

Patterns within protein/polyphosphoinositide interactions provide specific targets for therapeutic intervention¹

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ABSTRACT Signaling pathways involving the inositol polyphosphates and the polyphosphoinositides have become intricately linked with a number of disease states. More recently, this has principally involved the 3-phosphorylated products of phosphoinositide 3-kinase, an enzyme that itself shows oncogenic activity and has hence become of interest in the design of antitumorigenic drugs. The downstream effectors of phosphoinositide 3-kinase are involved in different aspects of cellular signaling and cytoskeleton and trafficking events that are linked to specific polyphosphoinositide binding properties of specific protein domains, which themselves have emerging roles in specific disease states. Our recent findings have demonstrated that there is a selectivity of the intracellular effects of extracellularly applied inositol polyphosphates in their abilities to inhibit a range of growth-related *in vivo* assay conditions, and that these can themselves be linked to the inhibition of the membrane localization of a green fluorescent protein (GFP) -tagged PH domain. We propose that GFP fusions of the polyphosphoinositides binding domains of specific proteins of interest can be used in high-throughput investigations of the therapeutic value of specific inositol polyphosphates analogs. Inhibition of *in vivo* membrane targeting of these domains from proteins involved in cell growth and tumorigenesis can thus be used in the search for new anticancer drugs.—Berrie, C. P., Falasca, M. Patterns within protein/polyphosphoinositide interactions provide specific targets for therapeutic intervention. *FASEB J.* 14, 2618–2622 (2000)

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INOSITOL POLYPHOSPHATES LEVELS AS THERAPEUTIC TARGETS

IT IS STILL just over 30 years since the higher inositol polyphosphates (IPPs)—namely, inositol (1, 3, 4, 5,

6)-pentakisphosphate (Ins13456P₅) and inositol hexakisphosphate (InsP₆; phytic acid)—were first reported to be naturally occurring components of mammalian cells. Since then, their extracellularly applied, intracellular effects have been noted (1); furthermore, early InsP₆ investigations demonstrated *in vivo* effects in diverse cell systems, including the enhancement of Ca²⁺ influx and aspartate release in cultured cerebellar neurons (2), the stimulation of Ca²⁺ uptake in cultured anterior pituitary cells (3), the priming of the stimulated respiratory burst in human neutrophils (4), and the excitation of rat medullary sympathoexcitatory neurons *in vivo* (5). Interest in the biological activities of the IPPs has thus further increased (see refs 6, 7 for reviews), with particular emphasis on the potential anticancer actions of InsP₆ emerging more recently (see ref 8 for review).

The use of lithium to potentially modulate cellular inositol and/or IPP levels via its action as an inhibitor of the inositol monophosphatases has been used both in the treatment of mood disorders and in numerous experimental systems. However, lithium also induces a rapid increase in phosphoinositide 3-kinase (PI3K) activity and Akt-1 phosphorylation in cerebellar neurons, an effect that is blocked by PI3K inhibitors (9). Furthermore, the identification of lithium regulation of a human enzyme (10) and a rat

¹ NOMENCLATURE: The generic forms of IPPs and PPIs are used with respect to the inositol polyphosphates and polyphosphoinositides, whereas the unspecified isomers of the IPPs are given without the phosphate position numbering [e.g., InsP₃; except in the case of InsP₆, which represents inositol (1,2,3,4,5,6)-hexakisphosphate, the only isomer of this IPP]; the indication of specific inositol (poly)phosphates and (poly)phosphoinositides are represented by the example of Ins145P₃ and PtdIns45P₂. PI3K is also used in the generic form to represent the family of phosphoinositide (PI) 3-kinases.

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gene (11) indicates that there indeed may be other targets in the mechanism of action of lithium. As such, and in the light of the emerging *in vivo* specificities of the inhibition of various signaling pathways by specific IPPs (see below), the mechanisms of these diverse *in vivo* effects of lithium now need to be reconsidered.

PtdIns 3-KINASE PRODUCTS, PH DOMAINS, AND MEMBRANE TARGETING

Along with the well-known lipid-signaling pathway that involves the 4- and 5-phosphorylation of phosphatidylinositol (PtdIns), which results in the production of phosphatidylinositol (4, 5)-biphosphate (PtdIns45P₂) at the plasma membrane, these lipids are also substrates for the newer family of PI3Ks. There have been three main classes of the PI3Ks described according to their *in vitro* lipid substrate specificities, structures, and regulation: Class I, which 3-phosphorylates PtdIns, PtdIns 4-phosphate (PtdIns4P), and PtdIns45P₂, and possesses both catalytic (e.g., p110 α , β , γ) and regulatory (e.g., p85 α , β) subunits; Class II, which 3-phosphorylates PtdIns and PtdIns4P selectively (e.g., PI3K-C2 α , β); and Class III, which 3-phosphorylates PtdIns selectively and is represented by the yeast Vps34p catalytic and the Vps15p regulatory subunits (see ref 12 for review). A Class IV of nonconventional PI3Ks has also been suggested (13). There are thus four different potential lipid products from the 3-phosphorylation of the phosphoinositides: PtdIns 3-phosphate (PtdIns3P), PtdIns (3, 4)-biphosphate, PtdIns (3, 5)-biphosphate, and PtdIns (3, 4, 5)-trisphosphate (PtdIns345P₃). Each of these PPIs appears to have its own specific targets and functions in the cell (see ref 14 for review), and include the mediation of signal transduction, cytoskeleton rearrangements and/or membrane trafficking, modulating these systems via specific interactions with certain emerging families of lipid recognition/binding domains (see refs 6, 7, 14, 15 for reviews), of which the pleckstrin homology (PH) domain is now the largest represented. The PH domain is a well-recognized, multifunctional structural domain consisting of some 100 to 120 amino acids that share a specific tertiary structure (see refs 16, 17 for recent reviews). Within a year of its initial definition, reports of the 3-dimensional structure appeared and the list of proteins containing PH domains began; a few hundred eukaryotic proteins with a variety of functions are now listed as containing PH domains (see ref 18 for further details). The common theme is that the PH domain itself has no catalytic properties and that its presence is generally associated with proteins that need to be positioned at or near membranes; this has led to the proposal that they are involved in the tethering of proteins to specific membranes, where the proteins are required for their function (19, 20).

In this context, whereby many diverse proteins can be activated via their recognition of and binding to specific membrane PPIs, and more particularly with respect to the generation of specific 3-phosphorylated PPIs that have now been implicated in the regulation of numerous cellular processes, such as growth, proliferation, survival, differentiation, and cytoskeleton rearrangements (20), a new field for drug design has opened. Thus, the ability to control the activities and specificities of the PI3Ks and their lipid products allows for the potential of interfering in any of these PI3K/PPI binding domain-mediated cellular processes. Furthermore, with the more recent reports indicating that the candidate tumor suppressor PTEN is not just a dual specificity protein phosphatase, but also a phosphoinositides 3-phosphatase (21), its involvement in these PI3K/PH domain-mediated cellular processes can also be targeted for drug design. However, whereas these approaches can allow for specificity at the level of the selection between pathways that do and do not involve the activation of PI3Ks (see, for instance, our recent report; ref 22), it is also clear that with the wide ranging effects of the PI3K-mediated cellular interactions, there is the need for a high level of inbuilt specificity in any drug design approach.

Therefore, PI3K is an attractive therapeutic target, and specific inhibitors of the kinase should prove useful in cancer therapy, particularly the oncogenic potential of PI3K. Our attention, however, is focused on the mechanisms of membrane targeting mediated by the lipid products of PI3K. Ligands of the main protein targets of these lipids, and more specifically the PH domain, have the potential to antagonize the activation of specific proteins by inhibiting their translocation to the plasma membrane (or to any specific membrane compartment). Indeed, as different PH domains possess different binding affinities toward different IPPs, this increases the possibility of specifically inhibiting a particular membrane-targeted protein. Hence, this introduces a novel aspect of antagonism of PI3K signaling pathways that could give us the potential to block a very specific downstream target without interfering with all the other PI3K-mediated signals. In combination with our recent work (22) and some slowly emerging (and often unexpected) patterns (see also below), we propose here that in the same way that each specific phosphate group around the head group of each specific PPI can promote unique functional roles in cells, the IPPs, the water-soluble head groups of these PPIs, can provide specific antagonists of these functions. Hence, it is possible to design specific drug structures in order to mimic, and thus inhibit, known protein structure recognition specificities.

FURTHER PROTEIN DOMAIN/POLYPHOSPHOINOSITIDE RECOGNITION PATTERNS

Although the PH domain as a structural protein motif is the most well defined, the first specific IPP/PPI binding motifs that were described were the short (10 to 20 amino acids) lysine/arginine-rich sequences that are present in a number of cytoskeleton proteins and in the C2 domains of particular proteins, such as the C2B domain of synaptotagmin (see refs 7, 23 for reviews). Although the actual sequences and specificities appear not to have always been very well defined, there may now be an emerging importance of these interactions in the regulation of cytoskeleton-plasma membrane adhesion by PtdIns45P₂ (24). More recently, and perhaps more important, after the initial report that PtdIns3P is involved in intracellular traffic in yeast (25) and the definition of a common structural motif in the membrane localization of specific proteins, the cysteine-rich, 'zinc finger' FYVE domain and its role in membrane trafficking events in the secretory and endocytic pathways has become established (see refs 26–28 for reviews).

At the same time, two additional sequences of ~ 20 amino acids have been proposed. The first of these has been named the HIKE motif, and was originally reported to be a candidate binding site for specific PH domains (29); its ability to also bind to the PPIs has been demonstrated in many cases because, as a motif, it includes the lysine/arginine-rich sequence of gelsolin and is included in the PH domain of Bruton's tyrosine kinase (Btk). The importance of mutations in HIKE regions of specific proteins has emphasized its involvement in various disease states (see ref 30 for review). The other new PPI binding region is present in the kinase domain of the PtdIns phosphate kinases; it determines both the enzymatic specificity and subcellular targeting of these kinases, and hence controls their signal specificity and function (31). The recent definition of a role for PtdIns4P 5-kinase α as a critical modulator of thrombin- and Rac-dependent actin assembly indicates the emerging importance of these enzymes (32).

PHYSIOLOGICAL TARGET SPECIFICITIES AND DISEASE

With the rising importance of the PPIs in signal transduction, cytoskeleton rearrangements, and membrane trafficking that began less than 10 years ago, there has been an increase in the number of reports of specific effects of these lipids in many diverse *in vitro* systems. Based on these known domain recognition/mutation patterns and with spe-

cific reference to known and emerging mutation/disease patterns, we aim to discuss some of these effects, their relationships to disease states and the specificities of protein/PPI interactions, and the evidence that they do indeed have relevance *in vivo*.

Mutation causes disease

In this context, perhaps the most well-defined system with regard to mutational analysis at the genetic level is the case of Btk. Btk plays a critical role in B cell development and proliferation, and some of the genetic mutations give direct links between PH domain membrane-targeting and protein function; some 20% (ca. 25) of the known genetic substitutions, insertions and/or deletions in the Btk gene have already been attributed to altered PH domain function in human X-linked agammaglobulinemia (XLA; 18). One of the most severe forms of XLA arises from a single amino acid substitution (Thr33Pro) in that part of the PH domain that coincides with the HIKE motif (18, 30). Although numerous other genetic mutations attributed to XLA also exist in the SH2, SH3, and kinase domains of Btk (18), this is a direct example of how interference with specific PH domain/membrane targeting can be used to inhibit the 'normal' activities of PH domain-containing proteins. As another direct linkage, faciogenital dysplasia (FGD; Aarskog-Scott syndrome) is a rare X-linked multisystemic disorder that leads to numerous physical abnormalities arising primarily from an insertion mutation in the FGD1 gene, which results in a frameshift and a termination signal that results in the loss of the carboxyl terminus half of the protein (18). This mutation leads to the loss of the two PH domains and the FYVE domain, with the remaining protein being nonfunctional. Even though the significance of these three potential lipid binding domains remains to be determined, this is an example where a PH domain is implicated in protein-protein binding, as a Cdc42-specific guanine-nucleotide exchange factor (33). In the case of the actin regulatory protein gelsolin, a single amino acid substitution in the HIKE motif has been described in a position just before the second of its two earlier defined lysine/arginine-rich, PPI binding regions (Asp187Asn and Asp187Tyr; 18, 30). These mutations lead to the Finnish-type hereditary amyloidosis and demonstrate the importance of the PPI binding domains and the membrane localization of this protein in its actin-severing activity. These examples of the importance of the PPI binding regions in the normal function of specific proteins and the emerging oncogenic potential of a number of proteins involved in such interactions lead us to the specific inhibition of pro-oncogenic signals.

Oncogenic inhibition

The activation of Akt (RAC/protein kinase B) by the PtdIns345P₃-dependent protein kinase (PDK1) regulates the downstream phosphorylation of further signaling proteins, which in turn leads to the choice of cellular proliferation or apoptosis (34, 35). The ability to inhibit this activity *in vivo* via our recent demonstration of the disruption of its membrane localization by selective IPPs (22) represents an important control point in the regulation of cell survival that can be exploited as an antitumor target due to the oncogenic activity of Akt. At the same time, many studies of the phospholipase C (PLC) family of enzymes have concentrated on the interactions of PLC δ with PtdIns45P₂, whereby a bisubstrate model of membrane localization by PH domain binding to PtdIns45P₂, followed by repeated hydrolysis of further PtdIns45P₂ molecules via the catalytic site, has been proposed (see ref 17 for review). However, with specific reference to tumor cell invasiveness, a central role for PLC γ has been proposed; as PLC γ signaling is promoted via a number of receptor-activation pathways, such as the EGF receptor and the PDGF receptor, and since these pathways also promote cell motility, the potential for PLC γ inhibition as an antitumor target has emerged more recently (36). Since this PLC γ activation is stimulated via PI3K-induced, PH domain-mediated membrane targeting (37), the ability to selectively inhibit this interaction via selective effects of the IPPs becomes a target for inhibition of tumor cell invasiveness.

INOSITOL POLYPHOSPHATE INTERACTIONS *IN VIVO*

Considering the IPPs themselves, early investigations into their biological activities arose principally as a result of the known second messenger actions and metabolic pathways of inositol (1, 4, 5)-trisphosphate, and the early observations of the suppression of cell proliferation and tumor formation by InsP₆ (8). After this, numerous *in vitro* systems were able to demonstrate that the higher IPPs, particularly InsP₅ and InsP₆, have various 'binding proteins' in cells (see refs 6, 7 for reviews). However, the mechanism of the action of InsP₆ remains to be satisfactorily explained (see ref 8 for review and further comment). Specific intracellular roles have now been proposed for extracellularly applied, cell permeant, hydrolyzable esters of other specific higher IPPs: inhibition of Ca²⁺-activated Cl⁻ channels by inositol (3, 4, 5, 6)-tetrakisphosphate (38), with the intracellular levels of this InsP₄ being controlled by inositol (1, 3, 4)-trisphosphate inhibition of its 1-phosphorylation (39), and inositol (1, 4, 5, 6)-tetrakisphos-

phate inhibition of EGF-induced inhibition of Ca²⁺-mediated chloride secretion in intestinal epithelia (40). Furthermore, with the demonstrations that InsP₆ (see ref 8) and inositol (1, 3, 4, 5)-tetrakisphosphate [Ins(1345)P₄; 22] can rapidly enter cells, our recently reported selective inhibition of PI3K-dependent cell growth by extracellularly applied Ins(1345)P₄ and Ins(13456)P₅ (22) confirms the potential for the use of these higher IPPs as anticancer drugs. Whereas a role for the IPPs in the inhibition of PI3K-dependent pathways has been suggested (14, 22, 40, 41), with the more recent evidence of their differing specificities for diverse physiological targets (see above), and with the newly emerging importance in disease of not just the PH domain, but also the FYVE domain and the HIKE motif (see above), we propose that the selectivity of these compounds is due to specific recognition sequences within these domains. Hence, by following these selectivities from the interactions of known IPPs/PPIs with specific protein domains/motifs, a new area of design of anticancer drugs emerges for the future.

CONCLUSIONS

Through the gathering and expansion of information around these protein/PPI interactions, we propose a unifying hypothesis that extends beyond the IPPs and leads to modifications around the known active IPP head groups. Thus, starting from an inositol head group with a known activity toward a specifically defined target for therapeutic intervention, the design of novel active compounds can begin with the search for chemically modified derivatives that display higher affinities and specificities toward this target protein. This approach will provide a novel framework for the orientation of future investigations that should result in the development of new active molecules. How can we test this hypothesis? As with our recent study (22), the use of GFP-fusion proteins to investigate the ability of IPP analogs to interfere with the *in vivo* membrane targeting of the selected therapeutic target provides a valuable tool in the study of their *in vivo* efficacy and selectivity. This, combined with biochemical studies of the protein activation and the pharmacokinetic properties of the new IPP analogs, can lead to the design of antitumorigenic drugs that act by mimicking known protein structure specificities at the level of their membrane localization through the protein/PPI interactions. **[F]**

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