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Phosphoinositide Signaling: From Affinity Probes to Pharmaceutical Targets

Review

Glenn D. Prestwich*
Department of Medicinal Chemistry
The University of Utah
419 Wakara Way, Suite 205
Salt Lake City, Utah 84108
Echelon Biosciences, Inc.
675 Arapeen Way, Suite 302
Salt Lake City, Utah 84108

Lipid signaling by phosphoinositides (PIP_ns) involves an array of proteins with lipid recognition, kinase, phosphatase, and phospholipase functions. Understanding PIP_n pathway signaling requires identification and characterization of PIP_n-interacting proteins. Moreover, spatiotemporal localization and physiological function of PIP_n-protein complexes must be elucidated in cellular and organismal contexts. For protein discovery to functional elucidation, reporter-linked phosphoinositides or tethered PIP_ns have been essential. The phosphoinositide 3-kinase (PI 3-K) signaling pathway has recently emerged as an important source of potential "druggable" therapeutic targets in human pathophysiology in both academic and pharmaceutical environments. This review summarizes the chemistry of PIP_n affinity probes and their use in identifying macromolecular targets. The process of target validation will be described, i.e., the use of tethered PIP_ns in determining PIP_n selectivity in vitro and in establishing the function of PIP_n-protein complexes in living cells.

Introduction to Phosphoinositide Signaling

Phosphoinositide (PIP_n) signaling networks are dynamically modulated by proteins with lipid recognition, kinase, phosphatase, and phospholipase activities. Lipid-protein interactions form the cornerstone for many signaling pathways, and the new discipline of functional lipidomics is defining many new targets for therapeutic intervention [1]. As illustrated in Figures 1A and 1B, PIP_ns are biosynthesized and interconverted within cells by the interplay of kinases [2] and phosphatases [3]. While the parent lipid phosphatidylinositol (PI) lacks additional phosphorylation on the inositol ring, the other seven known PIP_ns consist of three monophosphates, three bisphosphates, and one trisphosphate (Figure 1C). These charged lipids, particularly the 3-phosphorylated lipids [4], are minor components of cellular membranes but are vital as second messengers for diverse cellular functions [5]. PIP_ns are essential elements in tyrosine kinase growth factor receptor and G protein receptor signaling pathways [6, 7]. Furthermore, these lipid signals have important roles in membrane trafficking and endosome dynamics [8], including endocytosis, exocytosis, Golgi vesicle movement, and protein trafficking [9], in cell adhesion, polarization and migration [10, 11], in remodeling of the actin cytoskeleton [12], and in mitogenesis and oncogenesis [4, 13]. Activation of cellular signaling pathways occurs in response to spatially and temporally regulated production of particular PIP_ns in response to a stimulus. As outlined in Figure 2, a given PIP_n then recruits an assemblage of proteins, commonly referred to as a signalosome, to a given subcellular location to modulate a given signaling pathway [14–16]. The potential implications of modulation of the PI 3-K signaling pathway in developing new therapeutics will be a pervasive subtheme in this review.

For example, phosphatidylinositol 4,5-bisphosphate, or $PI(4,5)P_2$, plays a central role in many cellular functions [6]. First, $PI(4,5)P_2$ is a substrate for phospholipase C (PLC), and some isoforms contain pleckstrin homology (PH) domains that also bind to $PI(4,5)P_2$ [17]. Hydrolysis of the phosphodiester linkage releases the calcium-mobilizing second messenger $Ins(1,4,5)P_3$. Second, $PI(4,5)P_2$ itself recruits PH domain-containing proteins to membranes; PH domains occur in over 120 human proteins, and each exhibits distinctive PIP_n binding affinities and selectivities [16, 18]. Among these, the most well-known example is the specific binding of $PI(4,5)P_2$ to the PH domain of the δ_1 isoform of PLC [19]. Third, $PI(4,5)P_2$ binds to non-PH domain-containing proteins, e.g., gelsolin and profilin, and regulates actin polymerization [12]. $PI(4,5)P_2$ binding also modulates the GTPase activity of adenosine ribosylation factor (Arf) and the phospholipase activity of phospholipase D (PLD) [20, 21], recruits the human class II phosphoinositide 3-kinase (PI 3-K) to membranes via its C2-γ PX domain [22], binds PDZ domains in multiprotein complexes [23], and binds to the AP180 ENTH domain to mediate endocytosis via clathrin-coated pits [24]. The vanilloid receptors and calcium channels in neuronal membranes are also regulated by the binding or hydrolysis of $PI(4,5)P_2$ [25, 26]. Finally, $PI(4,5)P_2$ can be converted by PI 3-K to $PI(3,4,5)P_3$, which activates multiple downstream signaling events via interactions with numerous PH domains [4, 27], including the specific protein kinases PDK1 [28, 29] and Akt [30].

In addition to PH and ENTH domains, the PI(3)P-specific FYVE domains [15, 31] and phox homology (PX) domains [32–36] target proteins to intracellular membranes. In addition to interactions with membranes, the $InsP_n$s, and PIP_ns are part of a nuclear inositide pathway [37, 38], in which PIP_ns and $InsP_n$s regulate chromatin remodeling [39, 40] and reverse inhibition of RNA transcription by histones [41], assemble SWI-SNF-like BAF complexes [42], and regulate pre-mRNA splicing in nuclear speckles [43, 44].

Disruption of PI signaling is common to many disease states, including diabetes, cardiovascular disease, and cancer [45]. Because the activity of PI second messengers is determined by their phosphorylation status, the enzymes that act to modify these lipids are central to the correct execution of these signaling events [46]. For example, normal cellular levels of $PI(3,4,5)P_3$ are very

*Correspondence: glenn.prestwich@hsc.utah.edu

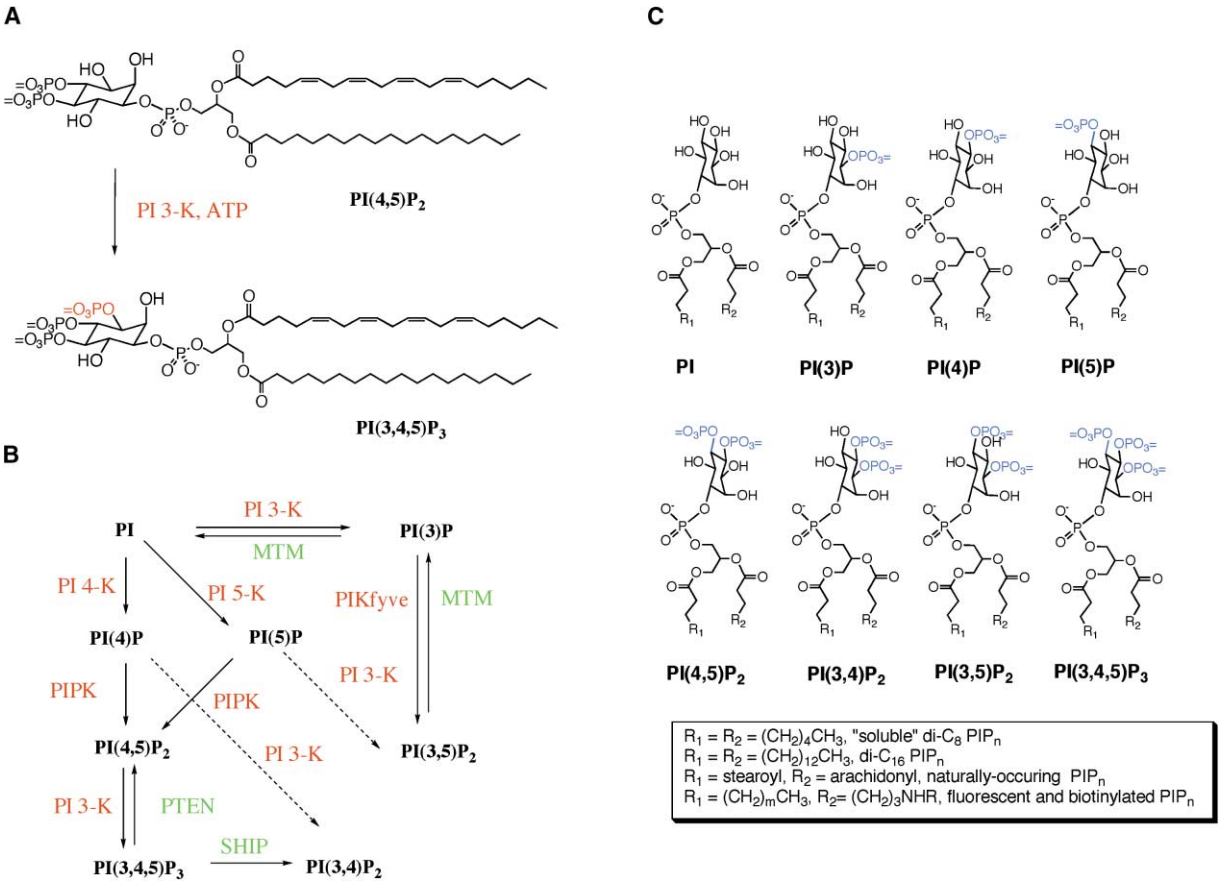

Figure 1. Chemistry of Phosphoinositide Metabolism

(A) PI 3-K reaction.

(B) Interconversions of PIP_ns by lipid kinases (red) and lipid phosphatases (green). Enzyme abbreviations are defined in the text.

(C) Structures of the phosphatidylinositol polyphosphates and synthetic analogs.

low, but rise dramatically following growth factor stimulation. Tyrosine kinase receptors activate class I PI 3-Ks to catalyze the formation of $PI(3,4,5)P_3$ via phosphorylation of $PI(4,5)P_2$ [4]. By increasing cellular levels of $PI(3,4,5)P_3$, PI 3-K induces the formation of defined molecular complexes that act in signal transduction pathways. Most notably, PI 3-K activity suppresses apoptosis and promotes cell survival through activation of its downstream target, PKB/Akt [30]. $PI(3,4,5)P_3$-regulated signaling is governed both by its formation by PI 3-K and by its conversion into phosphoinositide bisphosphates, such as $PI(4,5)P_2$ or $PI(3,4)P_2$. An interplay between $PI(3,4,5)P_3$ formation by PI 3-K and its removal by the 3-phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) controls eukaryotic cell polarization and movement [11]. Moreover, PI 3-K is a critical regulator of immune function [47, 48], particularly in T cell activation and mast cell function. Control of PI 3-K and $PI(3,4,5)P_3$ levels have emerged as important targets in cardiovascular disease and cancer [49, 50] as well as allergy and autoimmune disease [51] and diabetes, inspiring efforts to design novel isoform-selective inhibitors [52] and to use PI 3-K inhibitors in a chemical genomics approach to elucidate interconnected pathways in the proteome [53]. In sum, the PI 3-K signaling pathway has emerged as the target for development of a variety of new signal transduction modifying therapeutic agents [54, 55], such as targeting Akt with small molecule inhibitors [56].

In cancer cells, the activity of PI 3-K and the regulation of $PI(3,4,5)P_3$ levels is often defective [57, 58]. Elevated $PI(3,4,5)P_3$ levels appear to contribute to cancer progression in part through constitutive activation of PKB/Akt [59], promoting cell survival following detachment from the extracellular matrix [30]. Increased $PI(3,4,5)P_3$ levels are observed in cancer, and cellular transformation is PI 3-K dependent [60]. Elevated levels of $PI(3,4,5)P_3$ can occur through amplification of PI 3-K gene expression [61] or through alterations in the activity of the PIP_n phosphatases PTEN or SHIP (SH2-containing inositol phosphatase), which modulate $PI(3,4,5)P_3$ levels by converting $PI(3,4,5)P_3$ to $PI(4,5)P_2$ or $PI(3,4)P_2$ respectively, with concomitant sensitization to chemotherapeutic drugs [62].

PTEN, a PIP_n 3-phosphatase [63] that converts $PI(3,4,5)P_3$ to $PI(4,5)P_2$, negatively regulates PKB/Akt activation by PI 3-K [64]. The structure of a PTEN-Ins-$(1,3,4,5)P_4$ complex has been solved [65]. Loss of PTEN activity results in accumulation of $PI(3,4,5)P_3$ [66], abnormal activation of PKB/Akt, suppression of apoptosis

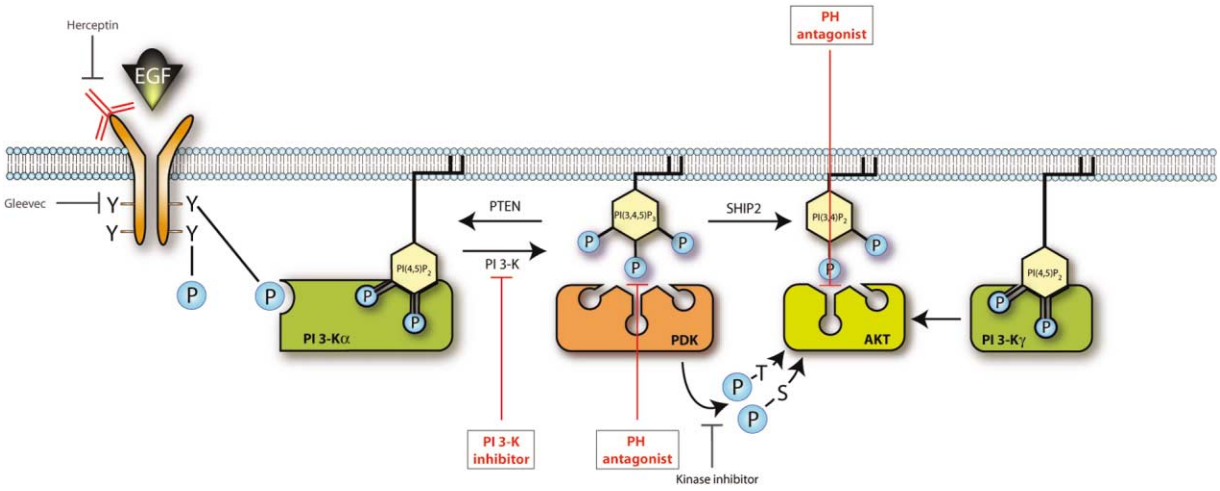

Figure 2. The PI 3-K/Akt Signaling Pathway and Potential Drug Targets for Cancer Therapy
Additional details on therapeutic targets are provided in several reviews [49–51, 55].

[67], and increased tumorigenesis in a number of human tissues. PTEN may be a candidate for targeted chemotherapy, as anticancer agents can preferentially destroy tumors with PTEN mutations [58]. Very recently, another protein tyrosine phosphatase, PTPRQ [68], was shown to have dual PI(3,4,5)P_3 3- and 5-phosphatase activity that regulates cell survival and proliferation. In addition, the 3-phosphatase myotubularin (MTM, MTMR) family [69] has been identified as important PIP_n phosphatases that contribute to lipid remodeling and are commonly mutated in genetic diseases [70]. Moreover, PTEN, Mdm2, and p53 are functionally interrelated, with PTEN acting as a negative regulator of an oncoprotein-tumor suppressor network to downregulate p53, and p53 acting to upregulate PTEN production [71].

Both PI 3-K and PTEN are involved in glucose homeostasis and tissue responsiveness to insulin [72]. Defects in PI 3-K response to insulin stimulation are associated with non-insulin-dependent diabetes mellitus (NIDDM), or "type II" diabetes. Inhibition of PI 3-K blocks insulin-stimulated glucose uptake as well as GLUT4 vesicle translocation [73]. Furthermore, mice deficient in PKB/Akt, which is activated by PI(3,4,5)P_3 generated in response to insulin, exhibit a diabetes-like phenotype [74]. As expected, PTEN also plays a role in control of insulin sensitivity by attenuating PI(3,4,5)P_3 levels in cells. Inhibition of PTEN activity stimulated GLUT4-mediated glucose transport and increased sensitivity to insulin in vitro [75]. Modulation of PI(3,4,5)P_3 levels by maintaining control of PI 3-K and PTEN activities is crucial for glucose homeostasis and regulating sensitivity to insulin in NIDDM.

The PIP_n 5-phosphatase SHIP2 [76], which converts PI(3,4,5)P_3 to PI(3,4)P_2, is also involved in insulin signaling and diabetes [77]. Homozygous deletion of SHIP2 in mice is fatal due to increased insulin sensitivity and severe hypoglycemia; animals heterozygous for the SHIP2 deletion exhibited increased glucose tolerance and insulin sensitivity [78]. Thus, SHIP2 appears to be an essential negative regulator of insulin signaling and sensitivity, and altered SHIP2 activity may be a contributing factor to the insulin resistance associated with NIDDM and obesity. Moreover, SHIP2—as with PI 3-K—is also a crucial regulator of immune function [79, 80].

In order to identify and characterize proteins important in the biology described above, chemical affinity probes were developed. The first section below will review the chemistries developed for preparing tethered $InsP_n$ and PIP_n ligands that display reporter groups with different molecular topologies. The second section will provide examples of the use of tethered PIP_ns for isolation of PIP_n binding proteins. The third section will then illustrate specific applications for fluorescent, photoaffinity, biotinylated, spin-labeled, and resin-immobilized probes for the characterization of the $InsP_n$ and PIP_n binding sites using in vitro biochemical and biophysical studies. The fourth and final section will highlight the uses of tethered PIP_ns for drug discovery and will briefly summarize the steps that will be involved in target validation in cell and organismal biology.

Chemistry of PIP_n Affinity Reagents

Different molecular topologies have been devised to bind proteins with different active site architectural features. The molecular recognition and targeting of lipid binding domains such as the C2, PH, PX, FYVE, PHD, ENTH, gelsolin, profilin, PI kinase, and PIP phosphatase domains have been thoroughly reviewed [16, 81]. From the variety of structures, it is now clear (which it was not when the first affinity probes were developed) that the location of the tether, the nature of the linker, and the mimicry of the diacylglyceryl (DAG) moiety needed to be varied in a given ligand to target and capture different proteins. A fishing analogy is relevant: different baits catch different fish. Moreover, a given bait, which cannot be optimal for all fish, will catch omnivorous prey as well as highly selective feeders. The different topologies are illustrated schematically in Figure 3 and with selected molecular structures in Figure 4. In the classical "recruitment" model, the DAG chains that would be buried in the lipid bilayer are minimally involved

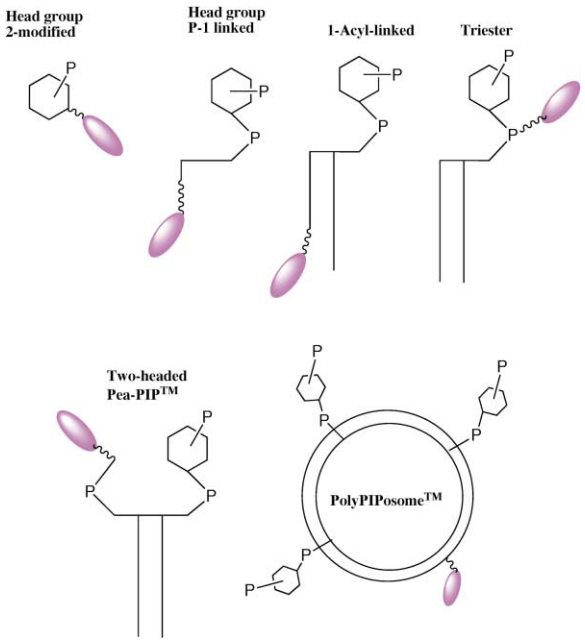

Figure 3. Molecular Topologies of Different Tethering Modes for $InsP_n$ and PIP_n Affinity Probes

in binding; the electrostatic and hydrogen-bonding interactions with the exposed, water-soluble phosphorylated inosityl head group were the dominant factors. This view has changed with increased understanding of the importance of length and unsaturation of the DAG moiety [82] and the clear identification of fatty acyl-protein contacts in FYVE [83] and PX [32] domains.

P-1 Tethered, Soluble $InsP_n$ Reagents

To identify and characterize protein targets of these signaling molecules, we first synthesized P-1 or P-2 tethered affinity probes of soluble $InsP_n$s [84–86] (Figure 4). (P-1 refers to the phosphate at the 1-position of the *myo*-inositol ring.) We adapted the Ferrier rearrangement of differentially protected methyl α-D-glucopyranosides to provide access to all of the possible head groups present in the known PIP_ns [84, 87–89]. These synthetic routes allowed introduction of affinity probes in a region of space that was generally well tolerated by most proteins studied. In later work, other P-1 tethered probes were independently prepared [90], and their affinity for the Ins(1,4,5)P_3 receptor was analyzed by surface plasmon resonance (SPR) spectroscopy. Hydrophobic and charged moieties linked to this position appeared to enhance the affinity for the Ins(1,4,5)P_3 receptor [91]. Finally, in some cases, e.g., the unnatural Ins(1,2,6)P_3, an experimental anti-inflammatory drug known as α-trinositol, a different tethering regiochemistry was required in order to preserve biological activity [92].

Ring-Modified $InsP_n$ Derivatives

The Ozaki group pioneered the synthesis of Ins(1,4,5)P_3 in which the 2-hydroxyl group was esterified to derivatives such as the *p*-azido or diazobenzoates or the (4-aminocyclohexyl)carboxylate. These 2-substituted derivatives were used to prepare affinity matrices that were then employed for protein purifications [93, 94] (Figure 4). One of these 2-substituted probes, which proved to be ahead of its time, was bifunctional, containing both a biotinylated tag and photoactivatable derivative [95] for the purpose of isolating peptide fragments of $InsP_n$ binding protein active sites. Attempts to modify the other nonphosphorylated C-6 position resulted in loss of activity. The uses of these materials for protein purifications are described below. Modifications of positions in other $InsP_n$ regioisomers have been little explored, with the exception of the placement of a 5-ester function in Ins(1,2,6)P_3 for attachment of a photoaffinity label that did not interfere with the activity or binding of α-trinositol [92].

Acyl-Modified Reagents

Having established a protecting group strategy for selective P-1 modifications, we next prepared each of the lipid-modified phosphoinositides shown in Figure 1. In each case, tethered forms were prepared by using an *sn*-1-*O*-(6-aminohexanoyl) derivative to which reporter groups could then be attached via an amide linkage. In this way, biotinylated [96] and fluorescent derivatives of PI(4,5)P_2 and PI(3,4,5)P_3 [87], PI(3)P and PI(4)P [88], PI(5)P and PI(3,5)P_2 [89], and PI(3,4)P_2 [97] were prepared. Subsequently, additional syntheses of biotinylated PI(3,4,5)P_3 [98, 99] and resin-immobilized PI(4,5)P_2 [100] and PI(3,4,5)P_3 were described. In addition, an [^{125}I]-labeled dioctanoyl PI(3,4,5)P_3 was employed to identify the role of different PIP_ns in the activation of the Rac-related GTPase Vav [101]. In this example, PI(4,5)P_2 inhibited Vav phosphorylation, while PI(3,4,5)P_3 enhanced phosphorylation and activation of Vav by the kinase Lck.

The main advantage of acyl tethering is that the reporter group is located in a region that is not likely to interact with the binding site for the PIP_n head group or with residues near the active site that interact with the glyceryl ester region of the DAG moiety. While this strategy is effective for many PIP_n binding proteins, three main drawbacks have been noted. First, the acyl-modified PIP_ns are often less hydrophobic, and the probe moiety often interferes with the insertion of the DAG group into a lipid bilayer. Second, modification of the size, shape, and physicochemical properties of the DAG moiety can alter the ability of the acyl-tethered PIP_n to act as a substrate or ligand. Third, when proteins dock to the head group in a lipid bilayer, the reporter group may be too distant or otherwise unavailable for direct interaction with the PIP_n binding protein. This has been a cause for failure in some attempted resonance energy transfer and photoaffinity labeling studies.

Ether lipids based on phosphoinositides have also been synthesized. For example, the 3-deoxy PIP_n ether lipid analogs [102] were prepared as potential inhibitors of PI 3-kinase or to bind PH domains. A tritium-labeled diundecyl ether analog of PI(4,5)P_2 was prepared as lipase-resistant tracer [103], and the 1-*O*-hexadecyl, 2-*O*-methyl ether of PI(3,4,5)P_3 was found to be a stable and biologically active analog with low serum albumin binding [98].

Triester-Modified Reagents

To address the inaccessibility of reporter groups in the acyl-modified PIP_n derivatives to residues adjacent to the head group binding pocket and to provide further flexibility in size and charge of the reporter group, we designed a series of phosphotriester probes [88, 97,

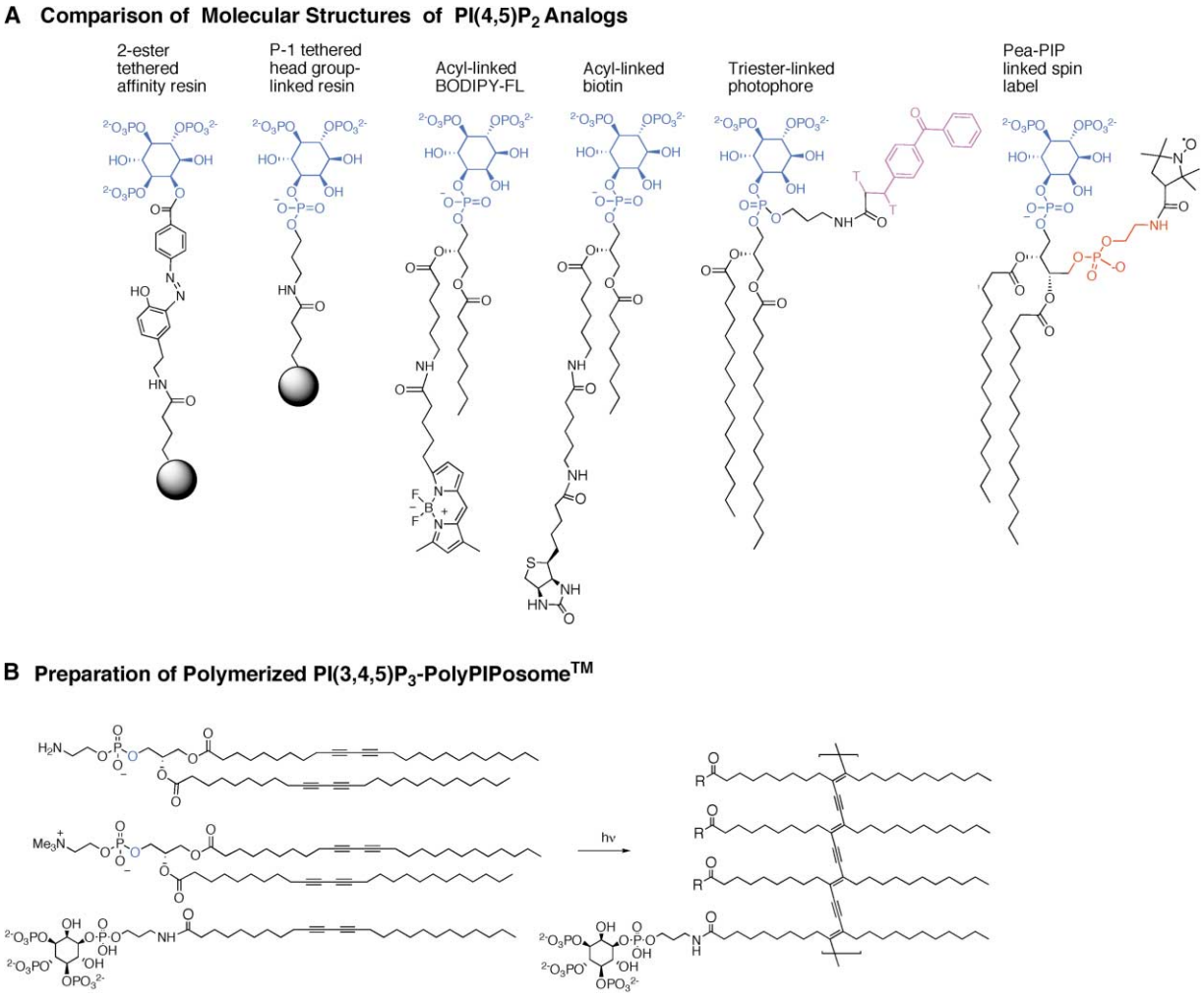

Figure 4. Chemistry of Tethered Phosphoinositide Topologies

(A) Comparison of $PI(4,5)P_2$ molecular types attached to a resin, lipophilic BODIPY fluorophore, biotin, benzoyldihydrocinnamoyl (BZDC) photophore, and proxyl spin label.

(B) Preparation of a $PI(3,4,5)P_3$ PolyPIPosome by irradiation of a polymerizable liposome containing a diyne-linked $Ins(1,3,4,5)P_4$ head group.

104]. In these derivatives, an additional P-1 phosphate modification adds a pendant aminopropyl moiety in the vicinity of the head group, while the DAG moiety is preserved intact. Unlike the acyl-tethered derivatives, these probes were specifically designed to sample the environment at the lipid-head group interface and to allow normal incorporation into a lipid bilayer. The $PI(4,5)P_2$, $PI(3,4)P_2$, and $PI(3,4,5)P_3$ triester photoaffinity probes have been used in a number of comparative labeling studies, as presented below. The photolabeling of human profilin by acyl, head group, and triester probes for the $Ins(1,4,5)P_3/PI(4,5)P_2$ head group provides a clear example of preferential labeling [105]. Still, these probes had the disadvantages of being sterically crowded near the head group as well as perturbing the normal phosphodiester linkage to the DAG moiety.

Hybrid Phospholipids

In order to address the problems of steric crowding and to restore the normal phosphodiester linkage to the DAG moiety, we developed a novel topology, which we called the "two-headed" or hybrid phospholipid approach. Thus, we synthesized a four-carbon threitol backbone with a readily derivatized phosphatidylethanolamine (PE or Pea) headgroup at one primary hydroxyl group, a PIP_n head group at the other primary hydroxyl, and two acyl chains on the internal 2,3-diol. The first synthetic hybrid lipids, or Pea-PIPs, were the NBD, fluorescein, biotin, and proxyl derivatives of the ethanolamine for Pea-$PI(4,5)P_2$ [106]. Subsequently, the technology has been expanded to prepare each of the eight head groups possible in a Pea-PIP, and additional fluorescent and immobilized derivatives are being evaluated as biophysical and cellular probes (P.W. Rzepecki, P.O. Neilsen, and G.D.P., unpublished results). The hybrid lipids appear to possess several characteristics that may make them preferable to the acyl-modified PIP_ns: (1) Pea-PIPs have improved water solubility, (2) many Pea-PIP derivatives translocate into cells and partition to cell membranes without intracellular delivery methods developed for acyl-modified PIP_ns [107], (3) Pea-PIP derivatives

give improved binding kinetics with PH domains, and (4) Pea-PIP derivatives are processed by the majority of lipid phosphatases and kinases examined. Details on these reagents will be presented in due course.

PolyPIPosomes

None of the molecular approaches above addressed the importance of the normal bilayer environment, in which an avidity effect arises from the proximity of multiple PIP_ns presenting head groups toward the cytoplasmic space. Using PIP_n-containing liposomes is a common method for measuring ligand binding to PIP_ns [108, 109]; the difficulty of preparing reproducible liposomes and the instability of the liposomes remain problematic. Thus, we devised a method of tethering $InsP_n$ head groups via the P-1 aminopropyl linked to the polymerizable 10,12-pentacosadiynoic acid. This surrogate PIP_n (5%) is combined with diyne-PE (29%), biotinylated-diyne-PE (1%), and diyne-phosphatidylcholine (PC) (65%), formed into liposomes, and crosslinked with UV light (254 nm) [110] to give polymerized PIP_n-containing liposomes. Designated PolyPIPosomes, these pale red, 100 nm nanoparticles are stable for many months in buffer suspension. Preliminary results indicate that the $PI(4,5)P_2$ and $PI(3,4,5)P_3$ PolyPIPosomes may be preferred to acyl-modified PIP_ns for obtaining binding kinetics using SPR (C.G. Ferguson, R. James, Y. Abdiche, D.G. Myszka, and G.D.P., unpublished results).

Protein Discovery with PIP_n Affinity Probes

Since the early 1990s, affinity probes for $InsP_n$s and PIP_ns have been used extensively to identify and characterize $InsP_n$ and PIP_n binding proteins. The specific examples that follow use affinity reagents for six main purposes: (1) isolation and purification of binding proteins, (2) domain identification and mapping of the active site, (3) biophysical measurements of binding affinity and ligand selectivity, (4) intracellular visualization with fluorescent lipids, (5) generation and use of anti-PIP_n antibodies, and (6) development of high-throughput screening (HTS) assays for drug discovery. In several cases, direct comparisons of probes with different molecular topologies is possible, e.g., comparing the affinity of the Grp1 PH domain [111, 112] for P-1 aminopropyl-linked $Ins(1,3,4,5)P_4$ [113], acyl-modified $PI(3,4,5)P_3$ [87, 114], Pea-$PI(3,4,5)P_3$ [106], and $PI(3,4,5)P_3$ PolyPIPosomes, as well as with noncovalently nitrocellulose-bound dipalmitoyl-$PI(3,4,5)P_3$ [115]. The chiral head group for each of these five molecular species was prepared by the Ferrier chemistry route [84, 113].

Both 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 head group of $PI(3,4,5)P_3$, was used to isolate the novel protein centaurin-α [116] from solubilized mouse cerebellar membranes. In contrast, tethered $PI(3,4,5)P_3$ captured a unique 35 kDa adaptor protein from over 200,000 in vitro expressed polypeptides [96]. Most recently, ING2, a zinc finger PHD nuclear protein involved in chromatin remodeling, was identified as a weak $PI(3,4,5)P_3$-interacting protein by expression cloning [117]. ING2 was subsequently found to prefer PI(5)P as a ligand, as determined by SPR and PIP Array [115] analysis, illustrating that the originally used tethered PIP_n may not uniquely determine the lipid selectivity of the protein captured. Below, three methods of using $InsP_n$ and PIP_n affinity matrices for purification of proteins from cell or tissue homogenates are described. First, affinity matrices have been used in classical purifications from tissue or cell homogenates. Second, cDNA expression libraries have been screened with immobilized or biotinylated PIP_n affinity probes. Third, a modification of PIP_n affinity supports will be described that allows isolation of PIP_n-complexed signaling complexes.

Classical Biochemical Isolations from Tissues or Cells

The first affinity purifications employed P-1 tethered $Ins(1,4,5)P_3$ and 2-modified $Ins(1,4,5)P_3$ that had been covalently linked to activated ester groups on agarose to give amide-linked ligands with >10 Å tethers. The P-1 tethered $Ins(1,4,5)P_3$ resin was first employed for purification of the $Ins(1,4,5)P_3$ receptor from mouse cerebellar membranes [118]. Independently, the 2-modified $Ins(1,4,5)P_3$ affinity matrices [93, 94, 119] were employed for purification of binding proteins from rat brain membranes [120], purified recombinant PH domains from PLC-δ1 [121], $InsP_n$ phosphatases [122], solubilized $Ins(1,4,5)P_3$ receptors from bovine liver microsomes [123], and a novel 130 kDa PLC δ_1-related protein (p130) that bound $Ins(1,4,5)P_3$. The p130 protein shared 38% sequence identity with PLC-δ1 [124] and was essential for normal function of $GABA_A$ receptors [125].

Next, P-1 tethered $Ins(1,3,4,5)P_4$ was used to isolate mouse cerebellar proteins with high affinity for either the soluble ligand or, as discovered later, for the head group of $PI(3,4,5)P_3$ [126]. The first protein, a putative $InsP_6$ receptor, was characterized as the α-subunit of assembly AP-2 [127]. This same purification afforded numerous other proteins, later characterized as centaurin α [116], AMP deaminase [128], and GCS1 [129]. Later, the $Ins(1,3,4,5)P_4$ affinity resin was used to isolate α-actinin from focal adhesions of PDGF-activated fibroblasts in a study of the role of $PI(3,4,5)P_3$ in regulation of cell motility and adhesion [130].

Two forms of tethered phytic acid were prepared and employed for purifications [85, 131]. Most purifications used the P-2 tethered $InsP_6$ [131], since by modifying the axial C-2 phosphate, the achiral *meso* nature of $InsP_6$ was preserved. This matrix was employed to purify an $Ins(1,3,4)P_3$ 5/6-kinase [132] and also an $InsP_6$-kinase that produces the diphosphate PP-$InsP_5$ ("$InsP_7$") [133], a molecule that launched a new frontier for inositol polyphosphate research [134]. The known protein kinase MAST205 could be purified using an $InsP_6$ resin [135].

In more recent studies, affinity matrices using tethered PIP_n derivatives have been employed. The first resin-immobilized biotinylated PIP_n derivatives were used for in vitro expression cloning from mouse spleen [117, 136]. In later studies, an amide-linked $PI(4,5)P_2$ matrix was synthesized by coupling an aminoalkanoyl PIP_n derivative to NHS-activated agarose [100]. This material was used to examine differential binding of traffic-related proteins to the $PI(4,5)P_2$ matrix or to a similarly prepared phosphatidic acid matrix [137]. Similarly, an amide-linked $PI(3,4,5)P_3$ resin was prepared and used to isolate

ARAP3, a novel 170 kDa protein with five PH domains, from pig leukocyte cytosol [138]. ARAP3 regulates both Arf and Rho GTPases and is important in cytoskeletal changes. Over 20 other known and novel proteins were isolated from this affinity purification, including Btk, Etk, centaurin, Cdc42-GAP, type II inositol polyphosphate 5-phosphatase, ezrin, cytohesin-4, PHISH/DAPP1, and rasGAP^{IP4BP}. The cytosol of pig platelets, sheep brain, sheep liver, and rat liver was also probed for proteins that bound to immobilized PI(3,4P)$_2$ (which bound vinculin), PI(3,5)P$_2$ (which bound α-tocopherol transfer protein), and PI(3)P (which bound type C phosphofructokinase) [138]. Proteins were digested with trypsin and identified by mass fingerprinting and sequencing.

Finally, rat lung cell cytosolic proteins were purified by PI(3,4,5)P$_3$ affinity chromatography [139], and two prominent protein bands were characterized by MS as the cytohesin family of Arf guanosine nucleotide exchange factors (cytohesin 1, ARNO, GRP-1) and Btk. Using the same affinity purification scheme with rat brain homogenates, however, yielded only p42^{IP4}, a protein identical to murine centaurin α. The cytohesins in rat brain surprisingly did not bind to the PI(3,4,5)P$_3$-affinity column. Gel filtration experiments of brain cytosol revealed that brain cytohesins are bound to large molecular weight complexes (150 to more than 500 kDa), while in lung cytosol, cytohesins occur as dimers. Thus, oligomerization of PI(3,4,5)P$_3$ binding proteins can modulate their function in a tissue-dependent manner [139]. This comparative approach foreshadowed the subsequent development of a pathway-wide signaling proteomics strategy for signalosome characterization, as discussed below.

In Vitro Expression Cloning (IVEC) Strategies

Beginning in 1996, the P-1 tethered Ins(1,3,4,5)P$_4$ resin was employed to screen mouse spleen proteins expressed from cDNAs pools using IVEC [96]. In the initial screens of 1000 expressed cDNAs per tube, a dozen high-affinity binding proteins, e.g., PDK1 and centaurin α/p42^{IP4}, were identified using 300 mM NaCl to suppress nonspecific binding. This screen then made the first use of the newly synthesized acyl-biotinyl-PI(3,4)P$_2$ and acyl-biotinyl-PI(3,4,5)P$_3$ anchored to streptavidin-agarose beads as an affinity matrix. A novel 35 kDa protein, PHISH, or 3-*ph*osphoinositide-*i*nteracting *s*rc *h*omology-containing protein [96], contained an SH2 domain, a PH domain, and a Tyr phosphorylation motif and was independently described from the human genome as DAPP1 [140]. Interestingly, PHISH was also identified during the same time frame from a human leukocyte cDNA library as SPIEL (*S*H2-containing 3-*p*hosphoinositide *i*nteractor *e*xpressed *i*n *l*eukocytes). SPIEL was found using TOPIS (*t*argets *o*f *P*I3K *i*dentification *s*ystem), a novel assay in the yeast *Saccharomyces cerevisiae*, which used a temperature-sensitive rescue approach based on a Ras-PH fusion that required membrane targeting by either PI(3,4)P$_2$ or PI(3,4,5)P$_3$ [141, 142]. Binding, photoaffinity labeling, and protein overlay assays confirmed the dual specificity of PHISH for PI(3,4)P$_2$ and PI(3,4,5)P$_3$ [96].

A protein isolated from this study was recently identified as a PI(5)P-selective binding protein [117]. This protein, ING2, belongs to the tumor suppressor and chromatin remodeling family of *p*lant *h*omeo*d*omain (PHD) finger-containing proteins with a nuclear localization signal (NLS). The PHD finger consists of two interlaced zinc binding motifs of Cys$_4$ and HisCys$_3$, both of which are required for proper folding in order to maintain PI(5)P affinity. This was confirmed using a structural model of the ING2 PHD (computed using a FYVE domain Zn-finger structure) followed by site targeted mutations to identify the 49-KXKXK-53 motif involved in lipid binding. Ligand specificity was demonstrated by SPR and protein overlay methods. Endogenous ING2 was shown to play a crucial role in p53-regulated apoptotic pathways, and expression of a construct of three tandem PHD fingers acted as a dominant negative to suppress ING2 function in etoposide-induced apoptosis of HT1080 cells by sequestering PI(5)P from its normal target proteins in the nucleus.

Finally, a *meso*-P-2-aminohexyl-linked biotinylated InsP$_6$ derivative [131, 143] was prepared and used to screen a P388D1 macrophage cDNA expression library. MAST205 kinase, a known Ser/Thr kinase in the lipopolysaccharide (LPS) signaling pathway linked to NF-kB activation, was obtained from this screen [135]. The binding of MAST205 to InsP$_6$ was attributed to a PDZ domain that is known to bind PI(4,5)P$_2$.; InsP$_6$ inhibited the phosphorylation of MAST205 [135].

Functional Lipidomics-Proteomics Approach

Solid supports that contained immobilized biotinylated PI(3,4)P$_2$ and PI(3,4,5)P$_3$ were used to capture novel proteins from chemokine-activated murine macrophages (C. Pasquali, D. Meier, K. Mechtler, F. Vilbois, C. Chabert, C. Arod, I. Xenarios, C. Ferguson, R. Booth, M. Camps, G.D.P., and C. Rommel, submitted). Immobilized biotinylated PI(4,5)P$_2$ served as the negative control. We then introduced a minor modification: the use of a disulfide-containing linker that provided reductively cleavable affinity ligands. This modified affinity method was then used to isolate PIP$_n$-associated protein complexes under nondenaturing conditions. Proteins of interest were identified directly by MS/MS, and both a sensitive radiometric lipid-protein binding assay and an in-gel protein kinase assay were used to evaluate signaling complexes. With very limited material, potential novel lipid binding proteins were identified, and algorithms were used to cluster the proteins functionally as enzymes, cytoskeletal-associated proteins, or membrane proteins. The mild elution conditions (DTT) from the disulfide-PIP$_n$ matrices allowed sensitive and selective dissection of multiprotein complexes from this single cell type as a function of chemokine-mediated activation of the PI 3-K γ-signaling pathway. This signaling-oriented proteomic approach has the potential to provide global data on the supramolecular composition of PIP$_n$-complexed signalosomes.

Biophysical and Cellular Studies with InsP$_n$ and PIP$_n$ Affinity Probes

Photoaffinity Labeling and Peptide Mapping

The success of P-1 modified InsP$_n$s as affinity reagents inspired other applications for protein characterization. The first method used photoaffinity labeling with tritium-labeled benzophenone derivatives [144, 145]. The 2,3-

ditritio 4-benzoyldihydrocinnamoyl (BZDC) probes [146] had proven superior to the radioiodinated azidosalicylamide derivatives employed initially, e.g., for the peptide mapping of the affinity–purified Ins(1,4,5)P_3 receptor [118]. In general, photolabeling with BZDC-PIP$_n$s provided three types of biological information: (1) identification of PIP binding proteins in mixtures, (2) relative affinities of different PIP$_n$s for a given protein, and (3) localization of the PIP$_n$ binding site(s).

After purification of mouse brain proteins on an Ins(1,3,4,5)P_4 resin, photoaffinity labeling [126, 147, 148] was used to confirm PIP$_n$ affinity and specificity. Centaurin α/p42^{IP4} (now centaurin α1) [116], the first member of centaurin family of PI(3,4,5)P_3 binding proteins [149], was identified using this approach. Rat adipocyte centaurin α2, a membrane protein with two PH domains, was 51% identical to centaurin αI and abundantly expressed in fat, heart, and skeletal muscle. Photoaffinity labeling with [^{3}H]-BZDC-PI(3,4,5)P_3 and [^{3}H]-BZDC-PI(3,4)P_2 probes confirmed the different ligand specificity for two isoforms [150]. Three other proteins were also obtained from this purification: AMP deaminase [128], GCS1 [129], and synaptotagmin. AMP deaminase is a tightly regulated brain enzyme in the adenylate pathway. Endogenous (rat) and recombinant human AMP deaminase specifically bound PI(4,5)P_2 in both photolabeling and liposome binding assays, and this binding could regulate localization and enzymatic activity in vivo [128]. GCS1, the mouse homolog of a yeast protein with ARF-GAP activity, is required in vivo for normal actin cytoskeletal organization [129]. The yeast homolog GCS1p was cloned, expressed, and photoaffinity labeled with [^{3}H]-BZDC-Ins(1,3,4,5)P_4 and [^{3}H]-BZDC-PI(3,4,5)P_3 probes [129].

A 4-(BZDC)-aminopentanoyl-linked Ins(1,2,6)P_3 derivative as well as a P-5-(BZDC-aminopropyl) linked Ins(1,2,5,6)P_4 derivative were used to search for putative α-trinositol receptors in human umbilical cord vascular smooth muscle cells and platelet membranes [92]. Only the 4-linked ester probe showed specific labeling of 43 and 55 kDa proteins, with Ins(1,2,6)P_3 but not Ins(1,3,4,5)P_4 showing competitive displacement of the binding. Unfortunately, these potential molecular targets for this novel antivasoconstrictive drug remain unconfirmed and have not yet been identified.

The PH domain but not the catalytic site of PLC δ1 isoform was efficiently photolabeled with [^{3}H]BZDC-Ins(1,4,5)P_3. The presence of high-affinity PI(4,5)P_2 and PI(3,4,5)P_3 binding sites in PLC δ1, β1, β2, and β3 isozymes was probed with photolabile BZDC analogs of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, InsP_6, PI(4,5)P_2, and PI(3,4,5)P_3 [19]. Only PLC δ1 was specifically labeled, with a single, high-affinity binding site found in the PLC δ1 PH domain. No photolabeling of the β1, β2, and β3 isoforms was detected. Binding was also probed in recombinant human phospholipase D2 (PLD2), which requires PI(4,5)P_2 for activation. Photoaffinity labeling with both acyl-[^{3}H]-BZDC-PI(4,5)P_2 and triester-[^{3}H]-BZDC-PI(4,5)P_2 revealed displacement by PI(4,5)P_2 but not by PI or PI(4)P [21] [151].

A number of proteins involved in PIP$_n$-regulated vesicular trafficking [152] have been studied with BZDC-labeled InsP_n and PIP$_n$s, and several will be described below. First, synaptotagmin II (Syt II), a synaptic vesicle protein involved as a mediator of exocytosis and vesicle recycling, contains two phospholipid binding C2A and C2B domains. The former acts as a Ca^{2+} sensor, while the latter binds inositol polyphosphates. Photoaffinity analogs of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and InsP_6 that contained the BZDC photophore were used to label GST fusion proteins of the Syt II-C2A and C2B domains [153]. The rank order of photocovalent modification paralleled the order of competitive displacement: InsP_6 (P-2-linked) > Ins(1,3,4,5)P_4 > Ins(1,4,5)P_3.

Second, clathrin coating of vesicles during endocytosis is regulated by InsP_n and PIP$_n$-mediated assembly proteins AP-2 [154] and AP-3 (aka, AP-180) [155]. AP-2 was isolated by affinity purification and photoaffinity labeling with Ins(1,3,4,5)P_4 affinity reagents [127]. Using a palette of PIP$_n$ photoprobes, the α subunit of AP-2 was labeled [156]. In a closely related project, a soluble BZDC-Ins(1,3,4,5)P_4 analog was used to label the N-terminal third of AP-180 [157], known to contain the PIP$_n$ binding site [155]. This region was later identified as the ENTH domain [24].

Third, profilin binds actin monomers, poly(L-proline), and PIP$_n$s and regulates actin filament dynamics that are essential for cell motility. Human profilin I was covalently modified using three [^{3}H]BZDC-containing photoaffinity analogs of PtdIns(4,5,)P_2 [105]. The P-1-tethered Ins(1,4,5)P_3 analog showed efficient and specific photocovalent modification of profilin I, which was competitively displaceable by PI(4,5)P_2 analogs but not by Ins(1,4,5)P_3. In contrast, the BZDC-acyl-PI(4,5)P_2 showed little protein labeling, while the BZDC-triester-PI(4,5)P_2 labeled both monomeric and oligomeric profilin I. [^{3}H]BZDC-Ins(1,4,5)P_3-labeled profilin I labeled the N-terminal 13 amino acid fragment, and the covalent modification of Ala-1 of profilin I was confirmed by Edman degradation. The PI(4,5,)P_2 binding site includes a bisphosphate interaction with a base-rich motif in the C-terminal helix and contact between the lipid moiety of PI(4,5,)P_2 with a hydrophobic inner patch of the N-terminal helix of profilin.

Fourth, photoaffinity labeling of three constructs—full-length, N-terminal, and C-terminal halves—of recombinant human plasma gelsolin was performed with four different *acyl*- and *triester*-[^{3}H]BZDC-PIP$_n$ probes [158]. Specificity was demonstrated by displacement with various short-chain PIP$_n$ analogs. In addition to the known PI(4,5)P_2 binding site in the N-terminal half, we located a novel PIP$_n$ binding site on the C-terminal half of gelsolin that required the intact protein. Proteolysis, purification, and sequencing led to the identification of the labeled region in residues 621–634 [158].

Fifth, coatomer, a complex of seven coat proteins (COPs) involved in the formation of specific Golgi intercisternal transport vesicles, binds Ins(1,3,4,5)P_4 and InsP_6 with subnanomolar affinity [159]. Phosphoinositide and subunit selectivity were examined using P-1-linked photoaffinity analogs of the soluble inositol polyphosphates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, a P-2-linked analog of InsP_6, and P-1-linked phosphotriester analogs of the polyphosphoinositides PI(3,4,5)P_3, PI(4,5)P_2, and PI(3,4)P_2. Labeling of the Golgi coatomer COPI complex showed a highly selective Ins(1,3,4,5)P_4-displaceable

photocovalent modification of the αCOP subunit by [^{3}H]BZDC-Ins(1,3,4,5)P_4 probe, while the P-2-linked [^{3}H]BZDC-InsP_6 probe labeled six of the seven subunits. Most importantly, [^{3}H]BZDC-triester-PI(3,4,5)P_3, the polyphosphoinositide analog with the same phosphorylation pattern as Ins(1,3,4,5)P_4, also showed completely specific, PI(3,4,5)P_3-displaceable labeling of αCOP [160]. In contrast, labeling by BZDC-PI(4,5)P_2 and BZDC-PI(3,4)P_2 showed no discrimination based on the PIP_n ligand. These data suggest the critical importance of the D-3 and D-5 phosphates in regulation of the recruitment of membranes during vesicle budding in signal transduction and thus implicate PI 3-K in this process [160].

Finally, three brief collaborative studies are illustrative of the scope of the photolabeling approach. In one example, PITP, a putative transfer protein for phosphatidylinositol, was labeled with triester [^{3}H]-BZDC analogs of PI(4,5)P_2 and PI(3,4,5)P_3, and competitive displacement was only observed using PI(3,4,5)P_3. This supported a role for PITP as a scavenger of PI(3,4,5)P_3 in stimulating the activity of PI 3-Kγ [161]. In a second collaboration, acyl-[^{3}H]-BZDC-PI(4,5)P_2 and [^{3}H]-BZDC-Ins(1,4,5)P_3 were used to label the holoprotein and the PH domain of the yeast oxysterol binding protein homolog Kes1p. Labeling was specifically displaced by PI(4,5)P_2 but not by other phospholipids, PIP_ns, or soluble InsP_ns [162]. Finally, an unusual tripartite interaction between the transmembrane heparan sulfate proteoglycan syndecan-4, the lipid PI(4,5)P_2, and protein kinase Cα was investigated. The PI(4,5)P_2-syndecan-4 interaction resulted in syndecan-4 oligomerization, an effect that was competable by InsP_6 but not by Ins(1,3,4,5)P_4. The region of the protein responsible for this regulation was identified by photoaffinity labeling with [^{3}H]-BZDC-Ins(1,3,4,5)P_4 and [^{3}H]-BZDC-InsP_6 [163].

Spectroscopic Studies with Spin-Labeled PIP_ns

The first spin-labeled PIP_n derivatives had proxyl groups attached to the acyl chain, while more recent spin-labeled Pea-PIPs [106] place the small, hydrophilic nitroxyl-containing group closer to the protein and near the aqueous interface occupied by the head group. Using ESR, acyl-proxyl-PI(4,5)P_2 was sequestered into lateral two-dimensional domains by the MARCKS polybasic peptide [164]. An analogous study with the SCAMP peptide compared acyl-proxyl PI(4,5)P_2 with proxyl-Pea-PI(4,5)P_2, in which the spin label interacts not with the lipid bilayer but with the peptide head group. The proxyl spin label in the Pea-PIP is in a completely aqueous environment and produces a greater signal than the acyl-linked proxyl group (D. Cafiso, D. Castle, P.W. Rzepecki, and G.D.P., unpublished results). Similarly, in NMR studies of paramagnetic line broadening due to the interaction of acyl-proxyl PI(3)P with the FYVE domain, Kutataladze and coworkers established the angle of bilayer insertion and identified hydrophobic residues in a loop that interacted with the lipid tails of PI(3)P [165].

Surface-Immobilized PIP_ns in SPR

The binding kinetics and ligand specificity for the interactions of a series of IP_n and PIP_n species with the C2B domain of synaptotagmin II (Syt II) was measured using SPR [166]. The on- and off-rates of His-tagged SytII-C2B (and C2B + C2A) were determined on BIAcore Pioneer B1 sensor chips that had been covalently modified by linking P-1 aminopropyl Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, or InsP_6 to NHS/carbodiimide-activated carboxymethyldextran. C2B and C2A+C2B domains had the highest affinity for the Ins(1,3,4,5)P_4 modified surface, and the short-chain analogs of PI(4,5)P_2 and PI(3,4,5)P_3 were more effective than head groups in displacing binding, suggesting that the SytII C2B domains use PIP_n interactions in docking and/or fusion of the secretory vesicles to the synaptic membrane.

Most recently, experiments have used BIAcore 2000 with CM5 sensor chips to compare acyl-linked PIP_ns with the new Pea-PIP and PolyPIPosome architectures for use in determining on-rates and off-rates for PH domain-PIP_n interactions (C.G. Ferguson, P.W. Rzepecki, P.O. Neilsen, Y. Abdiche, D.G. Myszka, and G.D.P., unpublished results). Streptavidin was immobilized to the chips and used to capture three biotinylated PI(3,4,5)P_3 probes [acyl-biotinylated PI(3,4,5)P_3, biotinylated Pea-PI(3,4,5)P_3, and biotinylated PI(3,4,5)P_3 PolyPIPosome]. The binding of the highly PI(3,4,5)P_3-selective Grp1-PH domain [111, 112] was analyzed in a glycerol and serum albumin-containing buffer at pH 7.4. Rapid flow rates minimized the effects of mass-transport limitations. Estimates of Grp1 binding affinity revealed that biotinylated Pea-PI(3,4,5)P_3 and biotinylated PI(3,4,5)P_3 PolyPIPosome surfaces were equivalent (K_D = 2.4 nM), while Grp1 bound acyl-biotinylated PI(3,4,5)P_3 with a 5-fold lower affinity (K_D = 12 nM). This suggested that the presentations of the PI(3,4,5)P_3 head group in the two-headed lipid and polymerized liposome were superior to that of the acyl-modified lipid.

Anti-PIP_n Antibodies

A method other than GFP-PH domains [167] was required to visualize specific PIP_ns in a number of cellular compartments. Although small lipids are in general poorly antigenic, liposomal preparations and bioconjugates of PIP_ns have allowed production of biologically useful anti-PIP_n reagents. Monoclonal antibodies (mAbs) against PI(4,5)P_2 were first developed over 15 years ago [168], and newer antibodies prepared against different immunogenic forms in the past four years [169, 170]. These antisera, prepared against liposomal PI(4,5)P_2, colocalized PI(4,5)P_2 with splicing factor SC-35, a known marker for nuclear speckles, implicating PI(4,5)P_2 in the assembly of a mitotically regulated particle important in pre-mRNA splicing [43]. Moreover, PI(4,5)P_2 was completely colocalized with SC-35 and partially colocalized with DAG kinase θ in MB-453, MCF-7, PC-12, and HeLa cells [44]. The nuclear PI(4,5)P_2 staining seen in normal mouse fibroblasts was absent in cells expressing the *beige* gene orthologous to the human Chediak-Higashi Syndrome gene [49].

When liposomes containing synthetic dipalmitoyl PI(3,4,5)P_3 were unsuccessful in eliciting an immune response, a bioconjugate was prepared by coupling over 100 succinyl-modified PI(3,4,5)P_3 molecules to the Lys residues of keyhole limpet hemocyanin (KLH) (Figure 5A). This immunogenic form of PI(3,4,5)P_3 was employed to obtain the first anti-PI(3,4,5)P_3 mAbs [171]; PIP_n selectivity was demonstrated by photoaffinity labeling and ELISA. Moreover, stimulation of neutrophils with fMLP led to a rapid (7 s) and transient peak of immunoreactive PI(3,4,5)P_3, while activation of NIH 3T3 fibroblasts with

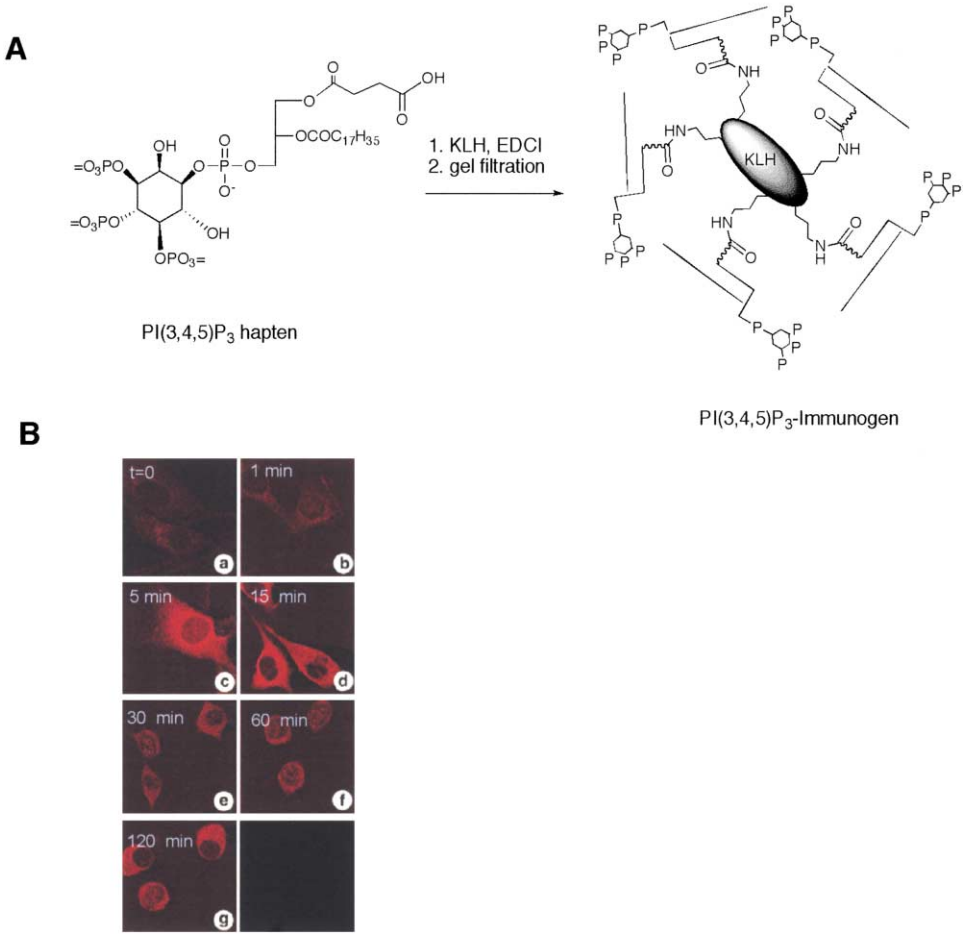

Figure 5. Immunodetection of Phosphoinositides

KLH, keyhole limpet hemocyanin; EDCI, ethyl *N,N*-dimethylaminopropylcarbodiimide.

(A) Preparation of an antigenic PI(3,4,5)P_3 bioconjugate.

(B) Visualization of PI(3,4,5)P_3 in stimulated fibroblasts [171].

PDGF showed maximal PI(3,4,5)P_3 in cytoplasmic compartments after 5–10 min with a slower return to baseline levels (Figure 5B). Interestingly, from 15–30 min, a peak of PI(4,5)P_2 production was observed in the nucleus. The anti-PI(3,4,5)P_3 IgMs have also been used to visualize spatiotemporal changes in PI(3,4,5)P_3 in bovine aortic endothelial cells (BAECs) in culture and hepatocytes of intact rat liver [172]. Treatment of the BAECs with either insulin or leptin increased PI(3,4,5)P_3, and this could be blocked with PI 3-K inhibitors [172]. Moreover, immunohistochemistry also showed preferentially increased PI(3,4,5)P_3 in cells of the acuate nucleus of the hypothalamus that contained insulin receptor substrate (IRS)-2, providing a mechanism for neuronal crosstalk between insulin and leptin signaling pathways [173].

In many types of cancer, upregulation of PI 3-K activity and abrogation of PTEN would be predicted to cause an overabundance of PI(3,4,5)P_3 in affected tissues [4]. Indeed, staining of uterine tissues from a hysterectomy showed massive amounts of PI(3,4,5)P_3 immunostaining in the metastatic side of the uterine wall but negligible staining in the unaffected contralateral side [57]. Similar results in breast cells and in ovarian tumors have been observed (B.E. Drees, S. Dhardmawardhane, G.B. Mills, and G.D.P., unpublished results).

In a related approach, polyclonal antibodies against Ins(1,4,5)P_3 were raised by immunizing rabbits with P-1-tethered Ins(1,4,5)P_3-BSA conjugates [174]. An ELISA using immobilized Ins(1,4,5)P_3 as the capture antigen showed sensitivity and selectivity: while most InsP_ns and PtdIns(4,5)P_2 did not compete for binding, Ins(1,3,4,5)P_4 had 33% of the affinity of Ins(1,4,5)P_3. The affinity-purified antibody displayed IC_{50} values of 12 nM for Ins(1,4,5)P_3. Follow-up studies using this antibody in biological contexts remain unreported. Because InsP_ns are water soluble, are highly metabolically labile in vivo, and are not localized to membranes, the applications of anti-InsPn mAbs in cell biology have appeared limited. Nonetheless, potential applications in metabolite analysis and in drug discovery suggest that efforts to obtain additional anti-InsP_n mAbs could provide useful new reagents.

Fluorescent PIP$_n$s in Biochemical and Biophysical Studies

The acyl-linked fluorescent PIP$_n$s were first developed as probes in model systems and for in vitro studies of

lipid binding proteins. The first use of NBD-PI(4,5)P_2 was to study the role of the MARCKS polypeptide in sequestration of PI(4,5)P_2 in two-dimensional lateral arrays [175]. In a lipid bilayer system, acrylodan-labeled MARCKS polypeptide colocalized with NBD-PI(4,5)P_2. Separately, the inhibition of PLC hydrolysis of PI(4,5)P_2 by MARCKS polypeptide was determined. Taken together, the results implicated the regulation of PLC activity by lipid clustering and sequestration by the MARCKS polypeptide.

A second application of acyl-linked fluorescent PIP_ns examined the effects of the PI(4,5)P_2-regulated proteins gelsolin, tau, cofilin, and profiln by using fluorescent PI(4,5)P_2 analogs in micellar or liposomal form (with PC) [176]. Gelsolin was unique in increasing the fluorescence of both NBD- and pyrene-PI(4,5)P_2 in both systems in a dose-dependent manner. Based on fluorescence resonance energy transfer (FRET) data, it appeared that PI(4,5)P_2 bound to a site formed by the juxtaposition of the N- and C-terminal domains. This corroborated the photoaffinity labeling results, in which a new PI(4,5)P_2 binding site was observed by photoaffinity labeling of the full-length gelsolin protein [158].

The most recent applications of fluorescent PIP_ns and fluorescent $InsP_n$s have been in the visualization of subcellular distribution of exogenously administered PIP_ns, and in the development of rapid HTS assays for detection of changes in PIP_n phosphorylation or levels. These applications are described in more detail below.

Intracellular Delivery of Fluorescent PIP_ns

We sought a method for intracellular delivery of PIP_ns that would not require photolytic uncaging of phosphate esters and enzymatic hydrolysis of masked hydroxyl groups [177]. We discovered that neomycin, histone H-1, and their fluorescent derivatives could deliver IP_n (n = 3,4) and PIP_n (n = 1,2,3) compounds into cells [107]. Fluorescent NBD-PI(4,5)P_2 was shuttled into plant (*A. thaliana*) root tip cells, mammalian cells (MDCK kidney cells, CHO ovary cells, and NIH 3T3 fibroblasts), bacteria (*E. coli*), protists (*Cryptosporidium parvum*), and yeast (*S. cerevisiae*) in the presence but not absence of a stoichiometric amount of carrier. In mammalian cells, shuttled NBD-PI(4,5)P_2 was localized to the plasma membrane, punctate cytoplasmic structures, and nuclear speckles, while shuttled NBD-PI(3,4,5)P_3 localized to the plasma membrane and cytoplasmic vesicular structures but remained outside of the nucleus. Histone-delivered PI(4,5)P_2 induced intracellular calcium mobilization in 3T3-L1 and NIH3T3 cells, while histone-shuttled BODIPY-PI(3,4)P_2 localized to the plasma membrane extensions in the same cells (see Figure 6).

Using the panel of small-molecule and histone-based translocases, we explored the parameters for optimal intracellular delivery of PI(4,5)P_2, PI(3,4)P_2, and PI(3,4,5)P_3 using fluorescently labeled lipids. The kinetics of uptake, the localization of specific PIP_ns, and their metabolic stability in fibroblasts, CHO, and MDCK cells have now been determined (D. DeWald, S. Ozaki, S. Malaviya, J. Shope, K. Manabe, L. Crosby, P. Neilsen, D. Johnston, and G.D.P., submitted). Specifically, dendrimer-shuttled PIP_ns appeared to enter through focal contacts, while other polycationic carriers caused uptake with entry first evident at 20 s and generally complete within 5 min. Intracellular NBD-PI(4,5)P_2 was stable for at least 30 min in unstimulated cells, but was metabolized to NBD-DAG and Ins(1,4,5)P_3 upon stimulation with PDGF, insulin, or bradykinin.

Chromophore-Assisted Laser Inactivation (CALI)

Laser inactivation studies with Ins(1,4,5)P_3-malachite green using CALI allows the study of in situ protein function by selective destruction of a target protein with high spatial and temporal resolution [178]. In particular, P-1 linked malachite green-Ins(1,4,5)P_3 was used to target the Ins(1,4,5)P_3 receptor (IP3R) using permeabilized smooth muscle cells. When the cells were treated with malachite green-Ins(1,4,5)P_3 followed by laser irradiation, the Ins(1,4,5)P_3-induced Ca^{2+} release rate was decreased in a concentration- and irradiation time-dependent manner. Similarly, the IP3R was inactivated with high spatiotemporal resolution in single B lymphoma cells and in a spatially distinct region of differentiated PC12 cells [179].

Finally, P-1 linked BODIPY-Ins(1,4,5)P3 was used to examine the localization and function of Ca^{2+} stores in isolated chromaffin cells of rat adrenal medulla using confocal laser microscopy. Binding sites for BODIPY-Ins(1,4,5)P_3 were perinuclear, particularly the endoplasmic reticulum, and at the cell periphery [180].

Drug Discovery and Target Validation

HTS Assays

Until very recently, all PI 3-K activity was determined by using tedious radiometric endpoint assays that relied on the biochemical conversion of PI to PI(3)P or of PI(4,5)P_2 to the corresponding PI(3,4,5)P_3 using γ^{32}P-ATP as the cosubstrate. After the reaction, phosphorylated lipids were separated by thin layer chromatography and quantified by liquid scintillation counting or phosphorimaging. A 96-well format version of this PI 3-K assay was recently described [181]. The discovery and cellular validation of the next generation of PI 3-K inhibitory compounds has been significantly accelerated by advances in two areas: (1) the development of HTS-compatible nonradioactive assays and (2) the availability of additional techniques to visualize the localization and effects of differentially phosphorylated lipids in cells [57].

Several formats have now been reported for nonradioactive PI 3-K assays in 96- or 384-well formats [114, 182], including ELISA, ALPHAscreen, and fluorescence polarization (FP) technologies, for which a variety of patents are currently pending. As illustrated in Figure 7A, these assays employ synthetic PIP_ns (or $InsP_n$s) labeled in the acyl chain with fluorophores (for FP) or biotin (for ALPHAScreen; not shown) [114]. Using these assays offers the possibility of accelerating the discovery of new inhibitors of PI 3-K, a selection of which are illustrated in Figure 7B and reviewed in more detail elsewhere [50].

Physiological Activity of Exogenous PIP_ns

Exogenous anionic PIP_n ligands can be delivered ("shuttled") across the membranes of living cells using specific polyamines as carriers [107]. The process occurs within a few minutes, even at reduced temperatures or in the presence of an endocytotic blocker. Moreover, fluores-

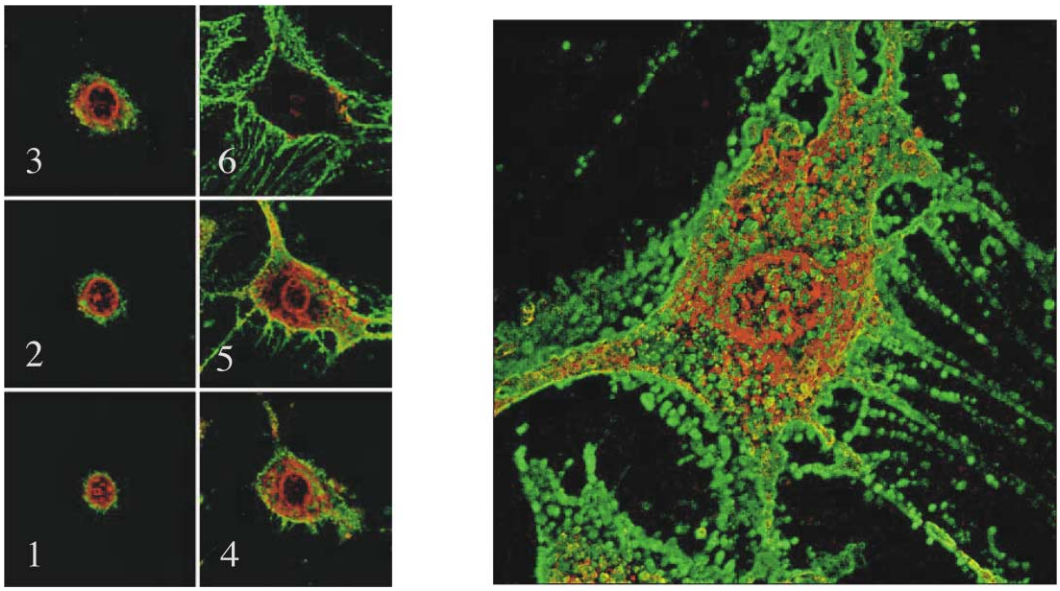

Figure 6. Confocal Images Illustrating the Uptake of NBD-PI(3,4)P_2 and Texas Red Histone in NIH 3T3 Fibroblasts Using Intracellular Delivery Methods

(A) Sequential optical z-sections (0.2 μm) were collected 5 min after addition of PI(3,4)P_2-histone complex. The montage shows six successive 1.5 μm steps from the top of the cell (1) to the attachment of the cell to the substratum (6).
(B) Pseudo-three-dimensional reconstruction. Images are courtesy of J. Shope and D.B. DeWald [107].

cently labeled PIP_n ligands could be shuttled into cells using fluorescently tagged histone or neomycin. Labeled PIP_ns 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 [57, 171]. Although we demonstrated that both shuttled Ins(1,4,5)P_3 and PI(4,5)P_2 could elicit calcium transients in living cells within 2–4 min, it was questioned whether shuttle-delivered exogenous PIP_ns would have general utility in manipulating cellular physiology. This has now been answered in the affirmative by a number of studies.

First, exogenous PI(3,4)P_2 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 [183]. Other shuttled PIP_n ligands were ineffective, and thus both PI(3,4)P_2 and PI(3,4,5)P_3 were required for full phosphorylation of Ser473 and Thr308 of Akt in SHIP null cells. Second, addition of exogenous PI(3,4,5)P_3 activated a PI 3-K- and Rho GTPase-mediated positive feedback loop that regulates polarization and migration of neutrophils [10]. Remarkably, within 3 min neutrophils began migrating when a histone-PI(3,4,5)P_3 complex was added to the medium; shuttling in PI(4,5)P_2 or omission of the carrier failed to activate polarization and migration. Third, in examining the role of $InsP_6$ in preventing tumor cell growth, coapplication of $InsP_6$ with histone induced apoptosis at a 10-fold lower dose than $InsP_6$ [184]. Induction occurred by inhibition of the Akt cell survival pathway. Fourth, in elegant work by the Yin group [185], intracellular delivery of PI(4)P but not PI(4,5)P_2 rescued the phenotype of cells in which RNA interference had knocked down the resident Golgi PI 4-K required for production of the endogenous PI(4)P supply. Fifth, exogenous PI(3)P was shown to induce plasma membrane translocation of the glucose transporter protein GLUT4, a PI 3-K-dependent process occurring downstream of activation of the insulin receptor [186]. Finally, the delivery of BODIPY-TR-PI(5)P to Balb-C 3T3 cells resulted in subnuclear localization of this lipid but not BODIPY-TR-PI(3)P. Importantly, exogenous PI(5)P ultimately colocalized and modulated the nuclear localization of the PHD zinc finger-containing protein ING2 and altered chromatin remodeling in these cells. ING2 binding to PI(5)P but not to PI(4)P caused ING2 to translocate from the chromatin to the nucleoplasm (O. Gozani, S. Field, C. Ferguson, C. Mahlke, L. Cantley, G.D.P., and J. Yuan, submitted). Since there are over 100 nuclear proteins that contain PHD fingers, additional roles of PIP_n in transcriptional regulation may emerge in the future.

Manipulation of PIP_n Signaling in Cells

The chemical rescue of PIP_n function in cells described above is the inverse of the more common pharmacological approaches that employ small molecule inhibitors, dominant negatives, cells from transgenic knockout animals, and more recently RNA interference to reduce the function of a given protein. Such approaches have of course been the mainstay of studies of PI 3-K signaling, but sadly only two low-selectivity PI 3-K inhibitors (see Figure 7B) have been available for the past ten years, as recently reviewed [49, 50]. There is widespread concern that many cell-based studies using LY294002 and wortmannin may require reinvestigation with isoform-selective inhibitors such as the published PI 3-Kδ inhibitor and the as yet commercially unavailable PI 3-K α, β, and γ inhibitors [50, 51].

An active area of drug discovery is the identification of Akt/PKB inhibitors using two main approaches. First,

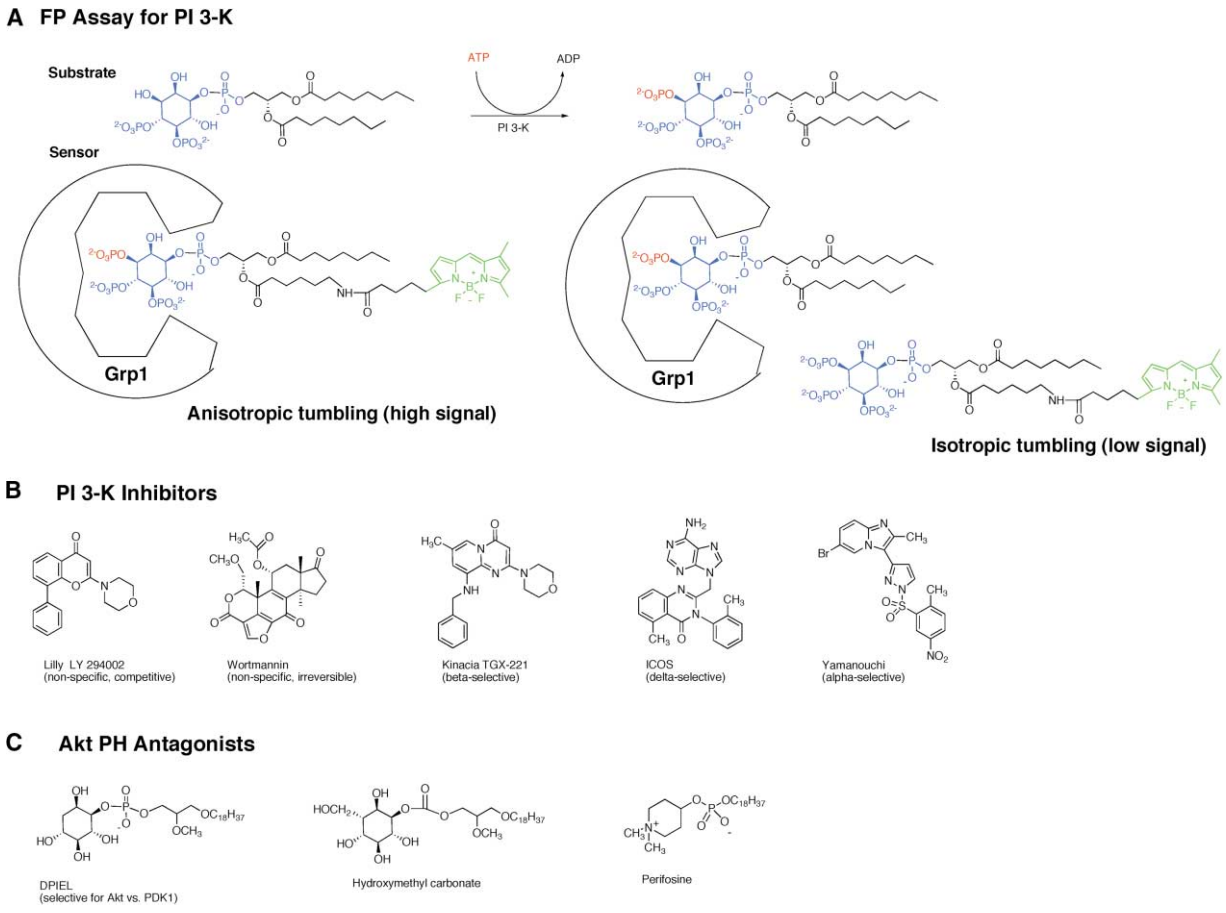

Figure 7. Drug Discovery in the PI 3-K Pathway

(A) Fluorescence polarization assay for PI 3-K [114].

(B) Selected inhibitors for PI 3-K [50].

(C) Akt PH domain antagonists [56].

traditional protein kinase inhibitors have become extremely important new cancer therapeutics [187]. Thus, an inhibitor could be targeted to the kinase domain phosphoryl transfer site of Akt, e.g., a heterocycle that would bind with high affinity and selectivity in the ATP pocket. An "Aktstatin" was rationally redesigned based on a PKA inhibitor with a modest potency and selectivity for Akt [188]. Second, although untested in therapy, selective antagonists of PH domains would be potential small molecule drugs. An Akt antagonist that "mimics" PI(3,4,5)P_3 could block signalosome assembly and thus activation of Akt by phosphorylation by PDK1 and an as yet unknown kinase with "PDK2" activity. Indeed, PDK1 inhibitors (or PDK1 PH domain antagonists) could also be potential targets for downregulation of Akt activation. A preliminary validation of this was accomplished by using antisense oligonucleotides to reduce the level of PDK1 in cancer cells, resulting in increased apoptosis [189].

In the Akt pathway, the Kozikowski group has used molecular modeling to predict the differential affinity of 3-phosphorylated PIP_ns and to provide a structural basis for the design of novel, high-affinity ligands that could modulate signaling by Akt [190]. For example, 3-modified inositol ether lipid phosphates and a carbonate (Figure 7C) were synthesized and found to inhibit both PI 3-K and Akt and to slow cancer cell growth [191, 192]. These and other D-3-deoxy-phosphatidyl-*myo*-inositol analogs appear to specifically inhibit membrane translocation via the Akt1 PH domain [193], thereby blocking Akt activation and inhibiting its prosurvival effect in cells [56]. This selective Akt inhibitor also reduces resistance to chemotherapeutic drugs and ionizing radiation in human leukemia cells [194]. The alkylphospholipid analog perifosine (Figure 7C), an orally bioavailable drug, has shown activity in vitro and in vivo in human tumor model systems and has entered phase I clinical trials. In PC-3 prostate cancer cells, perifosine does not inhibit PI 3-K but instead perturbs membrane translocation of Akt and other PH domain-containing proteins in the PI 3-K/Akt pathway [195].

There is a need for selective inhibitors of the 3-phosphatase PTEN and the 5-phosphatase SHIP in order to study responses of coupled PI 3-K signaling pathway components in cells and in animals. At present, no such selective inhibitors are commercially available. This is another area where both substrate-based design and pharmaceutical discovery of drug-like molecules is pro-

ceeding rapidly, with a robust demand for such inhibitors by the research community. As with nonradioactive HTS lipid kinase assays, lipid phosphatase assays using tethered PIPs as reporters for enzymatic turnover [114] offer the potential for increasing the rate of drug discovery.

In Vivo Target Validation

The next step is in many ways a leap into the unknown. For academic, biotech, and pharmaceutical researchers alike, there are many unanswered questions that must be addressed before the pathways revealed through the use of tethered PIP_n affinity probes will result in marketable drugs. Thus, despite the encouraging results for many of the PI 3-K enzyme isoforms and their downstream targets provided by genetic knockout animals [49–51], targeting the PI 3-K/Akt signaling pathway [54, 55] with drugs remains largely unvalidated in animals and still untested in humans or nonhuman primates. Therapeutic applications for modulation of this pathway include airway inflammation, rheumatoid arthritis, diabetes, cancer, and thrombosis, as recently reviewed [50]. The exploration of these complex issues for any particular pathophysiology is beyond the scope of this review, and thoughtful analyses of the situation in pharmacological manipulation of lipid signaling can be found in the reviews cited.

In closing, several of these key questions are addressed.

1. Can small molecule inhibitors be developed with all the necessary properties? There are four isoforms of type I PI 3-K in addition to the type II and III PI 3-K enzymes and more distantly related protein kinases, PIP_n kinases, and lipid kinases. The pharmaceutical industry routinely succeeds in optimizing solubility and pharmacodynamic properties for a given drug but succeeds less frequently in achieving a truly optimal selective inhibitor for a specific protein target.

2. How will these compounds be used clinically? For cancer, based on tumor xenograft models, it would be logical to combine a PI 3-K inhibitor with a cytotoxin such as taxol or doxorubicin. For diabetes, would a SHIP inhibitor be given alone or in combination with another treatment modality? Would such a drug be administered chronically or acutely? For inflammation, for rheumatoid arthritis, or for airway disorders, would PI 3-K γ or δ inhibitors be used separately or in combination with NSAIDS?

3. Prior to launching a clinical trial, what steps are required to ensure that an inhibitor will be safe and effective? How can we be confident that inhibiting an enzyme or an element of a signaling cascade required for normal physiology be free from life-threatening consequences in treating human pathophysiology? The animal models used in target validation are notoriously misleading in predicting effects in human patients. Distribution, metabolism, and target selectivity of drugs in people often differ dramatically from that in models.

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