.

One method of studying PtdInsP biology is to deliver synthetic PtdInsP analogs into living cells for chemical activation of functions normally mediated by endogenous PtdInsPs (Ozaki et al., 2000). This approach of “chemical rescue” has been used to discover and investigate diverse PtdInsP-biological activities; examples include activation of the proto-oncogene AKT/PKB, rescue of SHIP2 deficiency, trafficking of GLUT4, the establishment of cellular polarity, insulin-regulated F-actin stress fiber breakdown and golgi function (Derman et al., 1997; Franke et al.,

1997; Maffucci et al., 2003; Padron et al., 2003; Sbrissa et al., 2004; Scheid et al., 2002; Wang et al., 2002, 2003; Weiner et al., 2002). Moreover, the repertoire of potential chemical applications has been greatly expanded by the development of carrier systems that allow for fluorescent, biotinylated, spin-labeled or photo-activatable PtdInsP analogs to be introduced into cells (Ozaki et al., 2000). Previously, treatment of cells with exogenous PtdInsPs has not been employed to probe nuclear PtdInsP functions in live cells, primarily because there has not been a well-defined PtdInsP-binding domain that resides and functions in the nucleus. We now use the PHD finger as a physiologically relevant nuclear PtdInsP receptor to demonstrate that exogenous PtdInsPs can access and function within the nucleus of living cells.

Methods

Materials, plasmids and cell culture

All PtdInsPs were from Echelon Biosciences, Inc. (Salt Lake City, UT). Vectors: pEGFP-C and pDsRed2 (Clontech). BalbC-3T3 or NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin, and streptomycin, at 5% CO₂ at 37 °C. Transfections were carried out using LT1 (Mirus).

Confocal and time-lapse microscopy

Confocal microscopy was carried out as described (Gozani et al., 2003), using a BioRAD uRadiance2000 system. For PtdInsP treatment, BalbC-3T3 cells grown on coverslips were incubated in media containing the indicated concentration of PtdInsPs. After the incubation period, cells were washed with PBS and then fixed in 3.7% paraformaldehyde prior to analysis. Quantification was determined by counting multiple different fields in at least three independent experiments. Time-lapse microscopy was carried out as described (Kanai et al., 2001). Briefly, PtdInsPs were added directly to sub-confluent cultures to cells kept at 37 °C and images were taken every 30 s.

Synthesis of BODIPY-TR-PIPs

BODIPY-TR-PIP were synthesized with *sn*-2-O-palmitoyl and *sn*-1-O-(BODIPY-TR-aminohexanoyl) acyl chains to increase hydrophobicity to allow for self-shuttling into cells and enhanced native behavior in bilayers. (+)-1-O-[1-O-[6'-aminohexanoyl]-2-O-palmitoyl-*sn*-glycero-3-phosphoryl]-*myo*-inositol, 5-phosphate (1), or 3-phosphate (2), trisodium salt was prepared as previously described (Peng and Prestwich, 1998). Synthesis of BODIPY-TR labeled PtdIns(3)P, PtdIns(4)P and PtdIns(5)P (BODIPY-TR-PI(3)P; BODIPY-TR-PI(4)P; BODIPY-TR-PI(5)P): (+)-1-O-[1-O-[1-O-[6'-[6-[((4-(4,4-difluoro-5-(2thienyl)-4-bora-3a,4a-diaza-s-inda-

*cene-3-yl)phenoxy)acetyl)amino]hexanoyl]amino]hexanoyl]-2-O-palmitoyl-sn-glycero-3-phosphoryl]-myo-inositol 5-phosphate or 3-phosphate, trisodium salt. A solution of BODIPY-X-TR-SE (3.4 mg, 5.4 μ mol; Molecular Probes Inc.) in DMF (0.5 mL) was added to a solution of (1 or 2) (3.5 mg, 4.2 μ mol) in 0.25 M TEAB buffer (0.5 mL, pH 7.8) and stirred overnight at room temperature. The reaction mixture was dried and the residue was washed with ethyl acetate (5 \times 1.5 mL) to remove excess dye and *N*-hydroxysuccinimide. The crude product was dissolved in water (2 mL) and applied to a small column (12 \times 15 mm) of DEAE cellulose. The column was eluted with a step gradient (0.2 M steps) of 0.2 M to 2.0 M TEAB (2 mL portions) and finally with 3:7 MeOH:2 M TEAB. The product started to elute with 1.4 M TEAB and finished with the MeOH/TEAB mixture. After pooling and lyophilizing the desired fractions, the product was converted to the sodium salt with DOWEX 50 \times 8–100 resin (sodium form). The product was an indigo powder (yield: PI(5)P, 4.5 mg, 83%; PI(3)P, 4.0 mg, 74%).*

BODIPY-TR-PI(3)P: ^1H NMR (400 MHz, D_2O) δ 0.38–1.40 (m, 43H) 1.66–2.12 (6H), 2.61–2.99 (4H), 3.65 (quart, $J = 9.2$ Hz, 2H), 3.75–4.28 (10H), 5.08 (1H), 6.25–7.22 (8H), 7.57–7.96 (3H); ^{31}P NMR (161 MHz, $\text{D}_2\text{O}:\text{CH}_3\text{OD}$) δ 4.52, 0.48 (1:1); MALDI-MS m/z 1283.38 (M-H) $^-$.

BODIPY-TR-PI(4)P: ^1H NMR (400 MHz, D_2O) δ 0.57–1.55 (43H), 1.83–2.26 (6H), 2.81–3.09 (4H), 3.41 (t, $J = 8.8$ Hz, 1H), 3.60 (d, $J = 8.4$ Hz, 1H), 3.77 (t, $J = 11.2$ Hz, 1H), 3.86–4.38 (9H), 5.18 (1H), 6.42–7.14 (7H), 7.23 (2H), 7.78–7.95 (3H) ppm; ^{31}P NMR (161 MHz, $\text{D}_2\text{O}:\text{CD}_3\text{OD}$) δ 3.64, 0.53 (1:1) ppm; MALDI-MS m/z 1283.07 (M-H) $^-$.

BODIPY-TR-PI(5)P: ^1H NMR (400 MHz, $\text{D}_2\text{O}:\text{CD}_3\text{OD}$) δ 0.41–1.45 (m, 43H) 1.77 (2H), 1.87–2.06 (4H), 2.71–2.99 (4H), 3.40 (d, $J = 9.2$ Hz, 1H), 3.55–3.75 (3H), 3.77–4.22 (8H), 5.03 (1H), 6.28–7.21 (9H), 7.60–7.92 (3H); ^{31}P NMR (161 MHz, $\text{D}_2\text{O}:\text{CH}_3\text{OD}$) δ 4.55, 0.53 (1:1); MALDI-MS m/z 1283.41 (M-H) $^-$.

Results and discussion

Sub-nuclear targeting of the PtdInsP-binding PHD finger protein domain to nuclear foci

The majority of PHD-containing proteins are nuclear, and in many cases, mutations that disrupt the PHD finger have been shown to alter the sub-nuclear localization of these proteins (Sutherland et al., 2001). Consistently, we have previously reported that the fluorescent signal observed when the PHD finger of ING2 is fused to GFP (green fluorescent protein) is largely within the nucleus (Gozani et al., 2003). In addition, we have also previously found that endogenous ING2, which contains a nuclear localization signal (NLS) immediately upstream of the PHD finger, is also predominantly localized within the nucleus (see schematic Fig. 1A) (Gozani et al., 2003). Because the GFP-ING2_(PHD) fusion containing only the ING2 PHD finger (amino acids 200–280) is not entirely nuclear, we constructed GFP-ING2_(NLSPHD) (amino acids 180–280 of ING2), which includes the ING2 PHD

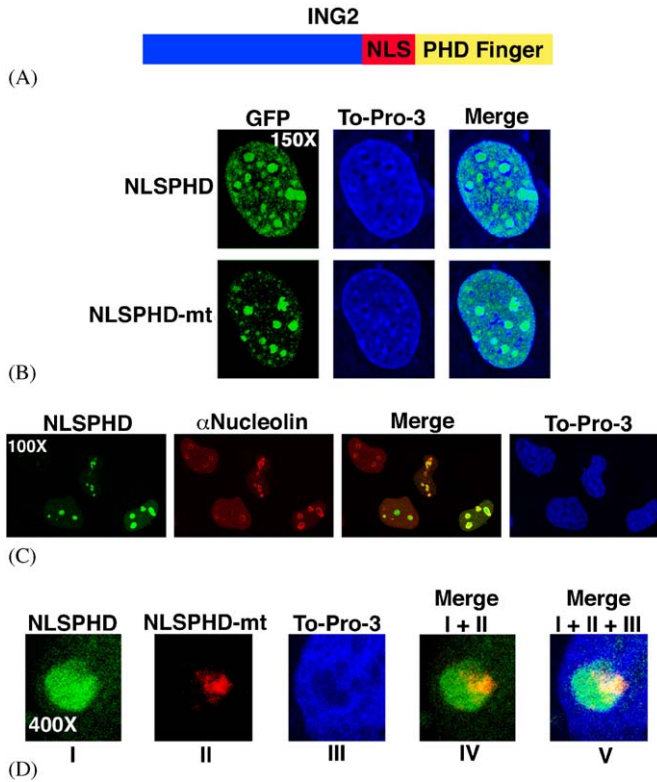


Fig. 1. GFP-ING2 NLS+PHD finger wild type and PtdInsP-binding mutant fusion proteins localizes to multiple foci within the nucleus. (A) Schematic of ING2 domains. Nuclear Localization Signal (NLS; amino acids 180–200) and plant homeodomain (PHD; amino acids 200–281) are indicated. (B) Subnuclear localization of wild type and PtdInsP-binding mutant GFP-ING2 NLS+PHD finger fusion proteins (amino acids 180–281 of ING2). Confocal images of the nuclei of BalbC-3T3 cells transfected with NLSPHD (GFP-ING2_(NLSPHD)) and NLSPHD-mt (GFP-ING2_(NLSPHD-mt)) and stained with To-Pro-3 nuclear dye (Molecular Probes). GFP signal (green); To-Pro-3 (blue). DNA dense areas are not stained by To-Pro-3. Magnification as indicated. (C) GFP-ING2_(NLSPHD) co-localizes with anti-nucleolin. Confocal images of the nuclei of HT1080 cells transfected with NLSPHD (GFP-ING2_(NLSPHD)) (green), immunostained against anti-nucleolin (red) and stained with To-Pro-3 nuclear dye (blue). Magnification as indicated. (D) PtdInsP-binding mutations in the PHD domain alter the subfoci localization of GFP-ING2_(NLSPHD). High magnification (400X) confocal image of a single nuclear foci from a BalbC-3T3 cell that has been co-transfected with NLSPHD (GFP-ING2_(NLSPHD)) and NLSPHD-mt (RFP-ING2_(NLSPHD-mt)) and then stained with To-Pro-3. I: ING2_(NLSPHD); GFP signal (green); II: ING2_(NLSPHD-mt); RFP signal (red); III: To-Pro-3 (blue); IV: merge of images I and II (overlap detected as yellow); V: merge of images I, II and III.

domain as well as the NLS. This fusion protein is found virtually exclusively in the nucleus. Within the nucleus, it is primarily detected in nucleoli (Fig. 1C) and other small nuclear foci that do not correspond to splicing speckles or PML bodies (Fig. 1B; data not shown). The punctate pattern is a result of overexpression and the

presence of the NLS (data not shown) (Scott et al., 2001), consistent with this, a derivative of the ING2_(NLS^{PHD}) fusion protein, with mutations in zinc coordination residues that disrupt PtdInsP-binding (ING2_(NLS^{PHD}mt)) [27], is similarly targeted to nucleoli and nuclear foci (Fig. 1B).

Interestingly, when cells were co-transfected with GFP-ING2_(NLS^{PHD}) and red fluorescent protein (RFP)-ING2_(NLS^{PHD}mt), the two fusion proteins typically co-localize to the same nuclear foci, but within these foci have different sub-foci localization patterns (Fig. 1D). We note that when the fluorescent tags were reversed (RFP-ING2_(NLS^{PHD}) and GFP-ING2_(NLS^{PHD}mt)) similar results were obtained (data not shown). These data indicate that although the overexpression of the NLS targets both fusion proteins to the same general area (nuclear foci), the precise intranuclear localization of the proteins varies in a manner dependent on whether the PHD finger is intact. We believe this difference in localization is due to loss of PtdInsP-binding, though it is possible that as yet unknown function of the ING2 PHD finger is regulating the localization of this fusion protein.

Exogenous administration of synthetic fluorescent PtdIns(5)P accesses the nucleus

We next asked whether long-chain synthetic PtdInsPs, which should have increased hydrophobicity and enhanced native behavior in bilayers, could access the nuclei of live cells grown in culture. We chose to examine PtdIns(3)P and PtdIns(5)P since we had previous evidence that both of these PtdInsPs bind to the PHD finger of ING2 in vitro and PtdIns(5)P interacts with the PHD finger of ING2 in vivo (Gozani et al., 2003; Sbrissa et al., 2004). The two fluorophore-labeled PtdIns(3)P and PtdIns(5)P derivatives (BODIPY-TR-PI(3)P) and (BODIPY-TR-PI(5)P, respectively) were generated by modifications of routes described previously (Fig. 2A; see Materials and Methods) (Peng and Prestwich, 1998; Prestwich, 1996). These molecules, containing *sn*-2-*O*-palmitoyl chains, were labeled in the *sn*-1-*O*-acyl position with the extended fluorophore BODIPY-TR, a polyunsaturated and highly lipophilic fluorescent probe that is predicted to facilitate cellular uptake of the whole molecule (Fig. 2A). The purity of the synthetic samples was confirmed by mass spectrometry and nuclear magnetic resonance (NMR) (see Materials and Methods) (Prestwich, 1996). To assess cellular entry, BalbC-3T3 cells were incubated with either BODIPY-TR-PI(3)P or BODIPY-TR-PI(5)P for different intervals prior to fixation and confocal microscopy analysis (Fig. 2B and 2C) (Ozaki et al., 2000). Both molecules rapidly crossed the plasma membrane and entered into the cytoplasm of live cells (Fig. 2C). Over time, BODIPY-TR-PI(5)P, and to a lesser extent BODIPY-TR-PI(3)P, were detected in the nucleus (Fig. 2C). We next synthesized BODIPY-TR-PI(4)P (Fig. 2A; see Materials and Methods) (Toker, 2002) and analyzed its ability to enter the nucleus (Fig. 2D). BODIPY-TR-PI(4)P can access the nucleus and does so with a frequency similar to BODIPY-TR-PI(5)P. Thus, while these exogenous and synthetically modified lipids cannot mimic all aspects of endogenous PtdInsP behavior or localization, this system nonetheless provides a means of examining exogenous PtdInsP functions within the physiologic milieu of the cell nucleus.

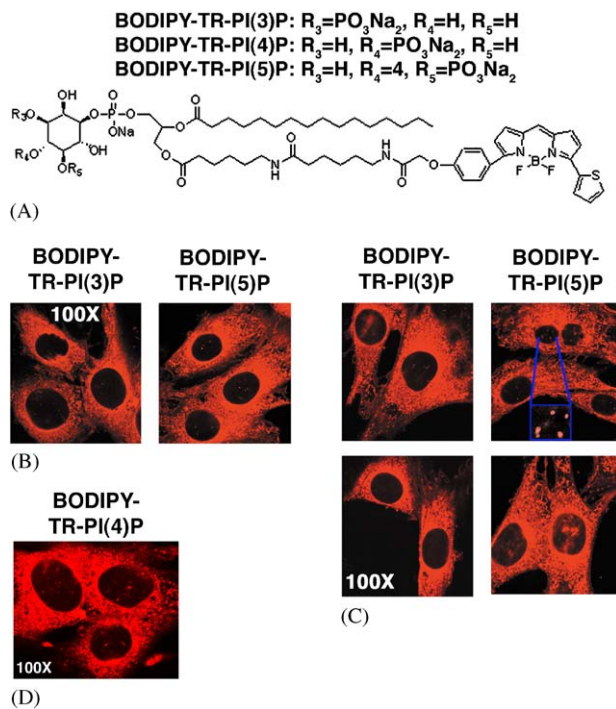


Fig. 2. Synthetic PtdInsPs enter live cells and access the nucleus. (A) Structure of BODIPY-TR-PI(3)P, BODIPY-TR-PI(4)P and BODIPY-TR-PI(5)P. (B) BODIPY-TR-PIPs enter into live cells. Confocal images of the fluorescent PtdInsP signal (red) in BalbC-3T3 cells incubated for 5' with 10 μ M BODIPY-TR-PI(3)P (left panel) or BODIPY-TR-PI(5)P (right panel) prior to fixation. Magnification as indicated. (C) Same as (B) except that incubation time was \sim 15'. Two images are shown for each fluorescent PtdInsP to demonstrate variability in nuclear access between BODIPY-TR-PI(3)P and BODIPY-TR-PI(5)P. The inset, denoted as the blue box at the right upper panel, shows at high magnification, small vesicle-like foci formed by BODIPY-TR-PI(5)P. (D) Same as in (C) except that BODIPY-TR-PI(4)P was used.

Co-localization between synthetic fluorescent PtdInsPs and the PHD finger of ING2 within the nucleus

To determine if, within the nucleus, synthetic BODIPY-PIPs retained the ability to interact with the PHD finger of ING2, we tested whether we could detect co-localization between the two in cultured cells. NIH3T3 cells were transfected with either GFP-ING2_(NLS_{PHD}) or GFP-ING2_(NLS_{PHD}mt), and after 24 h, incubated with either BODIPY-TR-PI(5)P or BODIPY-TR-PI(3)P for 15 min (Fig. 3A). The majority of cells incubated with BODIPY-TR-PI(5)P showed significant overlap in the nucleus between the fluorescent PtdInsP and GFP-ING2_(NLS_{PHD}) (\sim 70%) (Fig. 3A), suggesting recruitment by the ING2 PHD finger of BODIPY-TR-PI(5)P. In contrast, GFP-ING2_(NLS_{PHD}) rarely co-localized with BODIPY-TR-PI(3)P, though we note that (1) BODIPY-TR-PI(3)P does not access the nucleus as

efficiently as BODIPY-TR-PI(5)P; (2) we had previously found that PtdIns(3)P interacts less strongly with the ING2 PHD finger than PtdIns(5)P *in vitro* (Gozani et al., 2003); and (3) BODIPY-TR-PI(3)P may be metabolized differently than BODIPY-TR(5)P (Fig. 3A; see Discussion). The signals from the two fluorescent PtdInsPs failed to overlap with that of the mutant GFP-ING2_(NLS_{PHD}-mt) signal, in part, possibly due to decreased recruitment of the fluorescent molecules (Fig. 3). We next tested GFP-ING2_(NLS_{PHD}) and BODIPY-TR-PI(4)P co-localize (Fig. 3B). Consistent with the ability of the ING2 PHD finger to weakly bind to PtdIns(4)P in

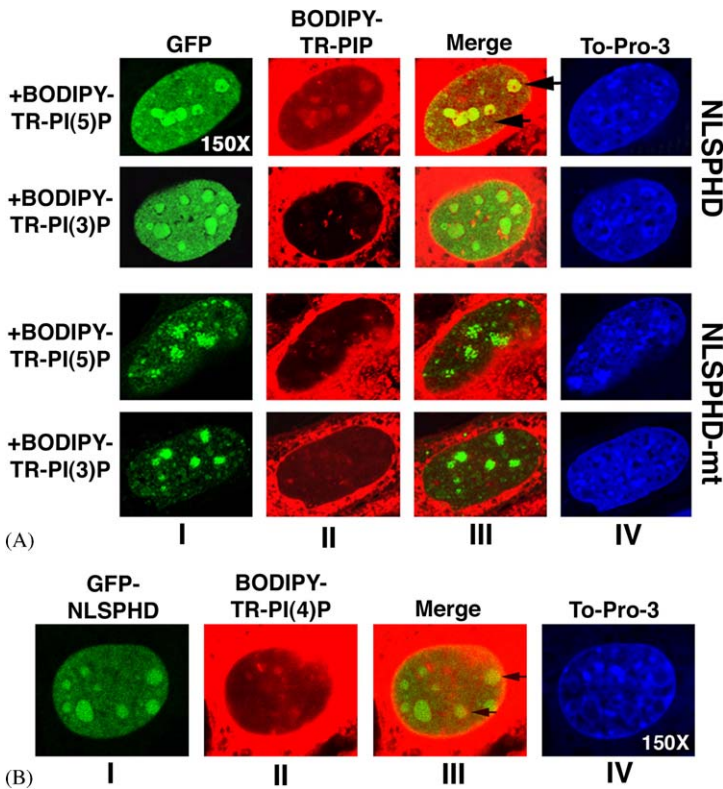


Fig. 3. Co-localization between synthetic fluorescent PtdInsPs and the PHD finger of ING2 within the nucleus. (A) Co-localization between BODIPY-TR-PI(5)P and GFP-ING2_(NLS_{PHD}) in the nucleus. Confocal images of BalbC-3T3 cells transfected with the indicated GFP fusion proteins (NLS_{PHD}, GFP-ING2_(NLS_{PHD}); NLS_{PHD}-mt, GFP-ING2_(NLS_{PHD}-mt)) and 24 h after transfection, incubated for 15' with 10 μ M of the indicated fluorescent PtdInsP (BODIPY-TR-PI(5)P or BODIPY-TR-PI(3)P). I: GFP signal (green); II: Fluorescent PtdInsP (red); III: merges of images I and II; IV: To-Pro-3 DNA staining (blue). Arrows indicate co-localization between GFP-ING2_(NLS_{PHD}) and BODIPY-TR-PI(5)P signals, detected as yellow in column III. Because BODIPY-TR-PI(3)P does not efficiently access the nucleus, only cells that exhibited positive staining of this PtdInsP in the nucleus were used for the analysis. To maximize detection of co-localization, the fluorescent PtdInsP signal was amplified to a greater extent than in Fig. 2. Magnification as indicated. (B) Partial co-localization between BODIPY-TR-PI(4)P and GFP-ING2_(NLS_{PHD}) in the nucleus. Same as in (A) except that the analysis was done with BODIPY-TR-PI(4)P.

vitro (Gozani et al., 2003) and the ability of BODIPY-TR-PI(4)P to access the nucleus (see Fig. 2D), we detected partial overlap in the two fluorescent signals (Fig. 3B). Thus, in the nucleus, GFP-ING2_(NLS_{PHD}) co-localized with exogenous, synthetic BODIPY-TR-PI(5)P, and this co-localization required that the PtdInsP-binding domain be intact. These data suggest that the PtdIns(5)P analog, under physiologic conditions in vivo, retains ligand specificity and affinity (see Discussion).

Synthetic PtdInsPs function within the nucleus of live cells

In the co-localization experiments, we observed that with prolonged incubation (25–30 min) of exogenous BODIPY-TR-PI(5)P, the GFP-ING2_(NLS_{PHD}) protein appeared diffuse rather than punctate within the nucleus. We hypothesized that this change in the localization of ING2_(NLS_{PHD}) protein may be a consequence of the interaction with the synthetic PtdInsP. To test this idea, cells transfected with either GFP-ING2_(NLS_{PHD}) or GFP-ING2_(NLS_{PHD}-mt), were incubated for 30 minutes in the presence of buffer control or synthetic dipalmitoyl (di-C₁₆) derivatives of either PtdIns(4)P or PtdIns(5)P, fixed, and protein localization of the transfected population was determined by confocal microscopy (Fig. 4A). As expected, under control conditions, the majority of cells transfected with ING2_(NLS_{PHD}) displayed a punctate nuclear pattern (Fig. 4A). In contrast, treatment of cells with di-C₁₆ PtdIns(5)P led ING2_(NLS_{PHD}) protein to lose its punctate distribution and instead the signal became diffuse throughout the nucleus (Fig. 4A, compare control to +PI5P panels). The PtdInsP-binding mutant ING2_(NLS_{PHD}-mt) protein retained a punctate pattern irrespective of treatment, indicating the requirement of an intact PHD finger for the altered localization to occur (Fig. 4A). Consistent with our co-localization data, the addition of di-C₁₆ PtdIns(4)P had an intermediate effect on ING2_(NLS_{PHD}) localization, likely reflecting binding of di-C₁₆ PtdIns(4)P to ING2_(NLS_{PHD}) and/or the ability of di-C₁₆ PtdIns(4)P to be metabolized to a product that can interact with ING2_(NLS_{PHD}) (Fig. 4A; see Discussion).

To ensure that re-localization of ING2_(NLS_{PHD}) occurs within the same cells and to rule out a fixation artifact, cells were transfected with GFP-ING2_(NLS_{PHD}) or GFP-ING2_(NLS_{PHD}-mt), treated with PtdIns(5)P and the GFP signal was visualized in live cells by time-lapse microscopy (Fig. 4B) (Kanai et al., 2001). As expected, ING2_(NLS_{PHD}) was initially punctate, but over the course of a 25 min incubation with PtdIns(5)P, ING2_(NLS_{PHD}) translocated out of the foci and became diffuse throughout the nucleus (Fig. 4B, NLS_{PHD}, compare 0' to 25' frames). In contrast, ING2_(NLS_{PHD}-mt) remained punctate regardless of time of incubation and concentration of lipid used, consistent with its inability to interact with PtdInsPs in vitro (Fig. 4B). We note that in addition to PtdIns(5)P, other synthetic PtdInsPs, though less efficient, also elicited movement of the GFP-ING2_(NLS_{PHD}). Thus, based on these data, we conclude that addition of synthetic PtdInsPs to cultured cells elicits functional consequences within the nucleus of live cells.

Synthetic PtdInsPs appear to have great potential as reagents to study nuclear processes regulated by PHD finger containing proteins and other PtdInsP-binding nuclear proteins. We have found that synthetic fluorescent tagged PtdIns(5)P will

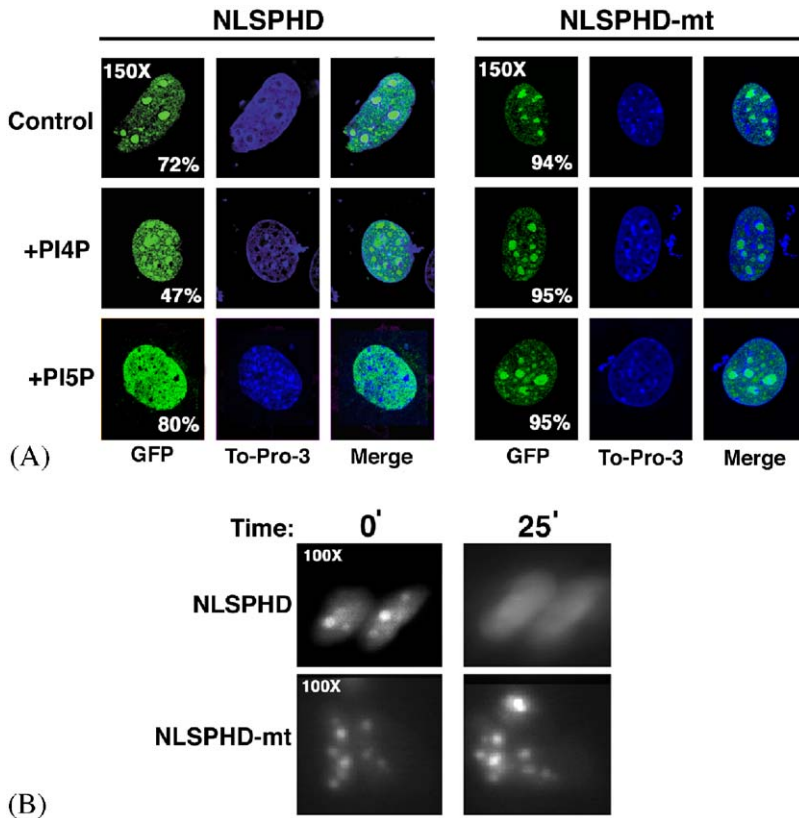


Fig. 4. Functional interactions between synthetic PtdInsPs and GFP-ING₂(NLSPHD) in the nucleus of live cells. (A) Re-localization of wild-type, but not PtdInsP-binding mutant, GFP-ING₂(NLSPHD) in cells in response to exogenous PtdInsP treatment. Confocal images of BalbC-3T3 cells transfected with the indicated GFP fusion plasmids (ING₂(NLSPHD) and ING₂(NLSPHD-mt)) and 24 h after transfection treated for 30' with the indicated di-C₁₆ PtdInsPs (control; PI4P, 50 μ M PtdIns(4)P; PI5P, 50 μ M PtdIns(5)P). GFP: green; To-Pro-3 DNA staining (blue); Merge: merge of GFP and To-Pro-3 images. The percent representation of the images is indicated in the lower right corner. Magnification is as indicated. (B) Time-lapse microscopy demonstrates movement of GFP-ING₂(NLSPHD) in response to exogenous, synthetic di-C₁₆ PtdIns(5)P treatment in the nuclei of live cells. NIH3T3 cells were transfected with the indicated GFP fusion plasmids (ING₂(NLSPHD), NLSPHD; ING₂(NLSPHD-mt), NLSPHD-mt) and incubated with 50 μ M of di-C₁₆ PtdIns(5)P for 25'. Images from the same field were captured every 30 s for 4' prior to treatment and for 25' after treatment. Shown are images captured at time of treatment (0') and the last image captured (25'). At the end of each incubation period, serial Z sections were acquired to confirm the plane of focus. Representative data from at least three separate experiments are shown. Magnification as indicated.

enter into the nucleus of cells and co-localize with GFP-ING₂(NLSPHD) (Fig. 3A). In addition, it appears that such interactions cause the GFP fusion protein to have an altered subnuclear localization (Fig. 4). Finally, both of these findings require that the PHD finger retain its ability to bind to PtdInsPs, suggestive of a direct interaction between the exogenous PtdInsP and the PHD finger of ING₂ within the nucleus.

In this study, we observed that BODIPY-TR-PI(4)P and BODIPY-TR-PI(5)P enter the nucleus more efficiently than BODIPY-TR-PI(3)P (Fig. 2). This is despite clear evidence that endogenous PtdIns(3)P is detected in the nucleus, specifically around nucleoli (Gillooly et al., 2000; Neri et al., 2002). These data may reflect differences in bioavailability of synthetic PtdInsP species for various biological processes. For instance, it is possible that in the cytoplasm there is greater binding-capacity for PtdIns(3)P than PtdIns(5)P and therefore the BODIPY-TR-PI(3)P is essentially sequestered before it has a chance to enter into the nucleus. Alternatively, BODIPY-TR-PI(4)P and BODIPY-TR-PI(5)P may more efficiently utilize endogenous PtdInsP cytoplasmic-nuclear transport systems than BODIPY-TR-PI(3)P. Finally, it is possible that the binding capacity for PtdIns(5) in the nucleus is greater than PtdIns(3)P, and that perhaps this is a reflection of ING2 binding to PtdIns(5)P. Regardless, our findings that GFP-ING2_(NLS^{PHD}) fails to co-localize with BODIPY-TR-PI(3)P indicate that either the BODIPY-TR-PI(3)P does not bind to the PHD finger of ING2 and/or is unable to efficiently access the nucleus. To circumvent differences in synthetic PtdInsP nuclear bioavailability, it will be necessary to develop shuttling systems that specifically aid entry of the synthetic lipids into the nucleus (Prestwich et al., 2002). For example, Histone H1 was first discovered to be a PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ shuttling agent and has subsequently been shown to deliver fluorescent PtdIns(4,5)P₂, but not fluorescent PtdIns(3,4,5)P₃, into subnuclear compartments (Chen et al., 2002).

The ability to discern which PtdInsP species is regulating a protein of interest or biological activity is fundamental for understanding PtdInsP signaling pathways (DiNitto et al., 2003; Payraastre et al., 2001). In our study, we observed movement of GFP-ING2_(NLS^{PHD}) in response to treatment of cells with a number of different synthetic PtdInsP species, with PtdIns(5)P being slightly more effective than other PtdInsP species (Fig. 4; data not shown). Therefore, based on these data alone, it cannot be concluded that endogenous PtdIns(5)P is regulating movement of the ING2 PHD finger. Furthermore, because the synthetic PtdInsPs are likely to be rapidly metabolized by endogenous PtdInsP kinases, phosphatases and lipases, it is difficult to unambiguously assign the chemical nature of the effector PtdInsP (Toker, 2002). These caveats notwithstanding, our previous work suggests that PtdIns(5)P regulates the subnuclear localization of endogenous ING2 via direct interactions with the PHD finger of ING2 (Gozani, et al., 2003), corroborating the results obtained in this study using synthetic PtdIns(5)P and its fluorescent analog. With further refinement of synthetic PtdInsPs, new generations of PtdInsP analogs have great promise as nuclear PtdInsP-biological research reagents. In this regard, metabolically stabilized PtdInsPs and polymerized PtdInsP-containing liposomes are currently being prepared for biochemical and physiological evaluation (Glenn D. Prestwich and Colin G. Ferguson, unpublished observations).

It has recently been reported that PtdIns(5)P functions to activate the myotubularin family of lipid phosphatases which triggers a positive feedback loop and leads to increased generation of PtdIns(5)P (Schaletzky et al., 2003). Similarly, other PtdInsPs can activate or inhibit the enzymes that regulate them; for example, PtdIns(5)P and PtdIns(4,5)P₂ have been shown to allosterically activate PTEN

(Campbell et al., 2003). Therefore, it is possible that synthetic PtdInsPs function in the nucleus and elsewhere by eliciting alterations in endogenous PtdInsP homeostasis via allosteric-mediated changes in the activity of PtdInsP regulatory enzymes.

Alternatively, synthetic PtdInsPs may function as competitive inhibitors of endogenous PtdInsPs. We visualized in live cells, with the GFP signal followed by time-lapse microscopy, that exogenous PtdInsPs dynamically and acutely triggered redistribution of GFP-ING2_(NLS^{PHD}) (Fig. 4). Based on these data, we propose that in our system the exogenous PtdInsPs may be competing with endogenous PtdInsP for binding to the PHD finger (see Figs. 3 and 4). This implies that one physiologic function for nuclear PtdInsP is to regulate subnuclear localization of PtdInsP-binding proteins. In such a model, the rapid generation of a specific PtdInsP species within a discrete subnuclear compartment could provide temporal and spatial cues to allow for highly regulated recruitment of the macromolecular machines that carry out different nuclear enzymatic processes. For instance, it is possible that PtdIns(4,5)P₂ generated at nuclear speckles may act as a docking station for exon-bound spliceosomes, and potentially contribute to exon synapsis during pre-mRNA splicing. Similarly, we postulate that PtdInsPs may function during synapsis of DNA during recombination and repair, possibly through recruitment of PHD finger containing proteins. Testing such hypotheses and other possible nuclear PtdInsP functions will demand new ways of thinking and tools to probe the nuclear functions and dynamics of PtdInsPs. In this study, we have demonstrated the utility of chemically generated PtdInsPs in investigating nuclear PtdInsP regulatory activity.

Summary

PtdInsPs are critical signaling molecules that regulate diverse cellular functions. One method to study PtdInsP biology involves using synthetic PtdInsP analogs to activate endogenous PtdInsP-mediated events in living cells. Such methodology has been successfully employed to explore the role of several PtdInsP-biological outcomes in the cytoplasm. However, this strategy has not previously been used to examine the function of PtdInsPs in the nucleus of live cells, primarily because there has not been a well-defined PtdInsP-binding protein to provide functional nuclear readouts. Here we have shown that synthetic PtdIns(5)P analogs access and function in the nucleus. We have found that these molecules modify the sub-nuclear localization of PHD finger-containing proteins in live cells and in real time. This work demonstrates that synthetic PtdInsPs and PtdInsP derivatives may be powerful tools for probing nuclear PtdInsP functions. Finally, our work supports a model that endogenous PtdInsPs regulate sub-nuclear localization and function of endogenous nuclear PtdInsP-binding proteins.

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