Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases

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Inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3) is a well-known messenger molecule that releases calcium from intracellular stores. Homologues with up to six phosphates have been characterized and recently, homologues with seven or eight phosphate groups, including pyrophosphates, have been identified. These homologues are diphosphoinositol pentakisphosphate (PP-InsP$_5$/InsP$_7$) and bis(diphospho)inositol tetrakisphosphate (bis-PP-InsP$_4$/InsP$_8$) [1], the rapid turnover of which [2] is regulated by calcium [2] and adrenergic receptor activity [3]. It has been proposed that the high-energy pyrophosphates might participate in protein phosphorylation [4]. We have purified InsP$_6$ kinase [5] and PP-InsP$_5$ kinase [6], both of which display ATP synthase activity, transferring phosphate to ADP. Here, we report the cloning of two mammalian InsP$_6$ kinases and a yeast InsP$_6$ kinase. Furthermore, we show that the yeast protein, ArgRIII, is an inositol-polyphosphate kinase that can convert InsP$_3$ to InsP$_4$, InsP$_5$ and InsP$_6$. We have identified a new family of highly conserved inositol-polyphosphate kinases that contain a newly identified, unique consensus sequence.

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Results and discussion

Peptide mass fingerprinting [7,8] has shown that purified InsP$_6$ kinase is the rat counterpart of the uncharacterized human KIAA0263 gene product [9] (Figure 1). By screening a mouse EST database with this information, matching sequences for probes to clone mouse InsP$_6$ kinase were obtained. Mouse InsP$_6$ kinase contains 433 amino acids and encodes a 50 kDa protein, which corresponds in size to the 54 kDa purified rat InsP$_6$ kinase [5]. The 97% amino acid homology between mouse InsP$_6$ kinase and

KIAA0263 indicates that the latter is human InsP$_6$ kinase. An insert of eight amino acids in the human protein might reflect alternative splicing.

Our database screen also revealed PiUS, so called because it stimulates the uptake of inorganic phosphate but lacks transporter features [10]. We cloned human PiUS, which displays 98% homology to the rabbit sequence and 48% and 67% amino-acid sequence identity and similarity, respectively, to mouse InsP$_6$ kinase (Figure 1). The sequences of InsP$_6$ kinase and PiUS are unique except for a conserved 25 amino-acid sequence that is also evident in InsP$_3$ kinases A and B [11–13]. A lysine residue, at position 262 in rat InsP$_3$ kinase A, that is essential for InsP$_3$ binding to the enzyme [14] is contained within the conserved sequence. This sequence, which also appears in two yeast proteins, KCS1 [15] and ArgRIII [16,17] (Figure 2), could therefore be a consensus sequence for inositol-polyphosphate kinases.

HEK293 cells were transfected with fusion proteins comprising glutathione S-transferase (GST) and InsP$_6$ kinase or PiUS. Western blotting of the purified proteins revealed molecular weights (MWs) that reflected the combination of the 26 kDa GST, together with the 50 kDa InsP$_6$ kinase or the 49 kDa PiUS (Figure 3). The transfected InsP$_6$ kinase and PiUS both displayed robust InsP$_6$ kinase activity, converting 60–80% of added [^{3}H]InsP$_6$ to PP-InsP$_5$, with no conversion evident by boiled enzyme (Figure 3). For InsP$_6$ kinase, K_m values for InsP$_6$ and ATP were 0.6 μM and 1.1 mM, respectively. The V_{max} was 0.76 μmol/minute/mg protein, thus resembling values for the purified enzyme [5]. For PiUS, K_m values for InsP$_6$ and ATP were 3.0 μM and 1.0 mM, respectively, and the V_{max} was 2.0 μmol/minute/mg. Both InsP$_6$ kinase and PiUS displayed no catalytic activity with [^{3}H]Ins(1,4)P$_2$, [^{3}H]Ins(1,4,5)P$_3$, [^{3}H]Ins(1,3,4,5)P$_4$ or [^{3}H]PP-InsP$_5$ (data not shown), thus demonstrating selective InsP$_6$ kinase activity. We therefore designated PiUS as InsP$_6$ kinase 2 and the InsP$_6$ kinase we originally purified as InsP$_6$ kinase 1.

KCS1 is a yeast protein, the deletion of which rescues the abnormality in recombination caused by mutating protein kinase C [15,18]. GST–KCS1, when transfected into HEK293 cells, had a MW of 150 kDa, reflecting the combination of the 119 kDa KCS1 and the 26 kDa GST (Figure 3). KCS1 stoichiometrically converted 50 pmoles of [^{3}H]InsP$_6$ to PP-InsP$_5$ (Figure 3) but was inactive with [^{3}H]Ins(1,4)P$_2$, [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]Ins(1,3,4,5)P$_4$

Figure 1
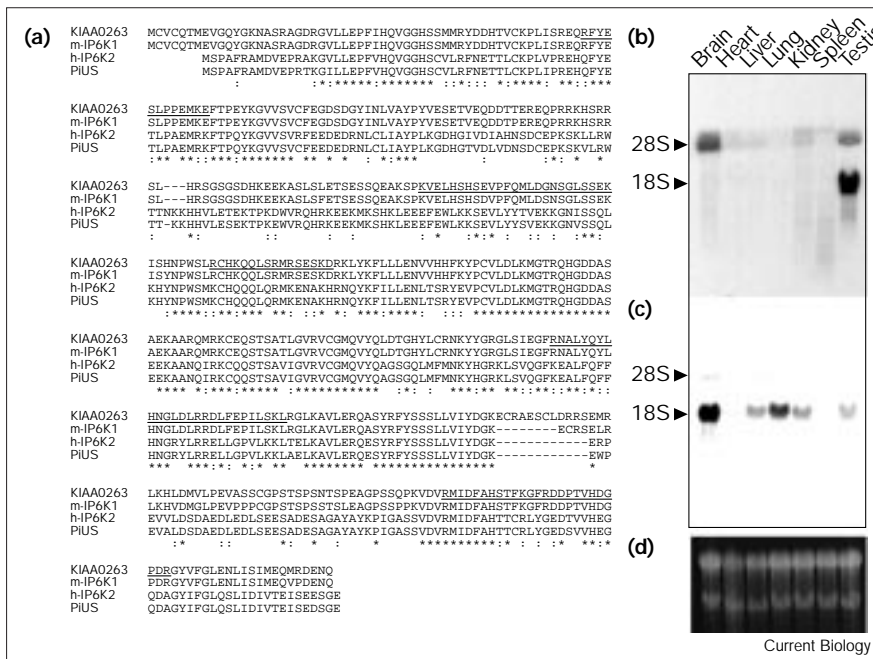
Sequence alignments and northern blot analysis for InsP$_6$ kinases 1 and 2. **(a)** CLUSTAL-W [23] alignment of mouse InsP$_6$ kinase 1 (m-IP6K1), human InsP$_6$ kinase 2 (h-IP6K2; human PiUS), human KIAA0263 and rabbit PiUS amino-acid sequences. The open reading frame (ORF) for mouse InsP$_6$ kinase 1 was cloned by reverse-transcription PCR from mouse brain mRNA. The cDNA for human InsP$_6$ kinase 2 was identified by screening a human EST data bank with the rabbit sequence for PiUS (Image clone 1610377; Genome Systems). Asterisks indicate identical amino acids; colons indicate conservative amino acid changes. Peptide regions identified by MALDI-TOF mass spectrometry [7,8] of peptides (nine in total) from purified rat brain InsP$_6$ kinase are underlined. GenBank accession numbers for mouse InsP$_6$ kinase 1 and human InsP$_6$ kinase 2 are AF177144 and AF177145, respectively. **(b,c)** Northern blot analysis of InsP$_6$ kinases 1 and 2. Total RNA (40 µg) from different mouse tissues, as indicated, was separated on a 1% agarose, 2.3 M formaldehyde, 40 mM MOPS gel. After protein transfer, the blot was hybridized with (b) a probe comprising the ORF for InsP$_6$ kinase1, and subsequently (c) with a probe comprising the ORF for InsP$_6$ kinase 2. **(d)** The gel was stained with ethidium bromide to check for equal loading.

(data not shown). We therefore designated KCS1 as yeast InsP$_6$ kinase (yInsP$_6$ kinase).

GST–ArgIII, when transfected into HEK293 cells, had a MW of 66 kDa, reflecting the combination of the 40 kDa

Figure 2

Alignment of the inositol-phosphate-binding motif of the different inositol polyphosphate kinases. Identical amino acids are shown in bold. The GenBank accession numbers of the different sequences are: rat InsP$_3$ kinase A, GI:124808; rat InsP$_3$ kinase B, GI:1170577; *Saccharomyces cerevisae* yInsP$_6$ kinase, GI:1078508; and IPMK (inositol polyphosphate multikinase), GI:114134. Numbers to the right and left of the sequences indicate their positions in the respective complete amino-acid sequences. The consensus sequence is written in Prosite format, where X represents any amino acid.

ArgIII and the 26 kDa GST (Figure 4a). It robustly phosphorylated Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ and weakly phosphorylated InsP$_6$. Incubation of [³H]InsP$_3$ with the purified enzyme led to the formation of a series of more polar higher inositol polyphosphates, one of which comigrated with authentic InsP$_6$. We therefore designated ArgIII as inositol polyphosphate multikinase (IPMK; Figure 4b–d).

Northern blot analysis in mice revealed that InsP$_6$ kinase 1 is highly expressed in brain and testis as a 5 kb transcript, whereas a 2 kb transcript occurs in testis, but is only faintly expressed in heart, kidney, liver, lung and spleen. InsP$_6$ kinase 2 was expressed most highly in brain as a 1.9 kb transcript, with robust levels in lung and lower values in liver, kidney and testis (Figure 1b–d), extending earlier reports of PiUS in kidney, intestine and liver [10].

We have described a newly identified family of inositol polyphosphate kinases with a unique consensus sequence. The consensus sequence we have described for InsP$_6$ kinases 1 and 2, yInsP$_6$ kinase and IPMK, and shared with InsP$_3$ kinases A and B, should help clarify the structure and function of inositol-phosphate kinases. Three of the kinases are selective InsP$_6$ kinases whereas IPMK, formerly designated ArgIII, is a multifunctional kinase that phosphorylates InsP$_3$ and InsP$_4$ and can presumably

Figure 3

Expression and enzymatic activity of recombinant GST fusion proteins containing InsP$_6$ kinase 1, InsP$_6$ kinase 2 (PiUS) or yInsP$_6$ kinase (KCS1). The fusion proteins were transfected into HEK293T cells and then purified using a glutathione resin. **(a)** A silver-stained 12% PAGE–SDS gel of the purified mammalian proteins. InsP$_6$ kinase activity was monitored using 10 ng of recombinant protein as described previously [5], using [^{3}H]InsP$_6$ as substrate and separating it from [^{3}H]PP-InsP$_5$ by thin-layer chromatography [24]. Both **(b)** InsP$_6$ kinase 1 and **(c)** InsP$_6$ kinase 2 displayed a time-dependent formation of PP-InsP$_5$. **(d)** A silver-stained 10% PAGE–SDS gel of purified GST–yInsP$_6$ kinase proteins. **(e)** The enzymatic activity of recombinant yInsP$_6$ kinase proteins was assayed at 37°C for 30 min. GST alone had no enzymatic activity. The data are the mean ± standard error (SEM) of three independent experiments.

convert InsP$_3$ to InsP$_6$. IPMK is probably identical to a purified, but uncloned, yeast enzyme with a similar substrate specificity and a similar MW [19,20]. IPMK has very little sequence similarity to InsP$_3$ kinases A and B, which act selectively on InsP$_3$. A kinase that adds phosphate at position 5 or 6 to Ins(1,3,4)P$_3$ but not to Ins(1,4,5)P$_3$ shows no homology to the proteins described here [21]. A yeast nuclear enzyme that converts InsP$_5$ to InsP$_6$, designated Ipk1p (*Saccharomyces cerevisae* gene YDR315c), also has no sequence homology to the family described here [22].
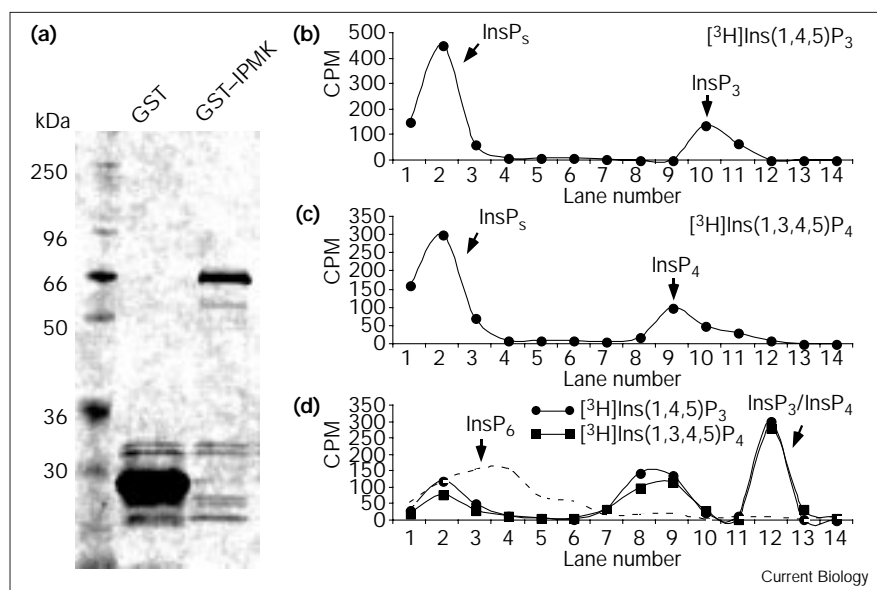
What might be the function of the PP-InsP$_5$ formed by InsP$_6$ kinases? Acting in the reverse direction, InsP$_6$ kinase 1 [5] and PP-InsP$_5$ kinase [6] are ATP synthases and so might act as energy reserves in selected intracellular sites, in a manner analogous to that of creatine phosphate. We
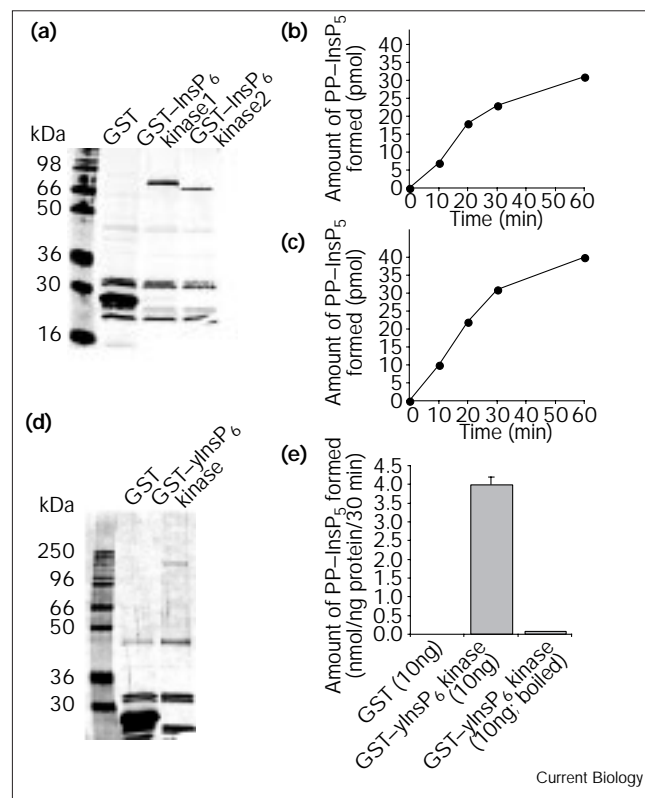
have obtained evidence that PP-InsP$_5$ directly phosphorylates selected proteins via a kinase distinct from InsP$_6$ kinase or PP-InsP$_5$ kinase and involves a small group of proteins unlike those phosphorylated by ATP [4]. It is conceivable that the inositol pyrophosphates are phosphate donors to discrete populations of proteins.

Figure 4

Expression and enzymatic activity of recombinant GST–IPMK (ArgRIII). GST–IPMK was transfected into HEK293T cells and purified using glutathione resin. **(a)** A silver-stained 12% PAGE–SDS gel of purified proteins. **(b–d)** The enzymatic activity of recombinant protein (10 ng) was assayed by incubation at 37°C for 30 min with different substrates. The reaction mixture was spotted onto a PEI-TLC plate and developed in 0.7 M HCl. The lanes were cut into 1 cm strips, and radioactivity was determined. (b) Reaction in the presence of 0.1 μM [^{3}H]Ins(1,4,5)P$_3$. (c) Reaction in the presence of 0.1 μM [^{3}H]Ins(1,3,4,5)P$_4$. (d) To properly separate higher inositol polyphosphate products (InsP$_s$), the reaction was developed in 1.0 M HCl using [^{3}H]Ins(1,4,5)P$_3$ (filled circles) or [^{3}H]Ins(1,3,4,5)P$_4$ (filled squares) as substrate. Dashed lines represent the migration of [^{3}H]InsP$_6$, which was used as standard. No activity was observed using GST alone. The data are representative of three similar experiments.

Materials and methods

Acknowledgements

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Supplementary material

Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases

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Supplementary materials and methods

Materials

$[^3H]Ins(1,4)P_2$, $[^3H]Ins(1,4,5)P_3$, $[^3H]Ins(1,3,4,5)P_4$, $[^3H]InsP_6$ and $[^3H]PP\text{-}InsP_5$ were supplied by NEN Life Sciences. Polyethyleneimine (PEI)–cellulose TLC plates were obtained from J.T. Baker. $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$ and $InsP_6$ were purchased from Calbiochem. Phospho-creatine, creatine kinase (EC 2.7.3.2), and all other reagents were obtained from Sigma.

Protein purification and characterization

Purification of $InsP_6$ kinase 1 was performed as previously described [S1]. Purified protein, obtained from about 500 rat brains, was loaded onto a 10% polyacrylamide-SDS (SDS-PAGE) gel. The protein was transferred on Protran BA 85 (Schleicher & Schuell) nitrocellulose paper at a constant voltage of 30 V. After Ponceau S staining, the protein band was cut out and subjected to *in situ* tryptic digestion as described [S2,S3]. The resulting peptide mixture was loaded onto a 2 µl bed volume of Poros 50 R2 (PerSeptive Biosystems) reverse-phase beads (packed into an Eppendorf gel-loading tip) and stepwise eluted with 4 µl of 16% (and then with 4 µl 30%) acetonitrile and 0.1% formic acid. The '16%' and '30%' peptide pools were each analyzed twice by matrix-assisted laser-desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), in the presence and absence of peptide calibrants [S3], using a REFLEX III (Bruker-Franzen) instrument equipped with a grid-less pulsed-extraction ion source and a 2 GHz digitizer, and operated in reflectron mode. Spectra were obtained by averaging multiple signals. After recalibration with internal standards, monoisotopic masses were assigned for the seven most prominent peaks and a peptide mass list generated to search a protein non-redundant database (NRDB; European Bioinformatics Institute) using the PeptideSearch [S4] algorithm with an accuracy requirement of 40 ppm.

Cloning of InsP₆ kinases 1 and 2, yIP₆ kinase and IPMK

The open reading frame (ORF) for mouse $InsP_6$ kinase 1 was cloned by reverse-transcription PCR (RT-PCR) from mouse brain mRNA purified using a FastTrak kit (Invitrogen). We used the sequence of the human gene KIAA0263 to screen a mouse EST database. We employed appropriate matching sequences to design mouse oligonucleotide probes for RT-PCR. The oligonucleotides used were: 5′-GCATGTC-GACAATGTGTGTTTGTCAAACC-3′ and 5′-GCTGCGGCCGCAGG-GCCTACTGGTTCTC-3′; the restriction sites *Sal*I and *Not*I contained within the sequence were used to clone the PCR product in the eukaryotic GST fusion vector pCMV-GST [S5]. cDNA for human IP₆K2 was identified by screening an human EST data bank with the rabbit sequence for PiUS. The human EST, GenBank accession number AA995457, EST Image clone 1610377, was purchased from Genome Systems. The clone was completely sequenced and the ORF for the human IP_6 kinase 2 was subsequently cloned as a GST fusion in a pCMV-GST vector using PCR amplification to create the *Sal*I and *Not*I cloning sites. The oligonucleotides used were: 5′-GCATGTCGAC-GATGAGCCCAGCCTTCAG-3′ and 5′-GCTGCGGCCGCTCACTC-CCCACTGACCTCA-3′. The yeast ORF IPMK (ArgRIII, ORF:YDR173C) and $yInsP_6$ kinase (KCS1, ORF:YDR017C) were cloned as GST fusions in the *Sal*I and *Not*I sites of pCMV-GST vector by genomic PCR, using the following oligonucleotides: 5′-GCATGTCGACCATG-GATACGGTAAACAATTA-3′ and 5′-GCTGCGGCCGCAAGGTAAA-CTTCACCTTCTA-3′ for IPMK, and 5′-GCATGTCGACTATGGATAC-CTCTCACGAA-3′ and 5′-GCTGCGGCCGCTTCTTTTCAATCACTAAC-3′ for $yInsP_6$ kinase. The clones were confirmed by sequence analysis using an Abi prism™ automatic sequencer (Perkin Elmer).

Figure S1

Alignment of the amino acid sequences of mouse $InsP_6$ kinase 1 and rat $InsP_3$ kinase A. The region of homology was identified using a Blast program and is represented in bold. An arrow indicates the Lys262 residue that is essential for $InsP_3$ binding to the rat enzyme.

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m-IP6K1  ----------------MC---VCQT--MEVGQY-GKNASR---AGDRGVLLEPFIH---   31
r-IP3KA  MTLPGHPTGMARPRGAGPCSPGLERAPRRSVGELRLLFEARCAAVAAAAAAGEPRARGAK   60
                      *    : ::    **:     :*           ** :

m-IP6K1  QVGG--HSSMMRYDDHTVCKPL-ISREQRFYESLP-PEMKEFTPEYKGVVSVCFEGDSDG   87
r-IP3KA  RRGGQVPNGLPRAAPAPVIPQLTVTSEEDVAPASPGPPDREGN-WLPAAGSHLQQPRRLS  119
         : **   :*   :  *  ** :: *:   : ** :*         *      :

m-IP6K1  YINLVAYPYVESETVEQDDTPEREQPRRKHSRRSLHRSGSG-SDHKEEKASLSFETSESS  146
r-IP3KA  TSSLSSTGSSSLLEDSEDDLLSDSESRSRGNVQLETSEDVGQKSHWQKIRTMVNLPVMSP  179
            * :       :**    : * :  :        *   * :: ::       *

m-IP6K1  QEAKSPKVELHSHSDVPFQMLDSNSGLSSEKISYNPWSLRCHKQQLS-RMRSESK-----  200
r-IP3KA  FKKRYSWVQLAGHTGS-FKAAG-TSGLILKRSSE-PEHY-CLVRLMADVLRGCVPAFHGV  235
         : :   *:*  *:  *:     ***  :* *  :   :: :*

m-IP6K1  -DRKLYKFLLLENVVHHFKYPCVLDLKMGTRQHGDDASAEKAAR-QMRKCEQSTSATLG-  257
r-IP3KA  VERDGESYLQLQDLLDGFDGPCVLDCKMGVRTYLEEELTKARERPKLRKDMYKKMLAVDP  295
         :*     :* *:::::  *  ***** *** * : :: ::    * ::**       ::

m-IP6K1  -VRVCGMQVYQLDTGHYLCRNKYYGRGLSIEGFRNALYQYLHNGLDLRRDLFEPILSKLR  316
r-IP3KA  EAPTEEEHAQRAVTKPRYMQWREGISSSTTLGFRIEGIKKADGSCSTDFKTTRSREQVTR  355
              :  :   *    : ::       : ***  :         * ::**        *

m-IP6K1  GLKAVLERQAS-YRFYSSSLLVIYDGKECRSELRLKHVDMG--LPEVPPPCGPSTSPSST  373
r-IP3KA  VFEEFMQGDAEVLKRYLNRLQQIRDTLEISDFFR-RHEVIGSSLLFVHDHC-HRAG--VW  411
         ::   :: :*  :  *    *     *   *  * :* :*  :*  *   *     :

m-IP6K1  SLEAGPSSP-PKVDVRMIDFAHSTFKGFRDDPTVHDGPDRGYVFGLENLISIMEQVPDENQ  433
r-IP3KA  LIDFGKTTPLP--DGQILDHRRPWEEGNRED---------GYLLGLDNLIGILANLAER--  459
         :: * ::* *  * *::*   :    :* *:*         **::**:*** *: :: :
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Current Biology

Table S1

Substrate specificity.

	Ins(1,4)P$_2$	Ins(1,4,5)P$_3$	Ins(1,3,4,5)P$_4$	InsP$_6$	PP-InsP$_5$
InsP$_6$ kinase 1	–	–	–	+++	–
InsP$_6$ kinase 2	–	–	–	+++	–
IPMK	–	+++	+++	+/–	ND
yInsP$_6$ kinase	–	–	–	+++	ND

A dashed line indicates no activity; +++ indicates that more than 50% of substrate is converted by 10 ng of recombinant enzyme at 37° in 30 min; +/– indicates less than 5% of substrate is converted at 37°C in 1 h. ND, not determined.

Expression and purification of recombinant enzymes
HEK293T cells, plated on 10 cm dishes and grown in DMEM supplemented with 10% fetal bovine serum, were transfected using the calcium phosphate precipitation method [S6]. Cells were lysed 24 h after transfection in 500 µl of 20 mM Hepes pH 6.8, 2 mM EGTA, 0.75 mM EDTA, 4 mM DTT, 5 mM NaF, 1.5 mM Na$_3$VO$_4$, 0.2% CHAPS, 1 mM phenylmethylsulfonylfluoride, 1 mg/ml leupeptin, 1 mg/ml chymostatin, and 1 mg/ml pepstatin A. Cell lysates were centrifuged for 10 min at 14,000 × *g*. Glutathione sepharose 4B resin (25 µl; Amersham Pharmacia Biotech) was added to 0.5 ml of the lysate supernatant. After a 1 h incubation with slow rotation, the GST fusion protein was recovered by washing five times with PBS and then resuspended in 50 µl of lysis buffer. Purified GST fusion proteins (5 µl) were loaded onto a 12% SDS-PAGE gel and visualized by silver staining. Protein concentration was determined by running a standard curve of BSA and GAPDH on the same gel.

Enzymatic assays
The enzymatic activity of InsP$_6$ kinase was assayed in 10 µl of reaction mixture containing 20 mM HEPES pH 6.8, 1 mM DTT, 6 mM MgCl$_2$, 5 mM Na$_2$ATP, 10 mM phosphocreatine, 40 U/ml creatine phosphokinase, 5 mM NaF, 5 µM InsP$_6$, and 20–60 nM [³H]InsP$_6$ and incubated at 37°C for 10–60 min. Reactions were terminated either by addition of 1 µl 1 M HCl or by immersion in an ice-cold water bath. Kinetic parameter determinations were done under initial rate conditions (< 10% of product formation).

Assay of [³H]InsP$_2$; [³H]InsP$_3$; [³H]InsP$_4$; [³H]PP-InsP$_5$ kinase activities were performed with the different substrates using the same buffer and conditions described for the InsP$_6$ kinase assay. The reactions were

Table S2

Inositol phosphate kinase family.

		Substrate specificity	PxxxDxKxG domain	References
IP$_3$Ks	IP$_3$KA	Ins(1,4,5)P$_3$	Yes	[S9,S10,S11]
	IP$_3$KB	Ins(1,4,5)P$_3$	Yes	[S12]
	IP$_3$(5/6)K	Ins(1,3,4)P$_3$	No	[S13]
	IPMK	Ins(1,4,5)P$_3$	Yes	This work
		Ins(1,3,4,5)P$_4$		
IP$_5$Ks	IpK1p	Ins(1,3,4,5,6)P$_5$	No	[S14]
IP$_6$Ks	IP$_6$K1	InsP$_6$	Yes	This work
	IP$_6$K2	InsP$_6$	Yes	This work
	yIP$_6$K	InsP$_6$	Yes	This work

IP$_3$Ks, InsP$_3$ kinases; IP$_5$Ks, InsP$_5$ kinases; IP$_6$Ks, InsP$_6$ kinases; IP$_3$KA and IP$_3$KB, InsP$_3$ kinases A and B; IP$_3$(5/6)K, InsP$_3$ 5- and 6-kinases; IpK1p; IP$_6$K1 and IP$_6$K2, InsP$_6$ kinases 1 and 2; yIP⁶K, yeast InsP$_6$ kinase.

performed in the presence of 0.1 µM InsP$_2$, InsP$_3$, InsP$_4$ or PP-InsP$_5$. We adapted methods used to separate InsP$_6$, InsP$_5$, InsP$_4$, InsP$_3$, InsP$_2$, ATP, and inorganic phosphate (Pi) using polyethyleneimine–cellulose thin-layer chromatography (PEI-TLC), which separates the different inositol polyphosphates on the basis of their overall polarity [S7]. The entire reaction mixture was spotted in 2.5 µl aliquots onto a PEI-TLC plate, which was developed in 1.5 M HCl to separate InsP$_6$ from PP-InsP$_5$, and developed in 0.5–1.0 M HCl to separate InsP$_2$, InsP$_3$ and InsP$_4$ from higher inositol polyphosphate products. The lanes were cut into 1 cm strips, and counted using 10 ml NEN Life Science Formula 963 scintillation cocktail.

Northern blot analysis
Total RNA from various mouse organs was prepared using LiCl precipitation methods [S8]. RNA (40 µg) was loaded onto a 1% agarose/formaldehyde/MOPS gel and transferred to Hybond™N+ nylon membrane (Amersham Pharmacia Biotech). ORFs for InsP$_6$ kinase 1 and InsP$_6$ kinase 2 were labeled with [α³²P]dCTP using oligo labeling as described [S8]. Hybridization and washing was carried out following the manufacturer's instruction.

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