Lipid Research Picks Up Speed on the Slopes of Taos

Meeting Review

Seth J. Field,[1,2,4] Katja A. Lamia,[1] Lucia E. Rameh,[3] and Lewis C. Cantley[1]
[1]Division of Signal Transduction
Beth Israel Deaconess Medical Center and
Department of Cell Biology
Harvard Medical School
77 Avenue Louis Pasteur
Boston, Massachusetts 02115
[2]Division of Endocrinology
Massachusetts General Hospital
55 Fruit Street
Boston, Massachusetts 02114
[3]Boston Biomedical Research Institute
64 Grove Street
Watertown, Massachusetts 02472

The 2002 Keystone Symposium on "Regulation of Cellular Responses by Lipid Mediators" provided a lively and active forum to discuss research in lipid signaling. This meeting review can provide only a glimpse into the diversity of research presented. Here we have chosen to highlight a group of exciting presentations describing novel features of the temporal and spatial regulation of phosphoinositides and their downstream targets.

The Keystone Symposium on "Regulation of Cellular Responses by Lipid Mediators" took place February 1–6, 2002, among the snow and the sagebrush of Taos, New Mexico. Nearby Taos Ski Valley's twisting and turning trails provided a suitable backdrop for a meeting that supplied surprising and intriguing twists and turns in our understanding of the regulation of lipid signals and their role in diverse cellular processes. Like a skier selecting one of many paths down the mountain, our review necessarily misses some of the thrills and vistas of this vibrant meeting. We have chosen to focus this review on four topics that represent recurring themes and/or particularly exciting new advances presented at the meeting. These include the following: (1) new insights into the function of phosphoinositide phosphatases, their role in regulating downstream signaling events, and their contribution to mammalian physiology, (2) discoveries about the mechanisms by which changes in phosphoinositides couple to the activation of small G proteins such as Rac, (3) accumulating evidence hinting at a role for phosphoinositides in the nucleus, and (4) broad interest in PI 3-kinase as a pharmaceutical target and the development of new PI 3-kinase assays amenable to high-throughput drug screens.

Phosphoinositide Phosphatases

In the past ten years, an overwhelming amount of information regarding the regulation of phosphoinositide synthesis by lipid kinases, mainly PI 3-kinase, has been published in the literature (see Figure 1). However, we

[4]Correspondence: sfield@alum.mit.edu

are only now beginning to explore how lipid phosphatases are regulated, how they contribute to phosphoinositide balance in cells, and how they affect cellular function.

The importance of these questions is emphasized by the finding that defects in phosphoinositide phosphatases are directly linked to human disease. Cowden's and Bannayan-Zonana syndromes are caused by genetic defects in the PTEN gene, and patients with X-linked myotubular myopathy and type 4B Charcot-Marie-Tooth syndrome carry mutations in the PtdIns(3)P 3-phosphatases MTM1 and MTMR2, respectively (Kim et al., 2002). In his keynote address, Jack Dixon showed that MTM1 and MTMR2 phosphatases have indistinguishable biochemical properties but that defects in these two proteins nevertheless lead to different disease syndromes. When overexpressed in COS cells, MTM1 and MTMR2 localize to different subcellular compartments, suggesting that they may target different pools of PtdIns(3)P. This could potentially explain their non-overlapping physiological functions.

A number of talks and posters addressed the functions of SHIP and SHIP2, 5-phosphatases, which convert PtdIns(3,4,5)P_3 into PtdIns(3,4)P_2. Christophe Erneux presented his group's work on SHIP2 knockout mice, which clearly demonstrated that SHIP2 is an important negative regulator of the insulin signal in animals (Clement et al., 2001). Homozygous disruption of the SHIP2 gene causes severe hypoglycemia and neonatal lethality. Heterozygous disruption of this gene leads to hypersensitivity to insulin and hypoglycemia. The most straightforward explanation of these results is that loss of SHIP2 reduces the rate of PtdIns(3,4,5)P_3 turnover, leading to increased PtdIns(3,4,5)P_3 levels in response to insulin receptor signaling, thus potentiating the effects of insulin. Direct measurement of phosphoinositide levels may help support this likely model. In any case, these results identify SHIP2 as a potential drug target in the treatment of diabetes mellitus.

While it is clear that PtdIns(3,4,5)P_3, the main substrate for SHIP2, is essential for Akt activation and, consequently, insulin signaling, the role of PtdIns(3,4)P_2, the product of SHIP and SHIP2, is still controversial. Two independent groups presented data suggesting that PtdIns(3,4)P_2 is a signaling molecule per se. Using bone marrow-derived mast cells from wild-type or SHIP knockout mice, Gerald Krystal showed that lack of SHIP leads to a dramatic increase in steel factor (SF)-induced PtdIns(3,4,5)P_3 levels and a decrease in PtdIns(3,4)P_2 levels, as would be expected from SHIP's biochemical activity (Scheid et al., 2002). His group found that by treating SHIP$^{-/-}$ cells with limited amounts of inhibitors of PI 3-kinase, such as LY294002, they could reduce the PtdIns(3,4,5)P_3 level to the level present in wild-type cells. This treatment also reduced PtdIns(3,4)P_2 levels to nearly undetectable. Thus, Dr. Krystal argued that comparison of wild-type cells to LY294002-treated SHIP$^{-/-}$ cells could be used to determine the distinct roles of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in downstream
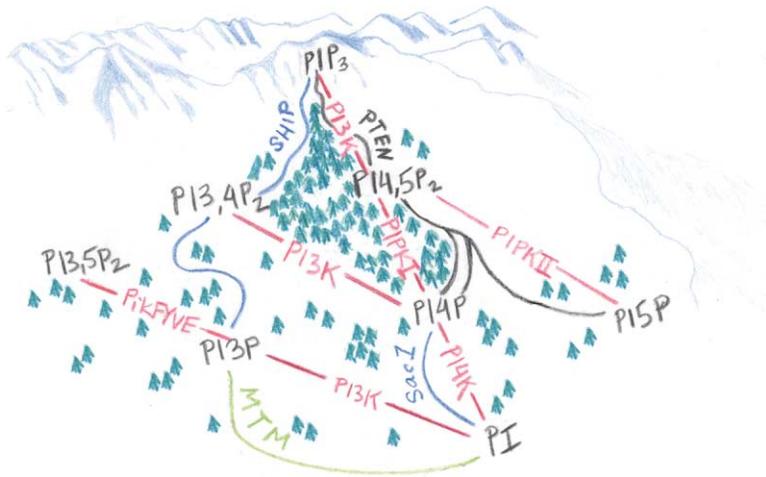
signaling. This approach was used to establish a correlation between SF-stimulated phosphorylation of Akt at Thr308 with PtdIns(3,4,5)P_3 levels. In contrast, Akt phosphorylation at Ser473 correlated with PtdIns(3,4)P_2 levels. Introduction of exogenous PtdIns(3,4)P_2 into SHIP$^{-/-}$ cells pretreated with PI 3-kinase inhibitors and stimulated with SF led to increased Akt phosphorylation at Ser473 and activation. Dr. Krystal proposed that PtdIns(3,4,5)P_3 is essential for the recruitment of Akt to the membrane and for Thr308 phosphorylation by PDK1, while PtdIns(3,4)P_2 is necessary for accumulation of phosphorylation at Ser473 by regulation of either a kinase or a phosphatase specific for this site.

Aaron Marshall discussed two novel proteins, TAPP1 and TAPP2, that are potential targets for PtdIns(3,4)P_2 generated during B cell stimulation. These Bam32-related proteins are expressed in many tissues but predominantly in lymphoid cells. Dowler et al. (2000) have previously shown that TAPP1 and TAPP2 PH domains bind specifically to PtdIns(3,4)P_2. Dr. Marshall and colleagues expressed GFP-tagged TAPP1 and TAPP2 in human B lymphoma cells as a probe for PtdIns(3,4)P_2 and found that these proteins are recruited to actin-rich membrane ruffles through their PtdIns(3,4)P_2 binding PH domain in a PI 3-kinase-dependent manner. When compared to other PH domains, TAPP1 and TAPP2 recruitment to the membrane is delayed and sustained. The authors hypothesize that TAPP1 and TAPP2 are targets for PtdIns(3,4)P_2 and may play a role in PI 3-kinase-dependent cytoskeletal rearrangement.

We learned from Christina Mitchell's presentation that SHIP2 is present in membrane ruffles and can associate with the actin binding protein filamin (Dyson et al., 2001). Binding to filamin is mediated through the C-terminal proline-rich domain of SHIP2 and may be an important mechanism to control the localization of SHIP2. SHIP2 could thereby regulate actin rearrangement by regulating local levels of its substrate PtdIns(3,4,5)P_3 or its product PtdIns(3,4)P_2 (or perhaps even the substrate PtdIns(4,5)P_2).

The above studies underscore the concept articulated most clearly by Scott Emr that phosphoinositide kinases and phosphatases always necessarily affect the levels of two phosphoinositides, the enzyme's substrate and product, each of which may have distinct downstream targets. In this context, perhaps the SHIP proteins are viewed best as a mechanism to simultaneously terminate PtdIns(3,4,5)P_3-initiated pathways and activate PtdIns(3,4)P_2-initiated pathways.

Interactions between Rac and Phosphoinositides in Ruffling

It has long been established that Rho family GTPases are involved in stimulating actin reorganization in response to various growth factors. However, the precise molecular links between receptor activation, small G proteins, and the actin machinery remain somewhat mysterious. A number of speakers presented evidence for the involvement of phosphoinositides and the enzymes that produce them in these pathways.

Richard Anderson discussed work from his lab demonstrating that phosphatidyl inositol 4-phosphate 5-kinase type I α (PIPKIα) acts in concert with Rac to stimulate actin reorganization. PIPKIα produces PtdIns(4,5)P_2, which many groups have shown to be involved in actin polymerization. Dr. Anderson showed that microinjection of PIPKIα induced actin reorganization in cells that were PDGF stimulated but not in serum starved cells. This effect was not blocked by inhibitors of PI 3-kinase and was enhanced by inhibitors of phospholipase C, suggesting that the observed effects were due to accumulation of PtdIns(4,5)P_2 produced by the microinjected enzyme. He also presented evidence that both activated Rac and the PIPKIα participate in and are required for PDGF-stimulated membrane ruffle assembly. Rac mutants that do not interact with PIPKIα poorly induce ruffling and do not show synergism with PIPKIα in PDGF-stimulated ruffle assembly. Further experiments will be necessary to demonstrate the precise ordering of these enzymes in a biochemical pathway leading to growth factor-induced actin reorganization.

Lew Cantley presented genetic evidence for positioning PI 3-kinase upstream of Rac or other small G proteins in signaling leading to the formation of lamellipodia. He showed time-lapse video microscopy of mouse embryonic fibroblasts (MEFs) ruffling in response to PDGF. MEFs lacking both isoforms of the regulatory subunit of PI 3-kinase (p85α and p85β) failed to ruffle in response

to PDGF. However, unstimulated $p85\alpha^{-/-}$;$p85\beta^{-/-}$ MEFs will ruffle when they are made to express high levels of Vav2, which is a GEF for Rho family small GTPases.

Len Stephens described the purification, cloning, and characterization of a novel Rac GEF, which his group has named P-Rex-1 (Welch et al., 2002). P-Rex-1 is regulated by both $PtdIns(3,4,5)P_3$ and $G\beta\gamma$ subunits and thus provides a potential mechanism for signal integration between multiple upstream pathways.

Heidi Welch, working in the joint laboratory group of Stephens and Phill Hawkins, found that addition of PI 3-kinase and $G\beta\gamma$ subunits to neutrophil lysates synergistically stimulates superoxide formation. Further, they found that the addition of $PtdIns(3,4,5)P_3$-containing vesicles alone stimulated the production of reactive oxygen species and that this stimulation was inhibited by the addition of dominant-negative N17-Rac. Finally, reactive oxygen formation was stimulated in a wortmannin-resistant manner by the addition of either $G\beta\gamma$ subunits or activated Rac-GTPγS. These data led to a model in which parallel pathways acting through PI 3-kinase and $G\beta\gamma$ subunits activate Rac.

Dr. Stephens' group began searching for a protein that would activate Rac in a $PtdIns(3,4,5)P_3$-dependent manner. Assaying blood fractions for the ability to activate Rac only in the presence of exogenous $PtdIns(3,4,5)P_3$ led to the isolation of P-Rex-1, a large protein with multiple domains that is highly expressed in leukocytes. Among the domains of P-Rex-1 are a PH domain, a GEF domain, and an inositol polyphosphate 4-phosphatase domain. The full-length protein binds to $PtdIns(3,4,5)P_3$ and activates exchange of guanine nucleotides on Rac1, Rac2, and Cdc42 in vitro. Furthermore, the Stephens group showed that $PtdIns(3,4,5)P_3$ and $G\beta\gamma$ subunits synergistically activate the GEF activity of P-Rex-1. Finally, overexpression of P-Rex-1 induces lamellipodia formation, indicative of Rac activation, in a PI 3-kinase-dependent manner.

Phosphoinositides in the Nucleus
A pervasive theme at the meeting was data hinting at a role for phosphoinositides in the nucleus. To set the stage, Susan Wente and John York summarized their past and ongoing genetic and biochemical experiments supporting a role for soluble inositol polyphosphates in the regulation of RNA transcription and export from the nucleus. Susan Wente reviewed her laboratory's genetic screen in *S. cerevisiae* to identify synthetic lethals in a strain mutated in the gene for the mRNA export factor Gle1p. The screen unexpectedly identified the genes for phospholipase C and two novel inositol phosphate kinases, Ipk1p and Ipk2p (York et al., 1999). Yeast Ipk2p is required to convert IP_3 to IP_4 and IP_5, while Ipk1p is required to convert IP_5 to IP_6. Wente's group believes that IP_6 is the crucial product required for nuclear pore function. An Ipk2p-GFP fusion protein localizes to the nucleus (Odom et al., 2000), and an epitope-tagged Ipk1p localizes to the nucleus and perinuclear cytoplasm. However, Dr. Wente went on to show that the nuclear localization of Ipk1p may not be required for its function. A version of Ipk1p tethered to the plasma membrane by fusion to Ste2p is capable of rescuing a *gle1-2 ipk1-4* synthetic lethal. This correlates with her

group's recent genetic data tying inositol polyphosphate metabolism to mRNA export events on the cytoplasmic face of the nuclear pore. As yet, there is no biochemical data to provide a detailed mechanism.

John York reviewed his lab's ongoing experiments implicating Ipk2p and one or both of its products, IP_4 and IP_5, as playing an important role in the regulation of gene transcription (Odom et al., 2000). Their data suggest that the Ipk2p protein but not kinase activity is necessary for assembly of the Arg80p-Mcm1p-Arg81p transcription factor complex on the DNA. The kinase activity to generate IP_4 and IP_5 is required for transcription of the target genes. Again, the detailed mechanism remains uncertain.

The above work suggests a role for soluble inositol polyphosphates in regulating nuclear events. A number of groups also presented work hinting at a role for lipid-bound phosphoinositides in the nucleus. Jeremy Thorner presented evidence for distinct roles for the PI 4-kinase Pik1 in the cytoplasm and nucleus in yeast. First, he demonstrated that a GFP-Pik1 fusion or myc epitope-tagged Pik1 can be detected in the nucleus. Next, like Susan Wente, he described experiments to determine whether Pik1 tethered in the cytoplasm could rescue a Pik1 deficiency. Intriguingly, when tethered to the plasma membrane by fusion to a CAAX box, Pik1 was unable to rescue a Pik1 deficiency. However, a Pik1 mutant Δ10-192 that localized purely to the nucleus but not to the cytoplasm also could not rescue a Pik1 deficiency. Importantly, coexpressing the exclusively cytoplasmic and exclusively nuclear mutants of Pik1 does rescue a Pik1 deficiency, arguing that Pik1 has required functions in both compartments. The nature of these distinct functions remains unclear.

There were also examples of research suggesting a role for lipid-bound phosphoinositides in the nucleus in mammalian cells. Richard Anderson provided evidence that PIPK1α and PIPKIIβ may be found in the nucleus (Boronenkov et al., 1998). Although many questions remain concerning these observations, they suggest novel roles for phosphoinositides in the nucleus.

PI 3-kinase as a Drug Target
One theme explored by a number of talks and posters was the importance of the PI 3-kinase pathway in human pathophysiology and, therefore, interest in it as a drug target. Dr. Cantley reviewed evidence implicating PI 3-kinase in the development of cancer. First, he reviewed the role of PI 3-kinase in promoting cell proliferation, cell motility, and anchorage-independent survival, all important features of a metastatic cancer. He also reviewed the many oncogenes that have been connected to the PI 3-kinase pathway, including early experiments identifying PI 3-kinase as a target of the polyoma middle T-transforming antigen and the identification of the protooncogenes AKT, myc, and cyclin D as downstream targets of the PI 3-kinase pathway. More recently, PTEN has been shown to function as a human tumor suppressor gene by antagonizing the PI 3-kinase pathway.

The role of PI 3-kinase in signaling downstream of the insulin receptor has been long appreciated. However, Dr. Cantley reviewed the surprising and intriguing results

from his lab that mice deficient for either the p85α or p85β regulatory subunits of PI 3-kinase exhibit increased insulin sensitivity (Fruman et al., 2000; Mauvais-Jarvis et al., 2002; Ueki et al., 2002). More investigation will be needed to provide a satisfactory mechanistic explanation for these unexpected results. Nevertheless, it is clear that pharmaceuticals that target the PI 3-kinase pathway may be valuable reagents in the treatment of some cancers and, possibly, diabetes mellitus.

Accordingly, a number of groups presented posters demonstrating their progress in developing novel PI 3-kinase assays amenable to high-throughput drug screens. Kinji Fuchikami et al. of Bayer, Henric Olsson et al. of Astra-Zeneca, Alexander Gray et al. of the University of Dundee, and Beth Drees et al. of Echelon all presented work showing progress in this area. Each of these groups has developed assays that detect the binding of a soluble short chain PtdIns(3,4,5)P$_3$ to a specific protein domain. For example, Olsson et al. utilize the Grp1-PH domain to bind specifically to PtdIns(3,4,5)P$_3$ and use Packard Bioscience's proprietary AlphaScreen technology to detect the binding interaction. The different groups use different readouts but employ similar principles. Echelon has been pivotal in developing and commercializing technologies to assist research in the field, and they voiced their intention to market their new PI 3-kinase assays as well. So far none of the groups have presented new small molecule modulators of PI 3-kinase, but, of course, these are anxiously awaited as novel research tools.

Conclusions

The above excerpts illustrate only the tip of the iceberg of exciting advances in lipid research presented at this year's Keystone Symposium on Lipid Mediators. Importantly, the collegial nature of the meeting fostered the free exchange of ideas at all levels. Undoubtedly, this meeting on the snowy slopes of Taos has produced an avalanche of new ideas in lipid research.

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