Visualizing signalling by phosphoinositide 3-kinase pathway lipids

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Abstract
Cells signal through lipids produced by phospholipid and phosphoinositide metabolism that involves three enzymic processes: (i) ester and phosphodiester hydrolysis by phospholipases; (ii) monophosphate hydrolysis by phosphatases; and (iii) phosphorylation of hydroxy groups by kinases. Unregulated enzyme activity correlates with specific pathologies, which are specific targets for therapeutic intervention. Three categories of reagents developed at the University of Utah and at Echelon Biosciences permit monitoring of in vitro enzyme activity and spatiotemporal changes in intracellular lipid concentrations, and identification of lipid-protein interactions.

Physiological activity of exogenous phosphoinositides
Exogenous anionic phosphoinositide ligands can be delivered (‘shuttled’) across the membranes of living cells using specific polyamines as carriers [1]. The process takes place within a few minutes, even at reduced temperatures or in the presence of an endocytotic blocker. Moreover, the shuttling can be accomplished using fluorescently labelled phosphoinositide ligands in conjunction with fluor-labelled histone or neomycin. Labelled phosphoinositides were observed in the nuclei and intracellular membranes, an observation now consistent with the use of newer fluorescent protein conjugates and anti-lipid antibody histochemistry [2,3]. Although we demonstrated that both shuttled Ins(1,4,5)P3 and PtdIns(4,5)P2 could elicit calcium transients in living cells within 2–4 min, it was questioned whether shuttle-delivered exogenous phosphoinositides would have general utility in manipulating cellular physiology. This has now been answered in the affirmative by a number of studies.

First, exogenous PtdIns(3,4)P2 was employed to restore full activation of Akt during steel-factor stimulation of bone marrow-derived mast cells obtained from mice genetically lacking the inositol 5-phosphatase SHIP [SH2 (Src homology 2)-containing inositol phosphatase] [4]. Other shuttled phosphoinositide ligands were ineffective, and thus both PtdIns(3,4)P2 and PtdIns(3,4,5)P3 were required for full phosphorylation of Ser173 and Thr308 of Akt in SHIP null cells. Secondly, addition of exogenous PtdIns(3,4,5)P3 activated a phosphoinositide 3-kinase- and Rho GTPase-mediated positive feedback loop that regulates the polarization and migration of neutrophils [5]. Remarkably, neutrophils began migrating within 3 min when a histone–PtdIns(4,5)P2 complex was added to the medium; shuttling in PtdIns(4,5)P2 or omission of the carrier failed to activate polarization and migration. Thirdly, in elegant work by the Yin group [6], intracellular delivery of PtdIns4P, but not PtdIns(4,5)P2, rescued the phenotype of cells in which RNA interference had knocked down the Golgi-resident PtdIns 4-kinase required for production of the endogenous PtdIns4P supply. Fourthly, exogenous PtdIns3P was shown to induce plasma membrane translocation of the glucose transporter protein GLUT4, a phosphoinositide 3-kinase-dependent process occurring downstream of activation of the insulin receptor [7]. Finally, the delivery of fluorescently labelled PtdIns5P to BalbC 3T3 cells resulted in subnuclear localization of this lipid, but not PtdIns3P or PtdIns4P (O. Gozani, S.J. Field, C.G. Ferguson, C. Mahlke, L.C. Cantley, G.D. Prestwich and J. Yuan, unpublished work). Importantly, exogenous PtdIns5P ultimately co-localized with and modulated the nuclear localization of the PHD (plant homeodomain) zinc-finger-containing protein ING2, and altered chromatin remodelling in these cells ([9]; and O. Gozani, S.J. Field, C.G. Ferguson, C. Mahlke, L.C. Cantley, G.D. Prestwich and J. Yuan, unpublished work).

Anti-phosphoinositide antibodies
A method independent of green fluorescent protein–pleckstrin homology domains was required to visualize specific phosphoinositides in a number of cellular compartments. To this end, antibodies against PtdIns(4,5)P2 were first developed over 15 years ago, with newer antibodies being prepared against different immunogenic forms in the past 4 years. The first anti-PtdIns(3,4,5)P3 antibody was introduced in 2002, and its phosphoinositide selectivity was demonstrated by photoaffinity labelling and ELISA [3]. Stimulation of neutrophils with IMet–Leu–Phe led to a rapid (7 s) and transient peak of immunoreactive PtdIns(3,4,5)P3, while activation of NIH 3T3 fibroblasts with platelet-derived

Key words: anti-phosphoinositide antibody, phosphoinositide shutting, tethered phosphoinositides
Abbreviations used: IVEC, in vivo expression cloning; PHD, plant homeodomain; SH, Src homology; SHIP, Src Homology 2-containing inositol phosphatase.
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growth factor resulted in maximal PtdIns(3,4,5)P_3 levels in cytoplasmic compartments after 5–10 min, with a slower return to baseline levels. Interestingly, at 15 min, a peak of PtdIns(4,5)P_2 production was observed in the nucleus. The nuclear PtdIns(4,5)P_2 staining seen in normal mouse fibroblasts was absent in cells expressing the beige gene, orthologous to human Chediak–Higashi Syndrome [10]. The anti-PtdIns(3,4,5)P_3 IgMs have also been used to visualize spatiotemporal changes in lipids in adipocytes stimulated with insulin and leptin [11].

Tethered phosphoinositides
Since the early 1990s, we have pioneered the use of affinity probes for inositol phosphates and phosphoinositides, including tethered biotin, spin labels, flours, and other reporter groups [12]. The types of proteins identified and the kinetics of interactions can be influenced by the tethering chemistry and the source of proteins. For example, tethered Ins(1,3,4,5)P_4, the headgroup of PtdIns(3,4,5)P_3, fished out centaurin-α [13] from a rat brain homogenate. In contrast, tethered PtdIns(3,4,5)P_3 captured the unique adaptor protein PHISH [3'-phosphoinositide-interacting SH-containing protein; also known as DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositides)], as well as other proteins such as phosphoinositide-dependent kinase 1, from among 500 000 proteins screened by IVEC (in vitro expression cloning) [14]. Most recently, a weak PtdIns(3,4,5)P_3-interacting protein obtained from the IVEC library was ING2, a zinc finger PHD nuclear protein involved in chromatin remodelling [9]. ING2 prefers PtdIns5P as a ligand, as determined by surface plasmon resonance and PtdInsP Array™ analysis, illustrating that the tethered phosphoinositide used originally may not uniquely determine the lipid selectivity of the protein captured. A similar result has been obtained recently in a proteomic survey of macrophage proteins using novel reductively cleavable bio-

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