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CELL BIOLOGY

Inositol Pyrophosphates as Mammalian Cell Signals

Anutosh Chakraborty,[1] Seyun Kim,[1] Solomon H. Snyder[1,2,3*]

Inositol pyrophosphates are highly energetic inositol polyphosphate molecules present in organisms from slime molds and yeast to mammals. Distinct classes of enzymes generate different forms of inositol pyrophosphates. The biosynthesis of these substances principally involves phosphorylation of inositol hexakisphosphate (IP_6) to generate the pyrophosphate IP_7. Initial insights into functions of these substances derived primarily from yeast, which contain a single isoform of IP_6 kinase (yIP_6K), as well as from the slime mold *Dictyostelium*. Mammalian functions for inositol pyrophosphates have been investigated by using cell lines to establish roles in various processes, including insulin secretion and apoptosis. More recently, mice with targeted deletion of *IP_6K* isoforms as well as the related *inositol polyphosphate multikinase* (*IPMK*) have substantially enhanced our understanding of inositol polyphosphate physiology. Phenotypic alterations in mice lacking *inositol hexakisphosphate kinase 1* (*IP_6K1*) reveal signaling roles for these molecules in insulin homeostasis, obesity, and immunological functions. Inositol pyrophosphates regulate these processes at least in part by inhibiting activation of the serine-threonine kinase Akt. Similar studies of IP_6K2 establish this enzyme as a cell death inducer acting by stimulating the proapoptotic protein p53. IPMK is responsible for generating the inositol phosphate IP_5 but also has phosphatidylinositol 3-kinase activity—that participates in activation of Akt. Here, we discuss recent advances in understanding the physiological functions of the inositol pyrophosphates based in substantial part on studies in mice with deletion of *IP_6K* isoforms. These findings highlight the interplay of IPMK and IP_6K in regulating growth factor and nutrient-mediated cell signaling.

INTRODUCTION

Inositol phosphates have been recognized as biologically pertinent molecules since the identification in the early 20th century of inositol hexakisphosphate (IP_6, also known as phytic acid) as an abundant constituent of plants that makes up their principal phosphate store. Interest in these substances escalated with the discovery in the mid-1980s of IP_3 [inositol 1,4,5-trisphosphate, also abbreviated as Ins(1,4,5)P_3] as a second messenger that releases calcium from intracellular stores (*1*, *2*). Inositol polyphosphate derivatives with highly energetic diphosphates (inositol pyrophosphates) were identified in the mid-1990s (*3*, *4*). The term "inositol polyphosphate" is often used to designate inositol with more than a single phosphate, whereas the energetic diphosphates are referred to as inositol pyrophosphates. Initial characterization of inositol pyrophosphate molecules revealed a rapid turnover, suggesting a role in cellular signaling (*3*–*5*).

New insights into the functions of the inositol pyrophosphates have been afforded by the identification of their synthetic enzymes (*6*), in particular by cloning of the cDNAs for these enzymes (*7*–*9*) and through their genetic deletion (*10*–*14*). Inositol pyrophosphates generated by IP_6 kinase 1 (IP_6K1) impact insulin homeostasis (*10*, *11*) by inhibiting the serine-threonine protein kinase Akt (*11*). Mice lacking *IP_6K1* are protected against age- and high-fat diet–induced obesity (*11*) and display improved neutrophil phagocytosis (*12*). IP_6K2 is a mediator of p53-determined cell death (*13*), and *IP_6K2*-deleted mice are predisposed to aerodigestive (upper respiratory and upper digestive) tract carcinoma (*14*).

The isomer of IP_7 generated by the three mammalian IP_6Ks and the yeast isoform (Kcs1, designated yIP_6K) is 5-IP_7 (5-diphosphoinositol pentakisphosphate) (*15*). A distinct form of IP_7, 1-/3-IP_7 (1-/3-diphosphoinositol pentakisphosphate, which has an unquantifiable mixture of 1- and 3-diphosphoinositol pentakisphosphates) (*16*), synthesized in yeast by the enzyme Vip1 (*17*), promotes phosphate homeostasis (*18*). Characterization of PP-IP_5K (diphosphoinositol pentakisphosphate kinase), the mammalian isoform of Vip1 (*16*, *19*, *20*), established that, in mammals, this enzyme is primarily responsible for IP_8 formation (*16*, *19*, *20*).

IPMK (inositol polyphosphate multikinase) was first identified as Arg82, a transcriptional regulator in yeast (*21*, *22*). IPMK generates inositol tetrakisphosphate (IP_4) [both Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4] and IP_5 [Ins(1,3,4,5,6)P_5] and thus acts upstream of the IP_6Ks (*7*, *23*, *24*) (Fig. 1). IPMK is also a physiologically important PI 3-kinase that forms PIP_3 [phosphatidylinositol (3,4,5)-trisphosphate] (*25*), which activates Akt (*26*). IPMK, in a catalytically independent fashion, activates mammalian target of rapamycin (mTOR) (*27*). Thus, IPMK and IP_6K1 participate in a network regulating growth factor– and nutrient-mediated signaling.

Inositol Pyrophosphate Metabolism

The complex metabolic pathway generating the pyrophosphates has been extensively reviewed elsewhere and is beyond the scope of this review (*28*–*32*). Hormones or growth factors activate phospholipase C to generate IP_3 from PIP_2 [phosphatidylinositol (4,5)-bisphosphate] (*28*). IP_3K [Ins(1,4,5)P_3 3-kinase] converts IP_3 to IP_4 (*33*). In addition, IPMK sequentially converts IP_3 to IP_5, albeit with species-specific isomeric preference (*7*, *23*, *24*, *34*–*36*) (Fig. 1). Cellular concentrations of IP_5 and IP_6, are substantially higher than those of IP_3. Because the techniques for measuring endogenous concentrations of inositol phosphates are limited, most studies assess concentrations of inositol phosphates by examining the conversion by cultured cells of [^{3}H]inositol into the relatively polar inositol phosphates, which can be separated by ion-exchange chromatography. The existence of pyrophosphates was initially postulated when several groups identified metabolites more polar than IP_6 (*37*–*40*) and speculated that they might represent diphosphates incorporating energetic pyrophosphate bonds. Subsequently, definitive evidence emerged that these more polar agents are diphosphorylated (*3*, *4*, *41*) and characterized as 5-IP_7 (*15*) and IP_8 (*4*, *16*, *19*, *20*, *41*, *42*). IP_5

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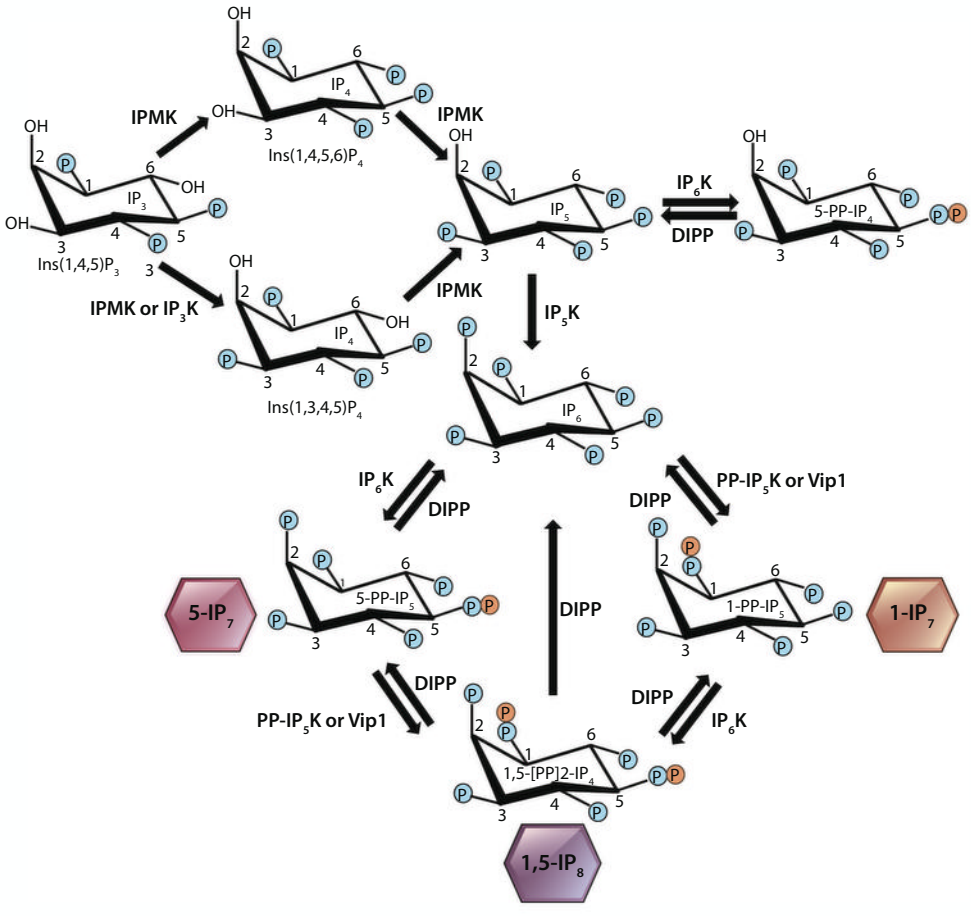

Fig. 1. Inositol polyphosphate biosynthetic pathway. IP_3K or IPMK phosphorylates IP_3 to generate IP_4 [Ins(1,3,4,5)P_4 or Ins(1,4,5,6)P_4]. Further sequential phosphorylation of IP_4 by IPMK and IP_5K yields IP_6 (*7*, *23*, *24*, *33*–*36*, *44*). IP_6Ks pyrophosphorylate the 5 position, generating 5-PP-IP_4 from IP_5, 5-PP-IP_5 (5-IP_7) from IP_6, and 1-/3-,5-(PP)2-IP_4 (1,5-IP_8) from 1-/3-PP-IP_5 (*7*, *17*, *19*, *20*). 5-IP_7 is the predominant inositol pyrophosphate in mammals. PP-IP_5Ks, the human Vip1 isoforms, pyrophosphorylate the 1 or 3 position to yield 1-/3-PP-IP_5 (depicted as 1-IP_7 for simplicity) from IP_6, or 1-/3-,5-(PP)2-IP_4 (1,5-IP_8) from 5-IP_7, and physiologically, they are primarily involved in generating IP_8. DIPPs dephosphorylate inositol pyrophosphates to IP_6 or IP_5 (*32*, *47*).

and IP_6 are the best-characterized precursor molecules for the inositol pyrophosphates. Diphosphoinositol tetrakisphosphate (PP-IP_4), synthesized by IP_6Ks from IP_5 (*30*–*32*, *43*) occurs endogenously in most mammalian tissues. IP_5K [Ins(1,3,4,5,6)P5 2-kinase] converts IP_5 to IP_6 (*44*), which is the substrate for IP_6Ks and Vip1 to synthesize 5-IP_7, 1-/3-IP_7 and 1-/3-,5-IP_8 (*7*, *17*, *19*, *20*) (Fig. 1). Diphosphorylated derivatives of IP_3 and IP_4 have also been reported (*45*). The enzymatic formation in vitro of a triphosphate derivative of IP_6 (*43*) raises the possibility that triphosphorylated IP_6 might exist in vivo (*46*). Diphosphoinositol-polyphosphate phosphohydrolases (DIPPs) dephosphorylate all inositol pyrophosphates including PP-IP_4 (*32*, *47*) (Fig. 1). The discovery of the DIPPs has provided a mechanism that accounts for the rapid turnover of inositol pyrophosphates (*3*–*5*).

Intracellular concentrations of IP_7 in most mammalian tissues are about 1 to 5 μM (*30*, *31*, *48*–*50*), whereas those of IP_6, the most abundant inositol phosphate, are 15 to 60 μM (*28*, *31*, *51*). The slime mold *Dictyostelium discoideum* has high concentrations of inositol pyrophosphates, about 10 μM in the disaggregated state and increasing to 100 to 250 μM during starvation and aggregation (*52*). The predominant form of IP_7 in *Dictyostelium* is 6-IP_7, which differs from yeast and mammalian isomers (*15*).

Mammalian inositol pyrophosphate concentrations are dynamic. IP_8 concentrations increase 1000 to 2500% within 20 to 30 min after exposure to sorbitol-induced hyperosmotic conditions (*53*, *54*) or heat shock (*55*). AICAR [*N*-(β-D-ribofuranosyl)-5-aminoimidazole-4-carboxamide], which is transformed in cells to an adenosine monophosphate (AMP) derivative, prevents the sorbitol-mediated increase in IP_8 independently of its ability to stimulate AMP kinase (AMPK), the latter a well-characterized action of AMP (*56*). Various cell stressors, including the anticancer drug cisplatin and broad-spectrum kinase inhibitors, increase intracellular IP_7 concentrations by activating IP_6K2 (*57*–*59*).

One classic criterion for putative intracellular second messengers, exemplified by IP_3 (*2*), is that their formation can be altered in response to intercellular stimuli such as neurotransmitters, hormones, and growth factors. Concentrations of IP_6 and IP_7 decline markedly after overnight serum starvation (*11*). Conversely, insulin-like growth factor 1 (IGF-1) stimulates IP_6 and IP_7 formation (*11*). IGF-1 increases the IP_7-to-IP_6 ratio in mouse embryonic fibroblasts (MEFs), an effect absent in MEFs lacking *IP_6K1*, which indicates a meaningful "second-messenger response" that depends on IP_6K1 activity. Similar alterations of inositol pyrophosphate concentrations occur in the hepatocellular carcinoma cell line HepG2 after IGF-1 or insulin treatment (*11*). IP_7/IP_6 ratios increase with age in primary hepatocytes. Moreover, IP_7 concentrations increase markedly during NIH3T3-L1 adipogenic differentiation (*11*). Modest increases in IP_8 but not IP_7 were observed after epidermal growth factor (EGF) treatment of DDT1 MF-2 smooth muscle cells (*53*). Inositol pyrophosphates are also responsive to chemokine signaling. Formyl-methionyl-leucyl-phenylalanine, a bacterial peptide that triggers chemotaxis, rapidly depletes IP_7 in the human promyelocytic leukemia cell line HL60 (*12*).

Regulation of the pyrophosphates is also determined by factors that regulate the biosynthesis of their precursor inositol phosphates. Several groups have reported increases in total inositol phosphate concentrations after treatment with various growth factors like EGF, IGF-1, platelet-derived

growth factor (PDGF), and the Wnt (for vertebrate Int and *Drosophila* Wingless) family (*60*–*64*). Concentrations of IP_5 and IP_6 increase after prolonged PDGF treatment of serum-deprived NIH3T3 cells (*63*). The Wnt family of signaling proteins participates in embryogenesis and adult tissue homeostasis with aberrations promoting degenerative diseases and cancer (*65*). Serum-starved F9 teratocarcinoma or human embryonic kidney–293 (HEK293) cells displayed increased IP_5 concentrations within 15 min of Wnt 3a treatment (*64*). Thus, inositol polyphosphate concentrations alter dynamically in response to diverse stimuli.

Table 1. PH domain–containing enzymes whose catalytic activity or membrane translocation, or both, are regulated by inositol phosphates or pyrophosphates. BTK, Bruton's tyrosine kinase.

Enzyme	Inositol phosphates	Regulation	References
Akt	IP5, IP6, IP7	Inhibition	(*11*, *12*, *69*, *73*, *115*, *125*)
PDK1	IP5, IP6	Inhibition	(*76*)
ITK or BTK	IP4	Activation	(*68*, *71*)

Molecular Mechanisms of Inositol Pyrophosphate Signaling

There appear to be two principal mechanisms whereby inositol pyrophosphates affect other cellular constituents: binding and pyrophosphorylation. Inositol polyphosphates physiologically bind to several proteins (*66*–*70*). Inositol polyphosphates bind to the membrane-targeted PH domains of several proteins (*68*, *69*, *71*–*76*) (Table 1). PH domains (*77*) bind phospholipids such as PIP_3 and PIP_2 (*78*, *79*) and so recruit signaling proteins to membranes. In *Dictyostelium*, PIP_3 binds the PH domain of the protein CRAC (cytosolic regulator of adenylate cyclase), which mediates cyclic AMP (cAMP)–related chemotaxis (*73*). IP_7 competes with PIP_3 for binding to CRAC and prevents the chemotactic response. Deletion of *IP_6K* depletes IP_7 and thereby augments slime mold sensitivity to cAMP and, consequently, aggregation. A critical regulatory role for inositol pyrophosphates in the chemotactic response to cAMP is evident by the marked elevation of inositol pyrophosphates in the amoeba elicited by cAMP (*73*). In mammals, one of the best-characterized examples is the recruitment of the kinase Akt, a PH domain–containing protein, to plasma membranes (*80*) downstream of hormone- or growth factor–mediated activation of PI 3-kinase to form PIP_3. 5-IP_7 blocks PIP_3-mediated activation of Akt (*11*). The other IP_7 isomer, 1-/3-IP_7, also exerts its physiological effects by binding and inhibiting the cyclin-cyclin dependent kinase (CDK) complex of yeast (*18*, *81*).

The pyrophosphate bond in IP_7 is as energetic as those of ATP (ATP), which raises the possibility that IP_7, like ATP, might phosphorylate proteins (*4*, *31*, *82*). Isolation and cloning of IP_6Ks so as to generate [^{32}P]IP_7, with the label in the β-phosphate anticipated to be donated, led to the demonstration that [^{32}P]IP_7 phosphorylates various proteins (*83*). The process is magnesium dependent, like ATP phosphorylation. However, the phosphate transfer by IP_7 is nonenzymatic and occurs in vitro without the intervention of protein kinases. IP_7-mediated phosphorylation has been characterized both in yeast and mammals (*83*–*85*), with the most extensive work done in yeast. Proteins that are particularly good targets for this phosphorylation process tend to be rich in serines adjacent to acidic amino acids and close to a stretch of basic amino acids, as exemplified by nucleolar yeast proteins, which are particularly good substrates (*83*, *84*). The importance of acidic amino acids is reminiscent of the consensus motif for phosphorylation by CK2 (casein kinase 2, an acidophilic serine-threonine kinase, discussed in detail in the cell death and development section).

Target proteins purified from bacteria or λ-protein phosphatase–treated proteins purified from mammalian systems are not phosphorylated by IP_7 (*83*, *84*). Although phosphorylation as a posttranslational modification in the bacterial proteome is increasingly appreciated (*86*), the relevant bacterial kinases differ markedly from their mammalian counterparts (*86*). These hints that something more than conventional phosphorylation was involved led to the discovery that IP_7 can only phosphorylate a protein that had been previously phosphorylated on the same serine, principally by CK2. Hence, IP_7 pyrophosphorylates proteins (*84*). Such a process provides a unique type of signaling event, distinguishing IP_7 phosphorylation from that involving ATP. There are other distinctions. Unlike ATP-dependent phosphorylation, pyrophosphorylation by IP_7 resists actions of most phosphatases while being more acid labile (*84*). Certain lines of evidence indicate that such protein pyrophosphorylation occurs in vivo. For instance, pyrophosphorylation in vitro of overexpressed nucleolar protein NSR1, a target of IP_7 phosphorylation, is enhanced when purified from yeast lacking *yIP_6K*, presumably because there is less endogenous pyrophosphorylation of the protein in the absence of yIP_6K and endogenous IP_7 (*83*). In mammals, pyrophosphorylation appears to inhibit physiological interactions between the β subunit of adaptor protein–3 (AP-3), a clathrin-associated protein, and the kinesin family motor protein Kif3A (*85*).

The energy of hydrolysis for inositol pyrophosphates is similar to that of ATP, which supports a physiologic role for inositol pyrophosphorylation (*87*). The pyrophosphorylating ability of 1-/3-IP_7 (*84*) supports the pyrophosphorylation potential of other inositol pyrophosphates such as IP_8.

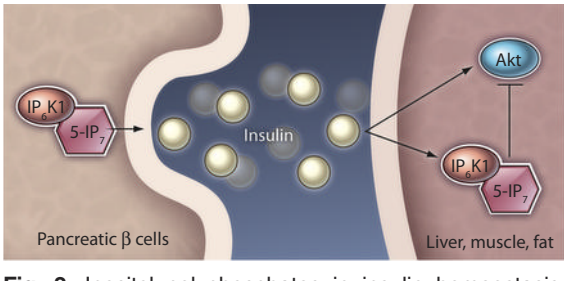

Fig. 2. Inositol polyphosphates in insulin homeostasis. Inositol pyrophosphates (5-IP_7) facilitate insulin secretion from pancreatic β cells (*50*). The secreted hormone signals through its receptors in insulin-responsive tissues and activates the protein kinase Akt through the PI 3-kinase pathway. Insulin also increases inositol pyrophosphate concentrations, which decreases Akt activity in liver, skeletal muscles, and adipose tissues (*11*) and thereby maintains homeostasis between insulin release and its signaling.

Physiologic Functions of Inositol Pyrophosphates

1. Nuclear dynamics.

Yeasts have been used extensively to characterize nuclear functions of inositol pyrophosphates. Overexpression of catalytically

Table 2. Physiological processes regulated by inositol pyrophosphate–synthesizing enzymes.

Functions	Enzyme	Mouse	References
Insulin sensitivity and resistance to weight gain	IP6K1	Mouse	(*10*, *11*)
Insulin release	IP6K1	Min6 cells	(*50*)
Neutrophil phagocytosis	IP6K1	Mouse	(*12*)
HIV-VLP release	IP6K1	HeLa, MEF cells	(*85*)
Dopamine release	IP6K1*	PC12 cells	(*94*)
Apoptosis and autophagy	IP6K2, yIP6K-Vip1	Cell lines, yeast	(*13*, *57–59*, *95–97*, *102*, *103*)
Development	IP6K2	Zebrafish	(*104*)
Telomere length maintenance	yIP6K	Yeast	(*88*, *89*)
DNA hyperrecombination	yIP6K	Yeast	(*90*)
Vacuole biogenesis	yIP6K	Yeast	(*91*)
Endocytosis	yIP6K	Yeast	(*92*)
Cell wall integrity, filamentous growth	yIP6K, Asp1	Yeast	(*91*, *93*)
Phosphate homeostasis	Vip1	Yeast	(*18*, *81*)
Chemotaxis	IP6K	Slime mold	(*73*)

*Indicates action of IP6K1 independent of its catalytic activity.

active yIP$_6$K leads to shortened telomeres, whereas its deletion lengthens them (*88*, *89*) (Table 2), which indicates that inositol pyrophosphates regulate telomere length. The ability of PP-IP$_4$ to shorten telomeres (*89*) requires Tel1, the yeast version of ATM, the kinase that is mutated in ataxia telangiectasia (*88*, *89*).

Mutations of yeast protein kinase C (PKC) lead to hyperrecombination of chromosomes; this augmentation of DNA recombination is reversed by mutating *yIP$_6$K*, which suggests a role for yIP$_6$K in mediating this process (*90*). The enhanced recombination linked to PKC mutations is associated with DNA damage, augmented transcription, and cyclin mutation and leads to a prolongation of the S phase. Catalytically active yeast or mammalian IP$_6$K both restore hyperrecombination (*90*).

Yeasts have evolved a complex system for regulating phosphate disposition during starvation. Phosphate starvation specifically increases the concentration of 1-/3-IP$_7$ (*18*), which binds and stimulates Pho81-mediated inhibition of the Pho80-Pho85 cyclin-CDK complex. Cyclin-CDK complex inhibition leads to reduced phosphorylation and nuclear accumulation of the transcription factor Pho4, which results in enhanced expression of genes from the phosphate-responsive signaling pathway (*PHO* genes) required to maintain phosphate homeostasis (*18*, *81*). Whether these findings are relevant to mammalian physiology is unclear.

2. Vacuole biogenesis, vesicular trafficking, and cellular morphology.
Studies deleting *yIP$_6$K* implicate inositol pyrophosphates in vesicular dynamics. Yeasts lacking *yIP$_6$K* display prominent vacuolar abnormalities (*46*, *91*). Yeasts with mutations of enzymes in the inositol pyrophosphate pathway display selective abnormalities of endocytic trafficking, which indicates that inositol pyrophosphates are physiologic enhancers of endocytosis (*92*). Generation of inositol pyrophosphates by yIP$_6$K regulates cellular morphology (*91*, *93*) and stimulates cell wall integrity in *S. cerevisiae* (*92*), whereas Asp1, a Vip1 homolog, enhances the dimorphic switch from single cell to filamentous invasive growth form in *Schizosaccharomyces pombe* (*93*).

Identification of GRAB [guanine nucleotide–exchange factor (GEF) for Rab3A] as an IP$_6$K1-binding protein revealed a role for IP$_6$K1 in synaptic vesicle exocytosis (*94*) (Table 2). GRAB inhibits neurotransmitter release via its GEF activity on Rab3A, which is a guanosine triphosphatase (GTPase) protein. IP$_6$K1 competes with GRAB for binding to Rab3A and thus stimulates neurotransmitter release. Catalytically inactive IP$_6$K1 also stimulates transmitter release (*94*).

Insulin release from pancreatic β cells involves exocytotic processes analogous to those mediating neurotransmitter release. Overexpression of the three isoforms of IP$_6$K stimulates insulin release, as does exogenous 5-IP$_7$ (*50*) (Fig. 2 and Table 2). Depletion of *IP$_6$K1* but not *IP$_6$K2* in pancreatic β cells inhibits exocytosis (*50*), and mice with targeted deletion of *IP$_6$K1* have reduced plasma levels of insulin (*10*, *11*).

The β subunit of AP-3 (AP3β1) is a clathrin-associated protein required for HIV-1 release from infected cells by means of binding to the kinesin motor protein Kif3A. Pyrophosphorylation of AP3β1 decreases its interaction with Kif3A, which reduces the release of HIV-1 virus–like particles (VLPs) (*85*) (Fig. 3).

3. Cell death and development.
Hints of a selective role for IP$_6$K2 in apoptosis came from studies of the mechanisms whereby interferon-β (IFN-β) suppresses proliferation of the ovarian cancer cell line OVCAR-3 by enhancing apoptosis (*95*, *96*) (Table 2). These studies showed that the abundance of IP$_6$K2, which was linked to cell death by antisense knockout (*95*), increased with IFN-β treatment. Moreover, overexpression of IP$_6$K2 increased the growth-suppressive and apoptotic influences of IFN-β, whereas a dominant-negative mutant of the inositol phosphate–binding domain prevented the actions of IFN-β (*96*). Subsequent work extended the involvement of IP$_6$K2 in apoptosis to other systems (*57*). Thus, transfection of *IP$_6$K2* in multiple cell lines substantially enhances the apoptotic actions of various cell stressors, which includes anticancer drugs such as cisplatin, etoposide, and staurosporine. Moreover, exposure to cisplatin enhanced IP$_7$ generation up to 400% (*57*, *58*). Overexpression of any one of the three isoforms of IP$_6$K increased sensitivity to the apoptotic actions of hypoxia and staurosporine. However, knockdown of *IP$_6$K2* by RNA interference markedly reduced cell death, whereas knockdown of *IP$_6$K1* and *IP$_6$K3* did not (*57*).

Under basal conditions, IP$_6$K2 is sequestered in the cytoplasm bound to the cytosolic heat shock protein 90 (HSP90). Cell stressors—such as cisplatin and novobiocin,

another anticancer drug—disrupt this binding, which liberates and activates IP_6K2 and leads to cell death (*58*). Single amino acid mutations of IP_6K2 that selectively inhibit its binding to HSP90 increase its catalytic activity and apoptosis (*58*). Thus, a substantial part of the apoptotic actions of drugs such as cisplatin and novobiocin, which also inhibit the ATPase activity of HSP90, may reflect disruption of IP_6K2-HSP90 binding.

IP_6K2-mediated cytotoxicity was associated with the translocation of IP_6K2 to mitochondria in HEK293 cells preincubated with staurosporine (*57*) but with its translocation to the nucleus in OVCAR-3 cells under IFN-β–treated conditions (*97*). Different cell types and other experimental conditions might explain these discrepancies.

In several mammalian cells, IP_6K2 selectively augments p53-related cell death (*13*). Somatic cell gene disruption of *IP_6K2* virtually abolishes IP_7 generation in HCT116 cells (an epithelial cell line derived from human colon carcinoma), which indicates that the IP_6K2 isoform is primarily responsible for forming IP_7 in these cells. Deletion of *IP_6K2* reduces apoptosis elicited by 5-fluorouracil, which acts through p53 but is not reduced by sulindac, an anti-inflammatory cyclooxygenase-2 inhibitor that elicits Bcl2-associated X protein (BAX)–dependent, but p53-independent, apoptosis (*13*). *IP_6K2* deletion causes cell cycle arrest associated with a marked increase in expression of proarrest genes such as *p21* and *14-3-3σ*. By contrast, there is no alteration in prodeath genes such as *PUMA* and *NOXA*. With moderate cell stress, p53 primarily activates proarrest genes, which enables the cell to repair damage, whereas, with grave insults, p53 activates proapoptosis programs. IP_6K2 appears to act by inhibiting expression of proarrest genes so that the prodeath program is activated seemingly by default. Overexpression of IP_6K2 markedly diminishes expression of proarrest genes without influencing that of prodeath ones (*13*).

How might IP_6K2 regulate p53? IP_6K2 binds directly to the p53 DNA binding core domain (*13*). This binding underlies IP_6K2's apoptotic actions, because dominant-negative constructs of IP_6K2, which prevent its binding to p53, block the proapoptotic influences of IP_6K2. Accordingly, drugs that block IP_6K2-p53 binding might prevent p53-mediated cell death and could diminish neuronal cell death in conditions such as stroke and neurodegenerative disease; IP_6K2's prodeath influences might also reflect its role in induction of the Apo2 ligand, Apo2L [TRAIL, tumor necrosis factor (TNF)–related apoptosis-inducing ligand] (*97*). Apo2L (TRAIL) is a member of the TNF superfamily of cytokines that preferentially induces apoptosis in cancer cells upon binding to death receptors 4 and 5 (DR4 and DR5) (*98*). Blocking antibodies to Apo2L (TRAIL) prevents the apoptotic influences of IFN-β–IP_6K2 (*97*).

IP_6K2's prodeath actions might also involve antagonism of the trophic influences of CK2 (*59*). CK2 is a protein kinase with prominent prosurvival functions. Its abundance is increased in many cancers, and its inhibitors display antitumor activity (*99*, *100*). CK2 stimulates angiogenesis to create a hospitable environment for tumor growth (*101*). The proapoptotic effects of CK2 inhibitors are reduced after IP_6K2 deletion (*59*). CK2 phosphorylates IP_6K2 to decrease its stability (*59*). Phosphorylation occurs at serines 347 and 356, whose mutations lead to marked increases in IP_6K2 abundance. The clinically beneficial effects of CK2 inhibitors could involve stabilization of IP_6K2 to augment its apoptotic actions.

Autophagy has been implicated in IP_6K-mediated cell death (*102*). Overexpression of the IP_6Ks increases the numbers of autophagosomes and proportionately enhances cell death (*102*), whereas depletion of *IP_6K2* by RNA interference diminishes both cell

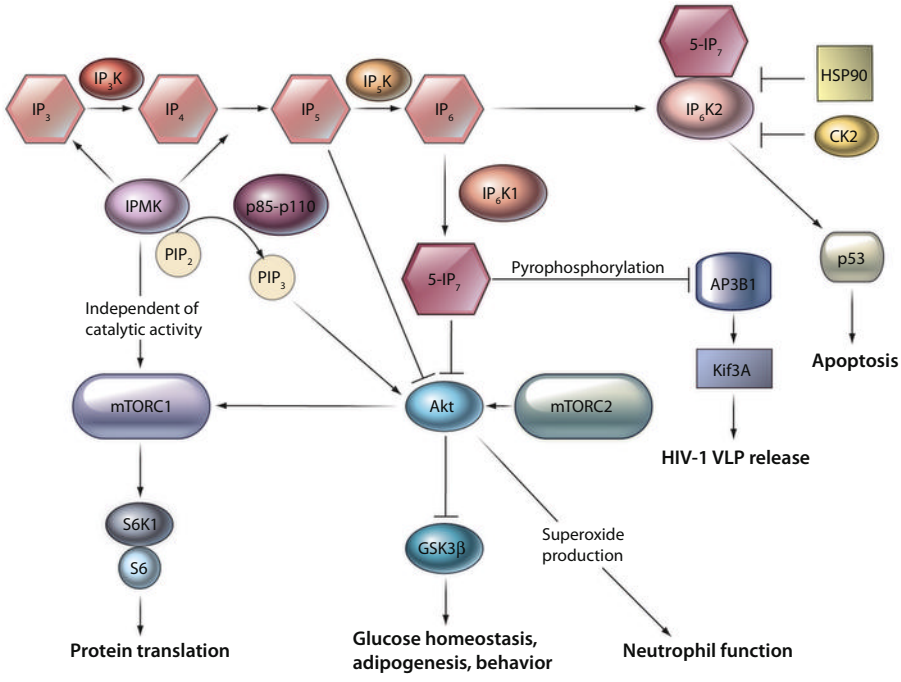

Fig. 3. Inositol poly- and pyrophosphates in mammalian cellular signaling networks. The combined soluble inositol phosphate kinase activities of IP_3K, IPMK, and IP_5K generate IP_5 and IP_6 from IP_3, which are precursors of inositol pyrophosphates. 5-IP_7 inhibits Akt activity, which decreases mTORC1-mediated protein translation and increases GSK3β-mediated glycogenolysis, adipogenesis (*11*), and behavior. IP_5 also inhibits Akt signaling (*124*, *125*). Inhibition by 5-IP_7 of Akt diminishes neutrophil phagocytic functions (*12*). 5-IP_7 regulates the release of virus like particles through its pyrophosphorylating activities (*85*). IPMK acts as a PI 3-kinase to activate Akt (*25*, *26*). In a manner independent of its catalytic activity, IPMK binds, stabilizes, and activates the mTORC1 complex (*27*). Thus, IPMK enhances or inhibits Akt signaling, respectively, by means of its PI 3-kinase and soluble inositol phosphate kinase activities. IP_6K2 is a proapoptotic protein regulated by HSP90, which binds and inactivates it (*58*), as well as by CK2, which phosphorylates IP_6K2 to enhance its proteasomal degradation (*59*). Apoptotic stimuli stabilize, activate, and facilitate nuclear translocation of IP_6K2, where it binds p53 and augments p53's apoptotic actions (*13*).

death and the numbers of autophagosomes.

Mice with targeted deletion of *IP$_6$K2* exhibit normal development, growth, and fertility (*14*). However, they display a 400% greater incidence of oral and esophageal tumors. Fibroblasts from these mice resist the antiproliferative effects of IFN-β (*14*).

IP_8 has also been implicated in cell death (*103*). Thus, yeast with *yIP$_6$K* or *Vip1* deleted display increased resistance to cell death induced by hydrogen peroxide. Furthermore, hydrogen peroxide elicits a rapid decrease in cellular inositol pyrophosphate concentrations in vivo and inhibits yIP$_6$K in vitro (*103*).

Inositol pyrophosphates have recently been linked to zebrafish development (*104*), where IP$_6$K2 activates the Hedgehog developmental pathway by PP-IP$_4$ generation. In zebrafish, deletion of *IP$_6$K2* impairs development of the craniofacial skeleton and muscle fibers, as well as the development and migration of neural crest cells (*104*). Similarly, *IP$_6$K2* depletion in NIH3T3 cells inhibits Hedgehog target gene expression, whereas overexpression of IP$_6$K2 elicits stimulatory effects (*104*). One might speculate about links between the apoptotic actions of IP$_6$K2 and its influences on Hedgehog signaling in mammalian nervous system development. Embryonic mammalian brain contains twice as many neurons as adult brain, as dropout of neurons is a prominent feature of brain ontogeny. Whether Hedgehog signaling interfaces with the neuronal dropout process and whether loss of neurons in development is related to IP$_6$K2 are unanswered questions.

4. Growth factor and cytokine signaling. Studies with *IP$_6$K1* knockout mice have established a role for IP$_7$ in regulating the growth factor–mediated Akt–mTOR–glycogen synthase kinase–3β (Akt-mTOR-GSK3β) signaling cascade (Table 2 and Fig. 3). Hints of the existence of this role came from evidence that IP$_7$ interferes with the binding of PIP$_3$ to the Akt PH domain (*73*). Akt enhances growth factor signaling, glucose uptake, glycogen synthesis, and protein synthesis (*80*). Akt influences critical regulatory proteins such as glucose transporter–4 (GLUT4), GSK3 α and β, and the mTORC1 complex (*80*). Akt overexpression in skeletal muscle leads to insulin sensitivity, skeletal muscle hypertrophy, and augmented hepatic fatty acid oxidation with reduced fat accumulation (*105*). Akt also exerts lipogenic effects. Akt1 and Akt2 double-knockout mice display reduced adipose mass and skeletal muscle atrophy (*106*). Conversely, Akt phosphorylates GSK3β, which inhibits its activity and which may affect lipogenesis, because GSK3β inhibition abolishes adipogenesis (*107*, *108*). In organisms with insulin resistance and obesity, Akt and GSK3β activities are reciprocally regulated. Thus, Akt and mTOR signaling are diminished, and GSK3β activity is increased, in insulin-resistant tissues of aged and obese mice (*109–111*). These evidently complex relationships of Akt and inositol pyrophosphates with insulin homeostasis (*11*, *50*) and lipid disposition (*11*) may reflect isoform- and tissue-specific influences (Fig. 2).

An initial link of IP$_6$K1 to Akt signaling came from observations that growth factors such as IGF-1 markedly stimulate IP$_7$ formation in serum-starved cells, with the increase abolished in *IP$_6$K1*-depleted cells (*11*). The *IP$_6$K1* knockout cells display markedly augmented IGF-1 and insulin-stimulated Akt signaling, with enhanced phosphorylation of Akt downstream effectors such as GSK3β, tuberin, p70S6 kinase1, and S6 itself. These effects reflect loss of IP$_6$K1 catalytic activity because they can be rescued by the wild-type enzyme but not by catalytically inactive IP$_6$K1 (*11*).

How might IP$_7$ regulate Akt? Inositol polyphosphate binding to various membrane-targeted proteins like Akt, phosphoinositide-dependent kinase–1 (PDK1), and interleukin 2–inducible T cell kinase (ITK) regulates their activity and membrane translocation (*68*, *69*, *71–76*) (Table 1). In the growth factor signaling cascade, extracellular receptor stimulation leads to activation of the p85-p110 PI 3-kinase, which generates the phospholipid PIP$_3$. PIP$_3$ activates Akt at the plasma membrane by facilitating its phosphorylation by the kinase PDK1. Growth factor stimulation of cells increases the membrane localization of Akt, with these effects substantially increased in cells lacking *IP$_6$K1*. The IP$_6$K inhibitor TNP {N^2-[*m*-(trifluoromethyl)benzyl], N^6-(*p*-nitrobenzyl) purine} (*112*) also increases growth factor stimulation of Akt, manifested as its phosphorylation at threonine-308, a process mediated by PDK1. 5-IP$_7$ inhibits the phosphorylation of Akt by PDK1 in vitro. In the absence of added PIP$_3$, IP$_7$ inhibits Akt phosphorylation with a median inhibitory concentration of about 20 nM, substantially more potent than any other known action of IP$_7$ (*11*). IP$_5$ and IP$_6$ are much less potent at inhibiting Akt phosphorylation by PDK1, whereas IP$_3$ and IP$_4$ are inactive (*11*). Inhibition is absent in Akt lacking the PH domain, which indicates that 5-IP$_7$ exerts its inhibitory effect through the PH domain of Akt. This fits with evidence that IP$_7$ interferes with PIP$_3$ binding to PH domains of several proteins (*73*). IP$_7$ inhibits PIP$_3$'s activation of Akt-T308 phosphorylation more potently when added before PIP$_3$ than when preparations are preincubated with PIP$_3$ (*11*), consistent with observations that preincubation with PIP$_3$ prevents IP$_7$ from displacing PIP$_3$ from Akt's PH domain (*113*).

Detailed mechanisms whereby IP$_7$ inhibits Akt are not fully resolved. IP$_7$ competes with PIP$_3$ for binding PH domains of Akt and other proteins with a potency similar to that of IP$_4$ (*73*). However, IP$_7$'s potency as an inhibitor of Akt phosphorylation is much greater (*11*), which suggests that its activity may depend on factors other than binding. Binding assays (*73*) used the Akt-PH domain, whereas the kinase assays (*11*) utilized full-length Akt, which may account in part for discrepant potencies. Similarly, full-length PDK1 binds phosphoinositides or inositol polyphosphates better than the PDK1-PH domain does (*76*). Variations in Akt conformation may contribute to the dual regulation of Akt by phosphoinositides and inositol pyrophosphates. Thus, PDK1-mediated phosphorylation depends on PH- and kinase domain–dependent conformational changes in Akt (*114*). In the PH-in conformation, the Akt activation loop is inaccessible to PDK1. Growth factor–mediated Akt binding to PIP$_3$ converts Akt to the PH-out conformation, which is available for PDK1 phosphorylation. Moreover, PDK1 and Akt form a complex in vivo (*114*). In this complex mode of PDK1-Akt-PIP$_3$-IP$_7$ regulation, 5-IP$_7$ appears to stabilize the inactive (PH-in) conformation of Akt, whereas PIP$_3$ does the opposite. Whether IP$_7$ influences this process by pyrophosphorylating Akt or PDK1 is unclear.

The increased negative charge of IP$_7$ may explain, in part, why it is preferred over other inositol phosphates for mediating certain biologic actions. Thus, the specificity for binding of IP$_5$ isomers to various PH domains depends on the number of basic residues in the domain. PH domains of Akt and pleckstrin bind more selectively to IP$_5$ isomers than does the general receptor for phosphoinositides–1 (GRP1) PH domain, which reflects the fewer basic residues of the PH domains of Akt and pleckstrin (*115*).

The intracellular distribution of inositol pyrophosphate–synthesizing enzymes

might affect inositol poly- and pyrophosphate-mediated PH domain regulation in vivo. Indeed, receptor-regulated compartmentalization of inositol pyrophosphate synthesis has been demonstrated (*116*). The mammalian IP_8-synthesizing enzyme PP-IP_5K1 has a cryptic PIP_3-binding PH domain, which also binds IP_6 to a lesser extent (*116*). PP-IP_5K1 translocates to the plasma membrane in response to PDGF treatment (*116*). Accordingly, IP_8 might influence the membranous disposition of Akt.

The enhanced Akt activation in response to insulin in *IP_6K1* knockout mice has notable physiologic consequences. Initial studies of *IP_6K1* knockout mice revealed reduced concentrations of blood insulin with normal plasma glucose, which implies insulin hypersensitivity (*10*). Enhanced glucose tolerance confirms the sustained insulin sensitivity of 10-month-old *IP_6K1* mutants (*11*). Moreover, glucose uptake into muscle and fat of the mutants is tripled. Consistent with the insulin hypersensitivity, *IP_6K1* knockout mice are resistant to obesity elicited by high-fat diets. These metabolic alterations in the *IP_6K1* knockouts appear to result from the increased Akt signaling, which leads to decreased GSK3β activity in consequence of phosphorylation by Akt of GSK3β. This also leads to increased glycogen abundance and reduced adipogenesis. Accordingly, IP_6K1 inhibitors may be useful in treating type 2 diabetes and obesity. The likelihood of adverse effects from such inhibitors can be inferred from the phenotype of the *IP_6K1* knockouts. They weigh about 15% less than controls, owing to less fat deposition, and males have reduced numbers of sperm, but otherwise the mice appear normal. Because of Akt's role in cell survival and proliferation (*80*, *117*), one might anticipate that the increased Akt signaling of the *IP_6K1* mutants would facilitate tumor formation. However, no spontaneous tumors have been observed in 2-year-old *IP_6K1* knockout mice, which corresponds roughly to 75 to 80 years of human life.

PI 3-kinase activity and PIP_3 signaling influence neutrophil chemotaxis and phagocytosis through NADPH (nicotinamide adenine dinucleotide phosphate) oxidase–mediated superoxide production (*118–120*). IP_6K1 appears to regulate neutrophil function through Akt inhibition (*12*). Thus, *IP_6K1*-depleted neutrophils display enhanced Akt activation and amplified superoxide production which leads to increased phagocytic and bactericidal activity (*12*).

IP_6K1 signaling may have behavioral consequences. Genetic abnormalities leading to decreased Akt abundance have been linked to schizophrenia (*121*). The mood-stabilizing actions of lithium have been attributed to direct GSK3 inhibition (*122*). In an alternative model, lithium dissociates a β-arrestin–protein phosphatase–Akt complex, which leads to augmented Akt activity, which in turn inhibits GSK3β (*123*). Thus, IP_6K1 inhibitors, by enhancing Akt signaling, might be therapeutic in diverse psychiatric diseases.

IPMK and IP_6K: A Molecular Switch in Growth Factor Signaling?

An interface between IP_6K1 and IPMK in regulating Akt is implied by the finding that IPMK has robust and selective PI 3-kinase activity in vitro (*25*). Initially, it was not clear how IPMK could contribute to cellular formation of PIP_3, as one can deplete PIP_3 with wortmannin, which inhibits PI 3-kinases but does not inhibit IPMK. The availability of *IPMK*-deleted cells permitted a resolution of this conundrum (*26*). Deletion of *IPMK* leads to a 50% decrease in PIP_3 generation. Moreover, IPMK purified from cells treated with wortmannin displays a 70% reduction in catalytic activity. Thus, in intact cells, wortmannin inhibits IPMK with potency similar to that for inhibiting the p110 PI 3-kinase. Presumably, PIP_3 formed by the p110 enzyme activates a kinase that phosphorylates IPMK to stimulate its catalytic activity. In support of this notion, dephosphorylation of IPMK with protein phosphatases reduces its catalytic activity to the same extent as wortmannin treatment of intact cells (*26*).

If IPMK is a physiologically important PI 3-kinase, then it should regulate Akt. Indeed, the stimulation of Akt by growth factors such as insulin, IGF-1, and EGF is substantially reduced in *IPMK*-deleted cells (*26*). IPMK's stimulation of Akt is due to its PI 3-kinase rather than its inositol phosphate kinase activity. Thus, in *IPMK*-deleted cells the loss of Akt signaling is rescued by overexpressing wild-type IPMK but not an IPMK isoform from *Arabidopsis* that has inositol phosphate kinase, but not lipid kinase, activity (*26*). Akt is a major determinant of cell proliferation and, as expected, the rate of proliferation in *IPMK*-deleted mouse embryonic fibroblasts is reduced about 50%. In U87MG glioma cells, depletion of *IPMK* by RNA interference also substantially reduces proliferation, which is augmented by overexpression of IPMK (*26*).

IPMK thus appears to be a required link in growth factor signaling via PIP_3. PI 3-kinases generate PIP_3, which, by means of unknown kinases, elicits phosphorylation and activation of IPMK, whose PI 3-kinase activity also synthesizes PIP_3 to stimulate Akt. Reasons for a cell to generate PIP_3 in two sequential steps are not clear. One likely possibility is the amplifying effect attendant upon a series of enzymatic steps. It is also possible that the PIP_3 that activates Akt derives in part from p110 and in part from IPMK. However, the 70% reduction in Akt activation after *IPMK* deletion suggests that p110 and IPMK act sequentially.

IPMK's deletion abolishes the formation of IP_5, IP_6, and IP_7, which indicates that it is rate limiting in the generation of inositol pyrophosphates (*26*). Like IP_7, IP_5 also inhibits Akt (*11*, *124*), although to a lesser extent than IP_7 (*11*), and IP_5 derivatives display antitumor activities (*125*). Because IP_5 can be converted by IP_6Ks to PP-IP_4, IP_5-mediated Akt inhibition is likely to involve generation of PP-IP_4 in vivo. IP_7 (and possibly IP_5), are thus opposing the influences exerted by the lipid kinase activity of IPMK. Accordingly, IPMK may exert two divergent actions, as its lipid kinase activity promotes Akt signaling, whereas its inositol phosphate kinase activity exerts the opposite effect (Fig. 3).

All of the above signaling influences of the IP_6K1-IPMK system involve the activation by growth factors of Akt and its downstream targets, such as mTOR. IPMK may be a key element regulating mTOR. mTOR was first isolated as a target for the immunosuppressive actions of the drug rapamycin (*126*, *127*) and subsequently has been shown to act by means of a complex of mTOR-binding proteins to serve as a major stimulant of protein synthesis (*128*). There are two mTOR complexes differentiated by the association of mTOR with raptor in mTOR complex-1 (mTORC1) (*129*), which is targeted by IPMK, and rictor in mTORC2 (*130*). mTORC1 is the principal determinant of protein synthesis and cell growth in response to nutrient amino acids. IPMK is bound to mTOR and raptor in the mTORC1 complex (*27*). IPMK appears to be a critical component of the complex, because depletion of *IPMK* reduces mTOR binding to raptor and the GTPase Rag without influencing interactions of mTORC2 with rictor. IPMK is critical for mTOR signaling, because its deletion reduces mTOR signaling in response to amino acids by about

60%. Overexpression of IPMK rescues this deficiency. Remarkably, catalytically inactive IPMK is equally effective in restoring mTOR signaling. Thus, IPMK acts in a noncatalytic fashion to stabilize mTORC1 and to facilitate protein translation. This conclusion is supported by findings that a dominant-negative construct that selectively blocks the binding of IPMK to mTOR abrogates nutrient-elicited mTOR signaling (*27*).

IP_6K1 and IPMK thus appear to be key regulators of metabolic signaling in response to growth factors and nutrients (Fig. 3). A fuller understanding of how IPMK switches between its lipid kinase and inositol phosphate kinase activities awaits identification of its regulation. We do not know whether an analogous regulation sequesters a pool of IPMK to act noncatalytically in stabilizing the mTORC1 complex for responses to altered amino acid availability. Indeed, it is not clear whether cells switch between devoting their signaling resources to coping with growth factors versus nutrients or whether the two activities occur in parallel without any crosstalk.

Conclusions

Delineation and genetic manipulation of key enzymes in the inositol pyrophosphate family have revealed major roles for these substances in diverse areas, especially metabolic regulation and the balance between cell growth and death. As intracellular messenger molecules, inositol pyrophosphates may well emerge as regulators comparable in importance to the cyclic nucleotides. The field is young and has been hampered by technical limitations. Thus, IP_7 and IP_8 are difficult to synthesize so that they are not readily available from commercial sources, although innovative approaches to their generation may overcome such hurdles (*131*, *132*). A recently developed polyacrylamide gel procedure affords facile separation of inositol pyrophosphates, which may simplify IP_7 purification and detection (*133*). Antibodies to the critical enzymes tend to lack adequate specificity for many purposes. It is hoped that these hurdles will be surmounted and unexploited areas addressed, such as roles of these substances in the brain and the function of IP_6K3. Major advances have been facilitated by the development of mice with deletion of inositol pyrophosphate biosynthetic enzymes. Some of the recent insights may have therapeutic relevance, especially in treating diabetes and obesity. IP_6K inhibitors such as TNP (*112*) may help clarify the functions of the inositol pyrophosphates and may also afford therapeutic benefit.

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