A role for rat inositol polyphosphate kinases, rIpk2 and rIpk1, in inositol pentakisphosphate and inositol hexakisphosphate production in Rat-1 cells

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Abbreviations: HPLC, high pressure liquid chromatography; V_{max}, maximal velocity attained with excess substrate; K_m, substrate concentration permitting a half-maximal velocity, PP-IP_4, diphosphorylinositol tetrakisphosphate; IP, inositol polyphosphate; I(1,4,5)P_3, inositol 1,4,5-trisphosphate; IP_3, inositol trisphosphate; IP_4, inositol tetrakisphosphate; IP_5, inositol pentakisphosphate; IP_6, inositol hexakisphosphate; PP-IP_5, diphosphorylinositol pentakisphosphate; ATP, adenosine 5’-triphosphate.

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ABSTRACT

Over 30 inositol polyphosphates are known to exist in mammalian cells, however the majority of them have uncharacterized functions. Here we investigate the molecular basis of synthesis of highly phosphorylated inositol polyphosphates (such as IP_4, IP_5 and IP_6) in rat cells. We report that heterologous expression rat inositol polyphosphate kinases rIpk2, a dual-specificity IP_3/IP_4 kinase, and rIpk1, an IP_5 2-kinase are sufficient to recapitulate IP_6 synthesis from I(1,4,5)P_3 in mutant yeast cells. Overexpression of rIpk2 in Rat-1 cells increased I(1,3,4,5,6)P_5 levels about 2-3 fold as compared to control. Likewise in Rat-1 cells, overexpression of rIpk1 is capable of completely converting I(1,3,4,5,6)P_5 to IP_6. Simultaneous overexpression of both rIpk2 and rIpk1 in Rat-1 cells increased both IP_5 and IP_6 levels. In order to reduce Ipk2 activity in Rat-1 cells, we introduced vector-based short interference RNA (siRNA) against rIpk2. Cells harboring the siRNA had a 90% reduction of mRNA levels and a 75% decrease of I(1,3,4,5,6)P_5. These data confirm the involvement of Ipk2 and Ipk1 in the conversion of I(1,4,5)P_3 to IP_6 in rat cells. Furthermore, these data suggest that rIpk2 and rIpk1 act as key-determining steps in production of IP_5 and IP_6, respectively. The ability to modulate the intracellular inositol polyphosphate levels by altering Ipk2 and Ipk1 expression in rat cells will provide powerful tools to study the roles of I(1,3,4,5,6)P_5 and IP_6 in cell signaling.
INTRODUCTION

Most of the over 30 inositol polyphosphates present in mammalian cells have unknown physiological functions (1-3). One of the well characterized inositol polyphosphates is a second messenger, inositol 1,4,5-trisphosphate (I(1,4,5)P₃) which participates in intracellular Ca²⁺ mobilization (4) and also serves as a precursor of highly phosphorylated inositol polyphosphates such as inositol tetrakisphosphate (IP₄), inositol pentakisphosphate (IP₅) and inositol hexakisphosphate (IP₆) (1-3). Recently, the inositol polyphosphate synthetic pathway in budding yeast, *Saccharomyces cerevisiae* has been identified (5-8). In yeast, I(1,4,5)P₃ which is hydrolyzed from phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) by phospholipase C, is sequentially phosphorylated to IP₆ by two inositol polyphosphate kinases, a multiple-specificity IP₃/IP₄ kinase (Ipk2) and an IP₃ 2-kinase (Ipk1). On the other hand, a synthetic pathway of IP₆ from I(1,4,5)P₃ in mammalian cells is thought to be more complex and to be a sequence of I(1,4,5)P₃ -> I(1,3,4,5)P₄ -> I(1,3,4)P₃ -> I(1,3,4,6)P₄ -> I(1,3,4,5,6)P₅ -> IP₆ (2, 3). In this pathway, IP₃ 3-kinases (2), inositol polyphosphate 5-phosphatases (9) and I(1,3,4)P₃ 5/6-kinase (3) appear to catalyze the first three steps to produce I(1,3,4,6)P₄, respectively. Additionally, the mammalian orthologs of IPK2 (also known as inositol polyphosphate multikinase (IPMK)) and IPK1 were cloned recently by several independent groups and partially characterized *in vitro* (10-13). These two kinases possibly catalyze the last two steps, respectively, however the possibility remains that IP₆ is synthesized from the sequential phosphorylation of I(1,4,5)P₃ by Ipk2 and Ipk1 in mammalian cells as it is in yeast cells. Currently, there is no definitive evidence describing the inositol polyphosphate synthetic pathways in mammalian cells (2, 3).

Several recent works suggest possible functions of highly phosphorylated inositol polyphosphates and/or their kinases. For example, IP₃ has been shown to modulate HIV-1 Gag...
protein assembly (14) and IP₆ has been reported to bind the clathrin assembly proteins AP2 and AP3 thus inhibiting clathrin cage assembly in vitro (15-18). Additionally, IP₆ stimulates non-homologous DNA end joining (NHEJ) of double strand DNA breaks by binding to the Ku70/80 subunits of DNA-PK in vitro (19-21). Yeast genetics data also suggest that these kinases and/or their inositol polyphosphate products are involved in transcription of specific genes, mRNA export and chromatin remodeling (5-7, 22-24). However, most of the data mentioned above were determined in vitro or in yeast and evidence in mammalian cells is still lacking. Additionally, some of the data obtained from in vitro studies may be experimental artifacts due to the effect of the highly polarized negative charge of these inositol polyphosphate molecules (25).

To address the synthetic pathway and the function of inositol polyphosphates in mammalian cells, it is important to establish a model system whereby the inositol polyphosphate levels can be modulated in vivo. In this study, we have analyzed the effects of either overexpression or knock down of inositol polyphosphate kinase(s) on inositol polyphosphate levels in Rat-1 cells. We show the accumulation of I(1,3,4,5,6)P₅ and IP₆ by overexpression of Ipk2 and Ipk1. We have also succeeded in decreasing cellular I(1,3,4,5,6)P₅ levels by RNA interference (RNAi). These data reveal the involvement of Ipk2 and Ipk1 in I(1,3,4,5,6)P₅ and IP₆ synthesis in vivo. Furthermore, the ability to modulate the intracellular inositol polyphosphate levels shown here by altering Ipk2 and Ipk1 expression in rat cells will provide powerful tools to study the roles of I(1,3,4,5,6)P₅ and IP₆ in eukaryotic cell signaling.
EXPERIMENTAL PROCEDURES

Cell culture and transfection

Phoenix, HEK293 and Rat-1 cells were grown in Dulbecco’s modified Eagles’ medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO₂. Culture medium was supplemented with 100 units/ml penicillin G and 100 µg/ml streptomycin. The cell lines expressing tetracycline inducible constructs were maintained in the same condition except using 10% Tet system approved FBS (Clontech). Retroviruses were produced by transient transfection of the plasmid into phoenix cells by the calcium phosphate method as described elsewhere (26). HEK293 or Rat-1 cells were infected with retroviruses containing media in the presence of 8 µg/ml of hexadiamethrine bromide (Sigma) for overnight. Thirty-six hours later, cells were selected in the presence of appropriate antibiotics for at least 2 weeks and then used for experiments. The concentration of antibiotics used for selection were: Geneticin 800 µg/ml, Hygromycin 400 µg/ml and Puromycin 1.5 µg/ml, respectively.

RNA preparation and cloning of rat IPK2 and rat IPK1 by RT-PCR

Total RNA was isolated according to the manufacture’s recommendations using the RNeasy Mini Kit (Qiagen). Briefly, 1 x 10⁷ Rat-1 cells were lysed into buffer RLT/1% 2-mercaptoethanol and then applied to the RNeasy mini column. After washing the column, total RNA was eluted with 40 µl of RNase free water. RT-PCR was performed using the RobusT I RT-PCR Kit (Finnzymes). Reverse transcription of 1 µg of RNA was performed at 42 °C for 1h in 50 µl of reaction mixture containing 1 x RobusT I reaction buffer, 1.5 mM MgCl₂, 40 units of RNase inhibitor, 0.8 mM dNTPs, 0.2 µM oligo(dT), 0.2 µM sense and antisense primer, 5 units of AMV reverse transcriptase and 1 unit of DyNAzyme EXT DNA polymerase. The subsequent
PCR reaction was done at 94 °C for an initial 2 min then 25 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 2 min and final extension at 72 °C for 10 min. The primers were designed to amplify the entire open reading frame of the rat ortholog of IPK2 (rIPK2) (GeneBank™ accession AY014898) (10). The primers for the rat ortholog of IPK1 (rIPK1) were designed based on the mouse IPK1 ortholog (GeneBank™ accession XM_283126). The primers used for rIPK2 were: sense 5’-TTT TTG TCG ACC ACC ATG GCC GCC GAG CCC CCA-3’ (underline shows SalI site) and antisense 5’-TTT TTG ATA TCG ATT CAA CTG TCC AAG ATA CTC CG-3’ (underline shows ClaI site). The primers used for rIPK1 were: sense 5’-AAC TCG AGC ACC ACC ATG GAA GAG GGG AAA AT-3’ (underline shows XhoI site) and antisense 5’-AAT CTA GAA AGC TTT TAG ACC TTA TGG AGA ACT AAT GTG CCC G-3’ (underline shows HindIII site). The RT-PCR products were cloned into pCR2.1 TA cloning vector (Invitrogen). The substitution of Asp127 to Ala in rIPK2 (rIPK2 D127A) was performed by PCR with the following mutagenic primers (underline shows D127A mutation): sense 5’-AAG CCC TGT ATA ATG GCC GTG AAG ATT GGG CGG-3’ and antisense 5’-CCG CCC AAT CTT CAC GCC GTG AAG ATT GGG CGG-3’. All constructs were sequenced by the ABI Prism Big Dye Terminator Ready reaction Kit (PE BioSystems).

**Plasmid construction**

The SalI/EcoRI fragment of pCR2.1/rIPK2 (and also D127A mutant) or the XhoI/EcoRI fragment of pCR2.1/rIPK1 were inserted into the SalI/EcoRI sites of pGST4 (27) to produce pGST/rIPK2 or pGST/rIPK1, respectively. The EcoRI fragments of pCR2.1/rIPK2 or pCR2.1/rIPK1 were inserted into the same site of pUNI10 (28) and then recombined with pRS426-CUP1-MYC3-LOXP (29) using the cre-loxP system (28) to produce the yeast expression
vector for rlpk2 or rlpk1, respectively. To introduce the N-terminus GFP or myc tag to the
tetracycline induced gene expression vector, pRevTRE (Clontech), the sequence for GFP or myc
tag were amplified by PCR using phGFP105-C1 (30) or pRS426-CUP1-MYC3-LOXP (29) as
template. The primers were: GFP sense 5’-TTT TTG GAT CCA CCA TGG TGA GCA AGG
GC-3’ (underline shows BamHI site), GFP antisense 5’-TTT TTG TCG ACT TGT ACA GCT
CGT CCA TGC C-3’ (underline shows SalI site), myc sense 5’-TTT TTG GAT CCA CCA TGG
GAT TCG AGC TAT GCG GC-3’ (underline shows BamHI site) and myc antisense 5’-TTT
TTG TCG ACC CTT CGA GAC TAG TGC GGC-3’ (underline shows SalI site), respectively.
The PCR products were digested by BamHI/SalI and inserted into the same sites of pRevTRE to
produce pRevTRE/GFP or pRevTRE/myc, respectively. The NheI/HindIII fragment of pEGFP-
C1 (Clontech) was also cloned into the same sites of pTRE2 (Clontech) to produce pTRE/GFP.
The SalI/ClaI fragment of pCR2.1/rlPK2 was inserted into the same sites of pRevTRE/GFP to
produce pRevTRE/GFP-rlPK2. The XhoI/HindIII fragment of pCR2.1/rlPK1 was inserted into
the SalI/HindIII sites of pRevTRE/GFP or pRevTRE/myc for pRevTRE/GFP-rlPK1 or
pRevTRE/myc-rlPK1, respectively. The BamHI/ClaI fragment of pRevTRE/GFP-rlPK2 or
pRevTRE/GFP-rlPK1 were inserted into BamHI/SalI sites of pBabePuro (31) to make
pBabePuro/GFP-rlPK2 or pBabePuro/GFP-rlPK1, respectively. The BamHI/SalI fragment of
pTRE/GFP was inserted into the same site of pBabePuro to produce pBabePuro/GFP. S.
cerevisiae IPK2 (and also kin-) (6) or IPK1 (5) were cloned by PCR using pUNI/ScIPK2 (kin-) or
genomic DNA as template, respectively. The primers were: ScIPK2 sense 5’-ACT CTA TAA
AGC TTT CTA TAA AAT GGA TAC GGT AAA CAA TTA TAG G-3’ (underline shows
HindIII site), ScIPK2 antisense 5’-ACT CTA GTC GAC AAG ACA AGG TAA ACT TCA CCT
CTC A-3’ (underline shows SalI site), ScIPK1 sense 5’-TTT TTG TCG ACC ACC ATG CAA
GTC ATC GGA CGT GG-3' (underline shows SalI site) and ScIPK1 antisense 5’-TTT TTT CTA GAA AGC TTC TGC CAG TAC CAA AGG TGG-3' (underline shows HindIII site), respectively. The PCR product of ScIPK2 was digested by HindIII/SalI site and cloned into the same site of pTRE/GFP to produce pTRE/GFP-ScIPK2. The BamHI/SalI fragment of pTRE/GFP-ScIPK2 was cloned into the same sites of pRevTRE to make pRevTRE/GFP-ScIPK2. The PCR product of ScIPK1 was digested by SalI/HindIII and cloned into the same sites of pRevTRE to make pRevTRE/GFP-ScIPK1. For the vector-based rIPK2 siRNA construct, pSUPER/rIPK2-3 which targeted nt 1075-1093 of rIPK2, synthetic DNA primers (sense 5’-GAT CCC CGC GGA AGT GCG GAT GAT AGT TCA AGA GAC TAT CAT CCG CAC TTC CGC TTT TTG GAA A-3’ and antisense 5’-AGC TTT TCC AAA AAG CGG AAG TGC GGA TGA TAG TCT CTT GAA CTA TCC GCA CTT CCG CGG G-3’) were annealed and then cloned into BglII/HindIII sites of pSUPER (32). All constructs were sequenced by the ABI Prism Big Dye Terminator Ready reaction Kit (PE BioSystems).

Expression and purification of recombinant GST fusion protein

The expression and purification of GST-rIpk2 and GST-rIpk1 was performed as described previously (29) with minor modifications. Briefly, competent BL21 E. coli cells were transformed by pGST/rIPK2 or pGST/rIPK1. One liter of LB was cultured at 37 °C to an absorbance of 0.8-1.0 at 600 nm. Expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration). The cells were grown for 20h at 20 °C, harvested by centrifugation at 4 °C, and resuspended in 20 ml of ice-cold phosphate-buffered saline (PBS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF) and one Complete™ Mini protease inhibitor cocktail tablet (Roche) per 10 ml of buffer). The cells
were lysed by 5 passages through a cell cracker (a high shear fluid processing system for cell rupture, Microfluidics Corp.). Triton X-100 was added to lysate as final concentrations of 1% and then subjected to centrifugation at 15,000 x g for 15 min at 4 °C. The supernatant was then incubated with 1 ml of 50% glutathione-Sepharose slurry (Amersham Biosciences) for 1h at 4 °C with shaking. The Sepharose was washed three times with 15 ml of ice-cold phosphate-buffered saline (PBS), and the GST fusion proteins were eluted from the Sepharose with 1 ml of 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) and stored at -80 °C in aliquots.

**Inositol phosphate kinase assays**

To synthesize \( [^3H]I(1,3,4,5,6)P_5 \), \( [^3H]I(1,4,5)P_3 \) was phosphorylated by 100 ng of recombinant *Arabidopsis thaliana* ortholog of Ipk2 (AtIpk2β) which phosphorylates I(1,4,5)P₃ to I(1,3,4,5,6)P₅ (29) for 30 min at 37 °C in the same buffer as kinase assay (see below) and the reaction was terminated by incubation for 3 min at 95 °C. The inositol phosphate kinase assay in vitro was described previously (29). Briefly, 10-100 ng of purified recombinant GST-rIpk2 or GST-rIpk1 were incubated independently with 1-10 μM \([^3H]\)inositol, \([^3H]I(1)P, [^3H]I(1,4)P_2, [^3H]I(1,3,4)P_3, [^3H]I(1,4,5)P_3, [^3H]I(1,3,4,5)P_4 \) or \([^3H]I(1,3,4,5,6)P_5 \) for 30 min at 37 °C in buffer containing 2 mM ATP, 50 mM Hepes-NaOH (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 10 mM phosphocreatine and 0.2 units/μl phosphocreatine kinase. To determine whether the IP₄ product of I(1,4,5)P₃ phosphorylation was I(1,3,4,5)P₄ or I(1,4,5,6)P₄, the reaction was terminated by incubation for 3 min at 95 °C and then dispensed in half. One half of the reaction was incubated with 100 ng of purified recombinant type I inositol polyphosphate 5-phosphatase (5-ptase) (9) for another 30 min at 37 °C. To determine whether the IP₅ product of I(1,4,5)P₃ phosphorylation was I(1,3,4,5,6)P₅, half of the reaction was incubated with 100 ng of purified recombinant GST-
AtIpk1, GST fused *Arabidopsis thaliana* ortholog of I(1,3,4,5,6)P$_5$ 2-kinase (Stevenson-Paulik *et al.* unpublished data), for another 30 min at 37 °C. To test whether rIpk2 or rIpk1 phosphorylated a range of different inositol polyphosphates, 100 ng of GST-rIpk2 or GST-rIpk1 were incubated independently with 10 µM I(1,4,5)P$_3$, I(1,3,4,5)P$_4$, I(1,3,4,6)P$_4$, I(1,4,5,6)P$_4$ or I(1,3,4,5,6)P$_5$ in 50 mM Hepes-NaOH (pH 7.5), 50 mM KCl, 10 mM MgCl$_2$ and trace amount of [$\gamma$-$^32$P]ATP (200,000 cpm) for 30 min at 37 °C. All reactions were terminated by the addition of 30 times the reaction volume of 10 mM NH$_4$H$_2$PO$_4$ (pH3.5) and analyzed by HPLC as described (5).

**Cell lysate preparation and western blotting analysis**

2 x 10$^5$ Rat-1 cells stably expressing the indicated plasmid were seeded in 60 mm culture dishes containing 5 ml of appropriate media with or without 2 µg/ml doxycycline and cultured for 2-3 days. Cells were washed twice with 5 ml of ice-cold PBS and then extracted with 200 µl of ice-cold 1% NP40-TNE buffer (10 mM Tris-HCl (pH 7.6), 1% NP40, 150 mM NaCl, 2 mM PMSF and one Complete™ Mini protease inhibitor cocktail tablet per 10ml of buffer). After a 15 min incubation on ice, the lysate was recovered by centrifugation (14,000 rpm for 15 min at 4 °C). The supernatants were subjected to protein assay using bovine serum albumin as standard. One microgram of cell lysates were separated by SDS-PAGE. Immunostaining of gels was carried out using an ECL Western blotting detection system (Amersham-Pharmacia) using a rabbit polyclonal anti-GFP antibody (Clontech).

**Fluorescent microscopy**

Rat-1 cells stably expressing GFP fusion constructs were washed with pre-warmed Hanks' balanced salt solution (HBSS) and observed under the Olympus IX70 inverted
microscope equipped with a confocal head using a LCPlan Fl 40x/0.60 N.A. lens. Images were recorded using UltraVIEW™ imaging software (Perkin Elmer).

[^H]myo-inositol labeling, isolation and analysis of soluble inositol phosphates

*S. cerevisiae* cells carrying expression plasmid (Table 1) were grown in 1 ml of complete minimal medium lacking uracil with 100 μM CuSO₄ and 40 μCi/ml [2-[^H]myo-inositol to late logarithmic phase. The cells were harvested and the soluble lysate containing the inositol polyphosphates was isolated according to York *et al.* (5).

1 x 10⁵ HEK293 or Rat-1 cells stably expressing the indicated plasmid were seeded into a 60 mm culture dish containing 3 ml of appropriate media. One day after incubation, cells were washed twice with 5 ml of pre-warmed Medium-199 and then labeled with 10-20 μCi/ml [2[^3]H]myo-inositol for 2 days in 2 ml of Medium-199 supplemented with dialyzed 10% FBS with or without 2 μg/ml doxycycline. For pulse chase experiment, cells were labeled with 50 μCi/ml [2[^3]H]myo-inositol for 4h and then chased in 10% FBS/DMEM for indicated time. Cells were washed twice with ice-cold PBS(-) and then harvested in 500 μl of 1N HCl. The soluble fraction containing the inositol polyphosphates was isolated as above mentioned. To determine whether the IP₅ product in the rIPK2 expressing Rat-1 cells was I(1,3,4,5,6)P₅, the lysate was dried using a SpeedVac and resuspended to 50 mM Hepes-NaOH (pH 7.5). The lysate was incubated with 100 ng of purified recombinant GST-AtIpkl for 30 min at 37 °C.

Northern blotting analysis

Northern blot analysis was performed as described by Stevenson *et al.* (33). Entire open reading frame of each gene was used as probe and labeled using the Ready-To-Go™ DNA
labeling beads (Amersham Biosciences) with $[\alpha^{-32}\text{P}]\text{dCTP}$. Hybridization was carried out using ExpressHyb hybridization solution (Clontech) following the manufacturer’s recommendations.

**Colony formation assay in soft agar**

5 x $10^4$ Rat-1 cells stably expressing the indicated plasmid were seeded into a 35 mm culture dish containing 1 ml of culture media with 0.4 % agar. The dishes had been coated with 2 ml of culture media containing 0.7 % agar. Cells were fed with 0.5 ml of culture media containing 0.4 % agar once a week. Colony formation was scored after 3 weeks at 37 °C in a humidified atmosphere of 5% CO$_2$. 
RESULTS

Cloning of rat IPK2 and rat IPK1 orthologs

To investigate the function of inositol polyphosphates in mammalian cells, we first cloned rat orthologs of IPK2 and IPK1. Saiardi et al. (10) have published the cloning of rIPK2, so we used this sequence information for cloning (see EXPERIMENTAL PROCEDURES).

When we started this project, the DNA sequence information of rIPK1 was not available. However, we could use both the human and mouse sequences of IPK1. These two orthologs possessed about 88% identity at the DNA level. Based on this information, we designed cloning primers for rIPK1 and performed RT-PCR (see EXPERIMENTAL PROCEDURES). The rat ortholog of IPK1 possessed 91.9% and 96.3% of amino acid identity to the human and mouse orthologs, respectively. The rIPK1 contained the conserved motifs EXKPK, CRXC, (F/Y)CPLDL and D(L/V)DLK(P/S)X(E/M) (13), the function of which are still unknown. Recently, the predicted open reading frame of rIPK1 was published in GeneBank™ (GeneBank™ accession XM_225201). This prediction was based on GenomeScan analysis of the rat genomic sequence (GeneBank™ accession NW_047490). Our cDNA differed somewhat from the predicted intron/exon splicing identified by the GenomeScan software. Therefore, we have submitted a new sequence, and GeneBank™ has issued a new accession number (###) for rIPK1.

Characterization of rat Ipk2 and rat Ipk1 in vitro

The cloning and characterization of rat and human orthologs of IPK2 has been reported by several independent groups (10-12). However, there is controversy about the substrate specificity among these reports. Therefore, we analyzed the rIpk2 in vitro. We incubated GST-
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rIpk2 with [3H]I(1,4,5)P₃ for 30 min and then analyzed the products by HPLC. Fig. 1A shows the elution pattern of the substrate, [3H]I(1,4,5)P₃. 10 ng of GST-rIpk2 phosphorylated I(1,4,5)P₃ to IP₄ and IP₅ (Fig. 1B). It was reported that both rat and human orthologs of Ipk2 preferred to phosphorylate the D3 position of I(1,4,5)P₃, although these data were based on the comparison of the elution position of products to standards on HPLC and lacked biochemical evidence (10, 11). Therefore, we identified these IP₄ and IP₅ isomers using enzymatic treatment. To identify the IP₄ isomer, we treated the reaction product with recombinant type I inositol polyphosphate 5-phosphatase (5-ptase) (9). The 5-ptase dephosphorylates I(1,3,4,5)P₄ to I(1,3,4)P₃ but does not dephosphorylate I(1,4,5,6)P₄. Upon 5-ptase treatment, the IP₄ was completely dephosphorylated to I(1,3,4)P₃ (Fig. 1C). These data show that the IP₄ intermediate is I(1,3,4,5)P₄. Next, we attempted to identify the IP₅ isomer generated by rIpk2. To do this, we treated the reaction products with AtIpk1, the Arabidopsis thaliana ortholog of I(1,3,4,5,6)P₅ 2-kinase (the gift of Dr. Stevenson-Paulik, York lab). The AtIpk1 phosphorylates the D2 position of I(1,3,4,5,6)P₅ to produce IP₆. Upon AtIpk1 treatment, the IP₅ was completely phosphorylated to IP₆ (Fig. 1D). These data demonstrate that the IP₅ isomer is I(1,3,4,5,6)P₅. Taken together, the rlpk2 phosphorylates I(1,4,5)P₃ to I(1,3,4,5,6)P₅ through I(1,3,4,5)P₄ in vitro. This activity differs from yeast Ipk2, which prefers to phosphorylate the D6 position of I(1,4,5)P₃ and then the D3 position to produce I(1,3,4,5,6)P₅ (6, 7).

To produce the kinase negative mutant of rIPK2, we changed Asp127 to Ala (rIPK2 D127A) by site-directed mutagenesis, which corresponds to the previously described D131A mutant of ScIpk2 (Scipk2 kin⁻) (6). The Scipk2 kin⁻ is completely kinase inactive but the rlpk2 D127A mutant retains roughly 1% of the wild type kinase activity towards I(1,4,5)P₃ (data not shown). We also tested the substrate specificity of rlpk2 using different inositol polyphosphates,
because it is known that the rat and human orthologs are able to phosphorylate several different inositol polyphosphate isomers (10-12). Among the substrates we used, 100 ng of rIpk2 phosphorylated I(1,4,5)P$_3$, I(1,3,4,5)P$_4$, I(1,3,4,6)P$_4$ and I(1,4,5,6)P$_4$ to I(1,3,4,5,6)P$_5$, but not inositol, I(1)P, I(1,3,4)P$_3$ and I(1,3,4,5,6)P$_5$. 100 ng of rIpk2 partially phosphorylated I(1,4)P$_2$ to an unidentified IP$_3$ (data not shown).

Next, we characterized the kinase activity and substrate specificity of rIpk1 in vitro. 100 ng of rIpk1 only phosphorylated I(1,3,4,5,6)P$_5$ to IP$_6$ (Fig. 1F) and no other inositol polyphosphates we tested (data not shown). These data are consistent with the recently reported characterization of human Ipk1 (13). Fig. 1E shows the elution of substrate the [³H]I(1,3,4,5,6)P$_5$.

Complementation of inositol polyphosphate production in mutant yeast cells

We tested if rIPK2 could rescue inositol polyphosphate synthesis in yeast cells lacking endogenous IPK2. In this experiment, the wild type (wt), ipk2 null (ipk2Δ), ipk2Δ strain expressing either rIPK2 or rIPK2 D127A mutant were metabolically labeled with [³H]inositol, and then the soluble inositol polyphosphates were analyzed by HPLC. In the wt strain, IP$_6$ was the major inositol polyphosphate (Fig. 2A). In the ipk2Δ strain, IP$_3$ accumulates because Ipk2 is the major kinase responsible for the conversion of IP$_3$ to produce higher inositol polyphosphates, such as IP$_5$ and IP$_6$ (Fig. 2B) (6, 7). The ipk2Δ strain harboring the rIPK2 expression plasmid complemented IP$_5$ and IP$_6$ production (Fig. 2C). These data confirm that rIpk2 is active in yeast and is capable of converting I(1,4,5)P$_3$ to I(1,3,4,5,6)P$_5$. Expression of the rIpk2 D127A mutant partially rescued IP$_6$ synthesis (Fig. 2D), consistent with our biochemical studies indicating that this mutation in the rat enzyme still possesses weak activity.
We next tested if rIPK1 could rescue IP₆ synthesis in mutant yeast cells. The yeast strain lacking IPK₁ (ipk1Δ) accumulates IP₅ and a pyrophosphorylated inositol polyphosphate, PP-IP₄ (Fig. 2E) (5). Overexpression of rIpk1 complemented this phenotype (Fig. 2F) indicating that the rIpk1 ortholog is also active in yeast.

Expression and intracellular localization of inositol polyphosphate kinase(s) in Rat-1 cells

After confirmation of the enzymatic activity of rIpk2 and rIpk1 in vitro and in budding yeast, we expressed these genes in rat cells. An inducible mammalian expression system (Tet-On™ gene expression system – Clontech) was chosen thereby enabling the control of protein expression by the tetracycline or tetracycline analogue, doxycycline (34). We expressed control GFP, yeast or rat IPK2, and yeast or rat IPK1 as GFP fusion proteins under the control of doxycycline in the Rat-1, rat embryonic fibroblast cells. Appropriate protein expression was confirmed using an anti-GFP immunoblot (data not shown). In the stable cell lines used, in the absence of doxycycline, there was some leaky expression of protein, but 2 µg/ml doxycycline induced expression levels of all of the fusion proteins (data not shown).

We investigated the intracellular localization of these proteins. The expression of the GFP-fusion proteins was induced by 2 µg/ml doxycycline, and living cells were observed under confocal microscopy. In contrast to the ubiquitous localization of GFP alone (Fig. 3A), GFP-rIpk2 was localized in the nucleus (Fig. 3C). To our knowledge, there is no other report describing rIpk2 localization, but it has been reported that the human Ipk2 localizes to the nucleus, and the nuclear localization signal (NLS) sequence was identified (11). This sequence is conserved between human and rat Ipk2, so this NLS sequence is likely also functional in rIpk2. It is reported that yeast Ipk2 is localized to the nucleus in yeast cells (6), but when we expressed
yeast Ipk2 in Rat-1 cells, it localized to the cytosol (Fig. 3B). This difference probably reflects the difference of nuclear localization mechanisms between yeast and Rat-1 cells. Yeast Ipk1 is localized to the nuclear envelope in yeast cells (5), but both yeast and rat Ipk1 were localized ubiquitously in Rat-1 cells (Fig. 3D and E).

**Inositol polyphosphate profiles in Ipk2 or Ipk1 overexpressing Rat-1 cells**

To address whether the overexpression of these genes effects inositol polyphosphate production, we labeled these cells using [3H]inositol, and then analyzed the soluble inositol polyphosphates by HPLC. In the untransfected (Fig. 4A) or GFP-expressing Rat-1 cells (Fig. 4B), IP₅ was the major inositol polyphosphate and it was about 16-18 % of the total inositol polyphosphates. IP₆ was about 7-8 % of total inositol polyphosphates in these cells. Both the yeast (Fig. 4C) and rat Ipk2 overexpression (Fig. 4E) increased IP₅ levels about 2-3 fold compared to control cells. Overexpression of ScIpk2 kin⁻ (Fig. 4D) did not show any increase of IP₅ levels but the rlIpk2 D127A mutant (Fig. 4F) expressing Rat-1 cells showed a subtle but consistent increase in IP₅ content compared with control cells. Our data indicate that increased Ipk2 activity, nuclear in the case of rIpk2 or cytoplasmic in the case of scIpk2, is sufficient to elevate IP₅ levels.

To confirm that the IP₅ isomer that accumulates in the Ipk2 overexpressing Rat-1 cells is indeed I(1,3,4,5,6)P₅, we prepared soluble radiolabeled inositol polyphosphate extracts from these cell lines and subjected the IPs to enzyme analysis. As mentioned above, AtIpk1 can phosphorylate I(1,3,4,5,6)P₅ to IP₆, but not other IP₅ species (data not shown). The IP₅ peak in the extract of cells expressing either GFP control or GFP-rIPK2 was completely phosphorylated to IP₆ by AtIpk1 *in vitro* (data not shown). These data show that rIpk2 synthesizes I(1,3,4,5,6)P₅ in
Rat-1 cells and also shows that the major IP₅ isomer in the Rat-1 cells is I(1,3,4,5,6)P₅ which is consistent with previous reports (35).

Next, we investigated the effect of Ipk1 overexpression on inositol polyphosphate production in the Rat-1 cells. Both the yeast and rat Ipk1 overexpression increased IP₆ level to about 20% of total inositol polyphosphates (Fig. 4G and H). This was about a 2.5-fold elevation of IP₆ levels as compared to control cells. Interestingly, the I(1,3,4,5,6)P₅ levels were decreased to undetectable levels in these cells, indicating that levels of endogenous rIpkl are highly regulated and/or rate determining.

Since all of the I(1,3,4,5,6)P₅ was converted to IP₆ when the cells were overexpressing Ipk1, and since I(1,3,4,5,6)P₅ was increased with Ipk2 overexpression, we attempted to generate high levels of IP₆ in Rat-1 cells by overexpressing Ipk2 and Ipk1 simultaneously. Figures 5A and C are HPLC profiles from Rat-1 cells expressing only myc-tagged rIpkl under the control of the tetracycline inducible system. In the absence of doxycycline, the HPLC profile was similar to control cells (Fig. 5A). The presence of doxycycline induced Ipk1 expression and the I(1,3,4,5,6)P₅ peak shifted to IP₆ (Fig. 5C). Figures 5B and D are HPLC profiles from Rat-1 cells which co-expressed GFP-tagged rIpkl2 with myc-tagged rIpkl1. Because the expression of GFP-rIpkl2 in these cells was not under the control of the tetracycline inducible system, I(1,3,4,5,6)P₅ was increased in the absence of doxycycline (Fig. 5B). With the doxycycline addition, we observed a substantial elevation in IP₆ (Fig. 5D). Taken together, these data suggest that Ipk2 and Ipk1 activities are key-determining steps in the production of IP₅ and IP₆ in Rat-1 cells.
Down regulation of rat Ipk2 using vector-based siRNA

The data we have shown so far are from overexpression of genes, that is to say, the effects of the gain of function. Another way of analyzing the function of gene products is by eliminating their expression to see the effects of the loss of function. To do this, we applied RNA interference (RNAi) (32, 36, 37). To avoid experiment-to-experiment variability due to different transfection efficiencies with the in vitro synthesized siRNAs, we used a vector-based siRNA expression system (32) to knock down the rIpk2 expression. Figure 6A shows the northern blotting data using the entire rIPK2 ORF as a probe. Cells expressing siRNA against rIPK2 had a 90% decrease of rIPK2 mRNA compared to cells expressing the vector control (Fig. 6A). Control actin amounts were not changed. To determine whether the knockdown of rIpk2 effected inositol polyphosphate production, we labeled these cells using [3H]inositol, and the soluble inositol polyphosphates were isolated and analyzed by HPLC. The cells expressing siRNA against rIPK2 had decreased I(1,3,4,5,6)P5 levels to about 20-25% of the control cells (Fig. 6B and C). The rIPK2 siRNA did not effect IP6 levels.

Overexpression of Ipk2 or Ipk1 activities is not sufficient to induce anchorage-independent growth of Rat-1 cells

Several studies have indicated that transformation of Rat-1 cells with the oncogene v-Src results in activation of inositol trisphosphate kinase activity and elevation of IP4 levels (38-40). We therefore tested whether or not gain of function of either Ipk2 or Ipk1 activities in Rat-1 cells enabled anchorage-independent growth (Fig. 7). Overexpression of either scIpk2 or rIpk2 increases IP3 and IP5 levels in cells; however soft agar analysis demonstrates these modified cells are not able to grow (Fig. 7B and 7C). In contrast v-Src transformed Rat-1 cells are able to grow.
Inositol Polyphosphate Synthesis in Rat-1 Cells

in soft agar (Fig. 7F). We next examined Rat-1 cells stably overexpressing either sclpk1 or rlpk1 in soft agar assays (Fig. 7D and 7E). Despite the elevated levels of IP₆ neither cell line was able to divide in an anchorage-independent manner.
DISCUSSION

There are several reports that have described functions for the highly phosphorylated inositol polyphosphates such as IP$_5$ and IP$_6$ in eukaryotic cells (1-3). However, the molecular route of their synthesis has remained unclear in higher eukaryotic cells. In order to elucidate the molecular basis for synthesis of these important messengers we established cell lines in which whose inositol polyphosphate levels were altered through overexpression or RNAi of inositol polyphosphate kinases. We find that rat Ipk2 and rat Ipk1 play a critical role in the synthesis of IP$_5$ and IP$_6$. Our data suggest that rIpk2 and rIpk1 activities are rate-determining steps in rat IP$_5$ and IP$_6$ production and appear highly regulated. Importantly, we demonstrate, both in vitro and in vivo, that rIpk2 and rIpk1 activities are sufficient to convert I(1,4,5)P$_3$ to IP$_6$, thus are the minimal two kinases required for IP$_6$ synthesis in rat cells. This observation is consistent with what we have reported in budding yeast, plants and flies.

Our data indicate that absolute levels of IP$_6$ in Rat-1 cells are tightly controlled, such that it appears that feedback mechanisms sense and control its levels. The simultaneous overexpression of rIpk2 and rIpk1, where the level of IP$_6$ appears to reach an upper threshold at which point it may be degraded support this hypothesis. For instance, the IP$_6$ levels in the Ipk2 and Ipk1 overexpressing cells did not proportionally elevate the levels of IP$_6$ as compared to Ipk1 overexpressing cells. In addition, decreasing cellular I(1,3,4,5,6)P$_5$ levels using siRNA against rIpk2 significantly altered the ratio of IP$_5$ to IP$_6$. This indicates that rIpk1 activity has been upregulated in order to compensate for IP$_6$ loss.

The substrate specificity of mammalian orthologs of Ipk2 is variable according to recent reports (10-12). Our data show that rIpk2 phosphorylates I(1,4,5)P$_3$ to I(1,3,4,5,6)P$_5$ through I(1,3,4,5)P$_4$ in vitro. Chang et al. reported that the human Ipk2 ortholog acts primarily as a
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I(1,3,4,6)P₄ 5-kinase using kinetics studies and yeast complementation assay, which showed that the human ortholog did not complement inositol polyphosphate production in ipk2Δ yeast (12). We find that rIpk2 is able to complement IP₅ and IP₆ production in ipk2Δ yeast demonstrating its ability to convert I(1,4,5)P₃ to I(1,3,4,5,6)P₅. The different specificities may arise from slight sequence variation as rIpk2 is 83.6% identical to human Ipk2 (12). Reports of two other groups have indicated that human Ipk2 is capable of converting I(1,4,5)P₃, although these studies only examined activity \textit{in vitro} and did not provide proof in cells (10, 11). It is also noteworthy that the IP₄ intermediate of I(1,4,5)P₃ to I(1,3,4,5,6)P₅ phosphorylation by rIpk2 was different from that of budding yeast, plant and fly Ipk2, all of which prefer to first phosphorylate the D6 position of I(1,4,5)P₃ and then to phosphorylate the D3 position to produce I(1,3,4,5,6)P₅ (6, 7, 29, 41). Moreover, the rIpk2 D127A mutant, which corresponds to the Scipk2 kin- (D131A) mutant (6), still showed weak but significant activity towards I(1,4,5)P₃ both \textit{in vitro} and \textit{in vivo}. Thus it appears that subtle changes in amino acids in the active site allows for significant alterations in substrate recognition between rat and yeast Ipk2. This feature will likely be highly useful for future studies aimed at designing substrate selective Ipk2 mutants.

Are there multiple pathways in place that enable IP₆ synthesis in mammalian cells?

Given the importance of these IP regulators, it is likely that alternate pathways may exist. Of interest, among the pathways proposed (see Fig. 8) the last two steps require Ipk2 and Ipk1 activities. In mammalian cells, one proposed alternate route involves two additional kinases and one phosphatase: 1) an IP₃ 3-kinase (which based on sequence similarity is a more recently evolved member of the Ipk2 superfamily); 2) an I(1,3,4)P₅ 5/6-kinase which generates either I(1,3,4,5)P₄ or I(1,3,4,6)P₄; and 3) an I(1,3,4,5)P₄ 5-phosphatase. Whether or not this route plays a role in IP₅ and IP₆ synthesis in Rat-1 cells is not known. Future studies of the rat I(1,3,4)P₅ 5/6-
kinase will be important for determining this in Rat-1 cells. Although, we clearly demonstrate in this study that rIpk2 is capable of bypassing these alternate three steps and directly phosphorylates I(1,4,5)P$_3$ to I(1,3,4,5,6)P$_5$ via I(1,3,4,5)P$_4$ in yeast and Rat-1 cells. However, since our RNAi studies indicate that loss of rIpk2 diminishes but does not eliminate IP production, it is possible that the alternate pathway is contributing in part to IP$_6$ production. It is also possible since the RNAi only partially knocked down rIpk2 that this accounts for the residual IP$_6$ synthesis. It is also tantalizing to speculate that the other reported roles of the I(1,3,4)P$_4$ 5/6-kinase, as a regulator of protein phosphorylation (42, 43) and I(3,4,5,6)P$_5$ metabolism (44), may function to control IP metabolism independent of I(1,3,4)P$_3$ phosphorylation. Given that rIpk2 functions as an I(1,4,5)P$_3$ 3-kinase in vivo, it remains an important question to determine the functional redundancy or difference between Ipk2 and IP$_3$ 3-kinases, which are only capable of phosphorylating the D3 position of I(1,4,5)P$_3$ (2). Do these two enzymes compete with each other for substrate or are they compartmentalized to access different pools of substrate in the cells? Three molecular studies that indicate that IP$_3$ 3-kinases are not involved in IP$_5$ and IP$_6$ synthesis: 1) Balla et al. suggested that overexpression of IP$_3$ 3-kinase did not increase higher inositol polyphosphates such as IP$_5$ and IP$_6$ in NIH 3T3 cells (45); 2) IP$_3$ 3-kinase mouse knockout cells reveal that IP$_5$ and IP$_6$ production is unaltered (46-48); and 3) in drosophila melanogaster loss of IP$_3$ 3-kinase isoforms does not decrease IP$_6$ levels but loss of dmIpk2 nearly ablate IP$_5$ and IP$_6$ (41).

Several reports have suggested the involvement of highly phosphorylated inositol polyphosphate production in cell proliferation and cell transformation (51-54). We could not detect a significant difference of proliferation rate and colony formation ability among control and our inositol polyphosphate kinase overexpression and Ipk2 knock down Rat-1 cells (not
shown). Thus it appears 2.5-fold elevation or 80% reduction of IP$_3$ does not sufficiently alter growth. It is likely that the 20% remaining activity in the RNAi knockdown cells is sufficient for survival, thus examination of Ipk2 or Ipk1 complete knockouts will be important future studies. Additionally, investigating the relationship between the production of highly phosphorylated inositol polyphosphates and Ipk2 and/or Ipk1 activity during the cell cycle, cell differentiation, proliferation and transformation may be equally important.

In conclusion, we have established an in vivo model system to modulate the inositol polyphosphate levels in this study. Importantly, we find that in Rat-1 cells, rIpk2 and rIpk1 are the minimal kinase activities required to synthesize IP$_6$ from I(1,4,5)P$_3$ via I(1,3,4,5)P$_4$ and I(1,3,4,5,6)P$_5$ intermediates. This now confirms that in S. cerevisiae (5, 6), Arabidopsis (29), drosophila melanogaster (41) and now rattus norvegicus these two kinases are functionally important. The ability to modulate the intracellular inositol polyphosphate levels will provide powerful tools to study the roles of I(1,3,4,5,6)P$_5$ and IP$_6$ in eukaryotic cell signaling.

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FIGURE LEGENDS

**Fig. 1. Kinase assay of rat Ipk2 and rat Ipk1 in vitro.** (A) HPLC trace of substrate, \(^{3}\text{H}]\text{I}(1,4,5)\text{P}_3\). (B) 10 ng of GST-rIpk2 was incubated with 10 µM \(^{3}\text{H}]\text{I}(1,4,5)\text{P}_3\) for 30 min at 37 °C. (C and D) To identify the inositol polyphosphate isomers, GST-rIpk2 phosphorylation products (B) were treated with 100 ng of Type I inositol polyphosphate 5-phophatase, which hydrolyzes \text{I}(1,3,4,5)\text{P}_4 but not \text{I}(1,4,5,6)\text{P}_4 (C) or 100 ng of GST-AtIpk1, which phosphorylates \text{I}(1,3,4,5,6)\text{P}_5 to \text{IP}_6 (D) for 30 min at 37 °C, respectively. (E) HPLC trace of substrate, \(^{3}\text{H}]\text{I}(1,3,4,5,6)\text{P}_5\). (F) 100 ng of GST-rIpk1 was incubated with 10 µM \(^{3}\text{H}]\text{I}(1,3,4,5,6)\text{P}_5\) for 30 min at 37 °C. The substrate and phosphorylation products were separated by HPLC using the Partisphere strong anion exchange column as described under “Experimental Procedures”. The elution position of each inositol polyphosphate isomer is indicated by the arrow.

**Fig. 2. Complementation of IP\(_6\) synthesis by rat IPK2 or rat IPK1 in S. cerevisiae lacking the IPK2 or IPK1 gene, respectively.** *S. cerevisiae* wild type, W303 strain (A), *ipk2* null strain (B), *ipk2* null strain expressing wild type rIPK2 (C) or rIPK2 D127A mutant (D), *ipk1* null strain (E) or *ipk1* null strain expressing wild type rIPK1, were grown to late-logarithmic phase in complete minimal medium lacking uracil with 100 µM CuSO\(_4\) and 40 µCi/ml \(^{3}\text{H}\text{inositol. The soluble inositol polyphosphates were extracted and then analyzed by HPLC using the Partisphere strong anion exchange column as described under “Experimental Procedures”. The elution positions of inositol polyphosphates were compared with the elution of known species and are indicated by the arrows.**
Fig. 3. Intracellular localization of GFP-fused inositol polyphosphate kinase(s) in Rat-1 cells. Rat-1 cells stably expressing pRevTRE/GFP (A), pRevTRE/GFP-ScIPK2 (B), pRevTRE/GFP-rIPK2 (C), pRevTRE/GFP-ScIPK1 (D) or pRevTRE/GFP-rIPK1 (E) were grown under normal growth conditions with 2 µg/ml doxycycline for 2 days to induce protein expression. Cells were washed with pre-warmed Hanks' balanced salt solution (HBSS) and observed by fluorescence microscopy as indicated in the “Experimental Procedures”.

Fig. 4. Inositol polyphosphate profiles of Rat-1 cells expressing inositol polyphosphate kinase(s). Approximately 1 x 10⁵ cells Rat-1 cells stably expressing mock plasmid, pRevTet-On (A), pRevTRE/GFP (B), pRevTRE/GFP-ScIPK2 (C), pRevTRE/GFP-ScIPK2 D131A (kin-) (D), pRevTRE/GFP-rIPK2 (E), pRevTRE/GFP-rIPK2 D127A (F), pRevTRE/GFP-ScIPK1 (G) or pRevTRE/GFP-rIPK1 (H) were seeded into a 60mm culture dish containing 3 ml of culture media. One day after growth, cells were labeled in 10% dialyzed FBS/Medium-199 containing 20 µCi/ml [³H]inositol for 2 days. 2 µg/ml doxycycline was added to the media to induce protein expression for the same period. The soluble inositol polyphosphates were extracted and then analyzed by HPLC using the Partisphere strong anion exchange column as described under “Experimental Procedures”. The elution positions of inositol polyphosphates were compared with the elution of known species and are indicated by the arrows.

Fig. 5. Inositol polyphosphate profiles of Rat-1 cells simultaneously expressing rat Ipk2 and rat Ipk1. Rat-1 cells stably expressing pRevTRE/myc-rIPK1 (A and C) or pRevTRE/myc-rIPK1 and pBabePuro/GFP-rIPK2 (B and D) were labeled in 10% dialyzed FBS/Medium-199 containing 20 µCi/ml [³H]inositol for 2 days with (C and D) or without (A and B) 2 µg/ml
doxycycline for 2 days. The soluble inositol polyphosphates were extracted from equal cell numbers and then analyzed by HPLC using the Partisphere strong anion exchange column as described under “Experimental Procedures”. The elution positions of inositol polyphosphates were compared with the elution of known species and are indicated by the arrows.

**Fig. 6. Northern blotting and inositol polyphosphate profiles of Rat-1 cells expressing vector-based siRNA against rat IPK2.** (A) Northern blotting analysis of the Rat-1 cells expressing the vector control (left) or vector-based rIPK2 siRNA (right). 20 µg of total RNA was separated using a formaldehyde gel, transferred to Nylon membrane and then hybridized with rIPK2 (top) or β-actin (bottom) probe. (B and C) Inositol polyphosphate profiles of Rat-1 cells expressing the vector control (B) or vector-based rIPK2 siRNA (C). The labeling, extraction and analysis were done as described under “Experimental Procedures”. The elution positions of inositol polyphosphates were compared with the elution of known species and are indicated by the arrows.

**Fig. 7. Anchorage-independent growth of Rat-1 cells expressing inositol polyphosphate kinase(s) in soft agar.** Approximately 5 x 10⁴ Rat-1 cells stably expressing pRevTRE/GFP (A), pRevTRE/GFP-ScIPK2 (B), pRevTRE/GFP-rIPK2 (C), pRevTRE/GFP-ScIPK1 (D) pRevTRE/GFP-rIPK1 (E) or v-src (F) were seeded into a 35 mm culture dish containing 1 ml of culture media with 0.4 % agar. The dishes had been coated with 2 ml of culture media containing 0.7 % agar. Colony formation was scored after 3 weeks at 37 °C in a humidified atmosphere of 5% CO₂.
Fig. 8. Molecular basis for inositol polyphosphate synthesis in Rat-1 cells. Phospholipase C activation results in the conversion of phosphatidylinositol 4,5-bisphosphate, PI(4,5)P₂, to I(1,4,5)P₃. In *S. cerevisiae*, *Arabidopsis* and *Drosophila melanogaster*, I(1,4,5)P₃ is phosphorylated predominately first on the D-6 position to generate I(1,4,5,6)P₄ and then the D-3 position to generate I(1,3,4,5,6)P₅ by Ipk2 (designated ScIpk2 for the original characterization in *S. cerevisiae*). Our studies of rat Ipk2 indicate that it prefers to convert I(1,4,5)P₃ to I(1,3,4,5,6)P₅ via an I(1,3,4,5)P₄ intermediate. IP₃ 3-kinase, a recently evolved relative Ipk2, also generates I(1,3,4,5)P₄ as its sole product. However, given data presented in this manuscript, previous published work in *drosophila melanogaster* (Seeds et al *JBC* 2004) and mouse knockout data of others, it does not appear that IP₃ 3K contributes to the direct synthesis of IP₃ and IP₆ in flies, rat or mouse cells. Similar to yeast, plant and flies, the rat Ipk1 acts as a 2-kinase to convert I(1,3,4,5,6)P₅ to IP₆. Finally, our work does not exclude the involvement of I(1,3,4,5)P₄ 5-phosphatase and I(1,3,4)P₃ 5/6 kinase activities in IP₆ production in mammals; however it does demonstrate that rIpk2 and rIpk1 activities are sufficient to convert I(1,4,5)P₃ to IP₆ in cells.
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Inositol Polyphosphate Synthesis in Rat-1 Cells

Figure 1.
Figure 2.
Figure 3.
Figure 4.

![Graph showing Inositol Polyphosphate Synthesis in Rat-1 Cells](image-url)
Figure 5.

[Diagram showing the synthesis of inositol polyphosphates (IPn) over time (min) with [3H] relative counts.]

A. IP_3, IP_4, IP_5, IP_6
B. IP_3, IP_4, IP_5, IP_6
C. IP_3, IP_4, IP_5, IP_6
D. IP_3, IP_4, IP_5, IP_6
Figure 6.
Figure 7.
Figure 8.
A role for rat inositol polyphosphate kinases, rIpk2 and rIpk1, in inositol pentakisphosphate and inositol hexakisphosphate production in Rat-1 cells
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