A Novel Inositol Hexakisphosphate Kinase: Identification and Characterization

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Running Title: Cloning of Inositol Hexakisphosphate Kinase3 (InsP6K3)
ABSTRACT

The inositol pyrophosphate disphosphoinositol pentakisphosphate (PP-
InsP₃/InsP⁷) is formed in mammals by two recently cloned inositol hexakisphosphate kinase, InsP₆K1 and InsP₆K2 (Saiardi et al. (1999) Curr. Biol. 9, 1323-1326). We now report the identification, cloning and characterization of a third InsP⁷ forming enzyme designated InsP₆K₃. InsP₆K₃ displays 50 and 45 % sequence identity to InsP₆K1 and 2 respectively, with a smaller mass, 46 kDa, and a more basic character that the other two enzymes. InsP₆K₃ is most enriched in the brain where its localization resembles InsP₆K1 and 2. Intracellular disposition discriminates the three enzymes with InsP₆K2 being exclusively nuclear, InsP₆K₃ predominating in the cytoplasm and InsP₆K1 displaying comparable nuclear and cytosolic densities.
Numerous inositol phosphates occur ubiquitously in biological tissues. The most extensively characterized, inositol 1,4,5-trisphosphate (InsP3), releases calcium from intracellular stores (1). Recently the function of some of the inositol tetrakisphosphate isomers has been elucidated. Inositol 3,4,5,6-tetrakisphosphate may modulate the InsP3 generated calcium signaling (2–4), whereas inositol 3,4,5,6-tetrakisphosphate is a physiologic inhibitor of calcium activated chloride channels (5, 6). Functions of other inositol phosphates have not been fully characterized (7). Recently, a group of higher inositol phosphates containing energetic pyrophosphate bonds and including disphosphoinositol pentakisphosphate (PP-InsP5/InsP7) and bis(diphospho)inositol tetrakisphosphate (bis-PP-InsP4/InsP8) has been identified (8). Additional inositol pyrophosphates have been described in which one of the hydroxyl groups of inositol is not phosphorylated as exemplified by disphosphoinositol tetrakisphosphate (PP-InsP4) (9). It has been suggested that the high-energy pyrophosphates participate in protein phosphorylation, while the high turnover rate of inositol pyrophosphates (10) could represent a molecular switching activity.

The high affinity binding of inositol hexakisphosphate (InsP6) and InsP7 to a variety of clathrin associated proteins including AP2 (11) and AP-180 (12, 13), as well to the yeast coatomer (14) suggest a role in vesicular trafficking. Yeast with deletion of InsP6K display an unusual vacuolar morphology (15).
Recently Morrison and colleagues (16) have suggested a role for InsP6K2 as a mediator of growth inhibition and apoptosis in response to interferon-β. Overexpression of InsP6K2 in ovarian carcinoma cells line reinforces the growth suppressive and apoptotic effects of interferon-β treatment (16).

Inositol pyrophosphates can be formed by several enzymes. InsP7 and InsP8 are synthesized by two inositol hexaphosphate kinases (InsP6K) designated InsP6K1 (17) and InsP6K2 (17). A protein with InsP7 kinase activity was purified but not cloned (18). PP-InsP4 is synthesized by InsP6K1 and InsP6K2 using InsP5 as substrate (15), as well by inositol phosphate multikinase (IPMK) an enzyme cloned from mammalian sources (19) and yeast (17).

Based on our identification of three distinct protein bands interacting with an antibody to InsP6K, we explored databases and uncovered a sequence that might represent a third InsP6K. We now describe the molecular cloning and characterization of human (hInsP6K3), a new member of the InsP6 kinases family with high densities in the brain and localizations in the nucleus and cytoplasm.
MATERIALS and METHODS

Materials. $[^3]H]$Ins(1,4,5)P$_3$ and $[^3]H]$InsP$_6$ were purchased from Perkin Elmer NEN (Boston, MA). $[^3]H]$Ins(1,3,4,5,6)P$_5$ was prepared by phosphorylating $[^3]H]$Ins(1,3,4,5)P$_4$ using yIPMK $^{17}$, $[^3]H]$PP-InsP$_5$ was prepared by phosphorylation of $[^3]H]$InsP$_6$ using mouse mInsP$_6$K$_1$ $^{15}$. All the radiolabeled inositol phosphates synthesized were purified by HPLC and desalted $^{9}$.

Generation of InsP$_6$K specific antibody. In collaboration with Affinity BioReagents, Inc (Golden, Co) immune serum was raised against the synthetic peptide PCVLDLKMGTROHGDDAS localized in the conserved catalytic domain of InsP$_6$Ks (see Fig.1 and 3). Affinity purification of the serum was subsequently performed using the antigenic peptide.

Identification, cloning, and expression of human InsP$_6$K3. We used the amino acid sequence of mInsP$_6$K1 and human hInsP$_6$K2 to screen the human genome data bank using the TBLAST program $^{20}$. We were able to localize InsP$_6$K1 and 2 genes on chromosome 3 and to identify on chromosome 6 a new gene with substantial similarity to the InsP$_6$Ks (see Results section). We used the sequence information to
synthesize the following gene specific oligonucleotide primer GSP1 5'-
GGTGGCTTCCATTATGTAGGAACTGATAG-3' and GSP2 5'-
GTGTCACAGTACACGCATCCCTGTGCC-3'. We used these primers to perform 5' and 3' Rapid Ar
of cDNA Ends (RACE) using a human brain Marathon-Ready" cDNA Kit (Clontech, Palo Alto, CA), following the manufactures instructions. The hInsP6K3 cDNA was
amplified from the 5'- and 3'-RACE products using the supplied AP1 oligo, and cloned
in NotI site of Bluescript SK+. The plasmid was sequenced on both strands using a
Perkin Elmer Abi Prism 310 genetic analyzer (Foster City, CA). The ORF for human
InsP6K3 was PCR amplified using the following primers: 5'-
GCGTCGACCATGGTTGTGCAAAACAGCGC-3' and 5'-GCGTCGACTCATTCTCCTCCTCTTGGATAT
and subcloned in the SalI site of the prokaryotic expression vector pGEX 4T-2
(Amersham Pharmacia Biotech, Piscataway, NJ). The ORF hInsP6K3 was also PCR
amplified using the following oligos 5'-
GCTAGATCTCCATGGTTGTGCAAAACAGCGC-3' and 5-GCAGAATTCTCATTCTCCTCCTCTTGGATAT
BglII and KpnI sites of the pTrcHisA expression vector (Invitrogen, Carlsbad, CA). We
used His-hInsP6K3 to mutagenize lysine 217 to alanine and serine 335 to alanine using
the following oligo: K(217)/A 5'-
CCCTGTGTCTGGATCTGGCCATGGGGACCCGGCAC-3' and complement;
S(335)/A 5'-CATACCCGGCTTCTATTCAGCGCTTCTCTTGGTATCTATG-3' and
complement. Transformation of E. coli (strain BL21), induction with isopropyl-1-thio-
-D-galactopyranoside, isolation of GST fusion proteins using glutathione agarose (Sigma, Saint Louis, Mo) and of poly(His)-tagged proteins using Talon resin (Clontech, Palo Alto, CA), were all performed according to the manufacturer’s recommendations.

**Enzyme Assays.** Recombinant hInsP6K3 (10-20 ng) was incubated at 37 °C in 20 µl of buffer containing 20 mM HEPES, pH 7.0, 6 mM MgCl, 1 mM dithiothreitol, 10 mM ATP, 1mM NaF, 20 mM phosphocreatine, 1 mM EDTA, 0.01 mg/ml phosphocreatine kinase (Calbiochem, San Diego, CA). About 5000 cpm of the specific [3H]-labeled inositol phosphate was added. Kinetic parameter determinations was done under initial rate conditions (<10% of product formation). Assays were quenched with ice-cold perchloric acid and neutralized before HPLC analysis as previously described (21). Briefly, the activity of recombinant enzymes was assessed using a 4.6x125mm Partisphere SAX column (Whatman Inc., Clifton, NJ). The column was eluted with a gradient generated by mixing Buffer A (1 mM Na2EDTA) and Buffer B (Buffer A plus 1.3 M (NH4)2HPO4, pH 3.8, with H3PO4) as follows: 0-5 min, 0% B; 5-10 min, 0-30% B; 10-60 min, 30-100% B; 60-75 min, 100% B. Fractions (1 ml) were collected and counted using 5 ml of Ultima-Flo AP LCS-cocktail (Packard, Downers Grove, IL).

**Northern Blot Analysis.** Total RNA was isolated from various rat organs using LiCl precipitation methods (22). RNA (20-40 µg) was loaded on 1% agarose/formaldehyde/MOPS gel and transferred to Hybond™N+ nylon membrane.
ORFs for hInsP6K3 were labeled with \([\alpha^{32}P]dCTP\) using oligo labeling as described \(22\). Hybridization and washing was carried out following the manufacturer’s instructions.

**In Situ Hybridization.** Fresh-frozen mouse brain sections (2-3 months old, C57/Black6 male) were fixed with 4% paraformaldehyde/PBS, permeabilized, and hybridized in 50% formamide, 5% SSC with 100 ng/ml of unhydrolyzed digoxygenin-labeled probe overnight at 55ºC. Sections then were washed, blocked, and incubated overnight at 4ºC in 4% normal goat serum in TBS with antidigoxygenin-AP antibody (Roche Molecular Biochemicals, Indianapolis, IN) at 1:5000 dilution. After washing in TBS, slides were developed with 1 ml of color development solution, containing 3.375 mg/ml nitroblue tetrazolium, 3.5 mg/ml BCIP, and 0.24 mg/ml levamisole, in the dark. The color reaction was allowed to run 48 h at room temperature. The reaction was stopped in ddH2O, and the slides were sealed in Aquapolymount. Unique probes for hInsP6K3 were generated from cDNA corresponding to the ORF of hInsP6K3 subcloned in the SalI site of Bluescript SK+ plasmid (Stratagene, La Jolla, CA). Templates were generated by T7 and T3 RNA polymerases. Sense control probes used at equal concentration generated no specific signal.

**Subcellular localization of mInsP6K1, hInsP6K2 and hInsP6K3.** The ORF of InsP6K1, 2 and 3 were subcloned in the sites XhoI and EcoRI of Enhanced Green Fluorescent
Protein pEGFP-C1 plasmid (Clontech, Palo Alto, CA). GFP-mInsP6K1, GFP-hInsP6K2
and GFP-hInsP6K3 plasmids were transient transfected into 70% confluent HEK 293
cells using Lipofectamine 2000 (Life Technologies, Rockville MD). The cells were grown
in 5% CO2 at 37°C and maintained in DMEM (Gibco BRL, Bethesda MD) supplemented
with 1% (w/v) glutamine, 1% penicillin/streptomycin and 10% (v/v) fetal-calf serum.
After 24 h, transient transfected cells were fixed for 30 min with 4% formaldehyde in
PBS and washed three times in PBS. Nuclei were stained with 100 ng/ml 4',6-
diamidino-2-phenylindole (DAPI) for 10 min. Images of fluorescent cells were
obtained on a Zeiss 510 cofocal microscope.
RESULTS

Identification and Molecular Cloning of hInsP6K3:

mInsP6K1 and hInsP6K2 display about 60% similarity in amino acid sequence. A sequence of 18 amino acids, located in the catalytic domain of the enzymes, is identical in mInsP6K1 and hInsP6K2. In western blot analysis of rat brain, an antibody raised against this conserved domain (Fig. 1A) reveals a major broad band at about 49 kDa and a faint band at about 60 kDa (Fig. 1B). Preimmune serum interacts with the 60 kDa band but not with the prominent 49 kDa band. Since InsP6K purified from rat brain migrates as a 50 kDa band (23), and the calculated molecular weight of cloned mInsP6K1 and hInsP6K2 is about 49 kDa (17), we conclude that our antibody specifically recognizes InsP6Ks. Western blot analysis of multiple tissues reveals the same broad and prominent band with highest density in whole brain, cerebellum and spleen, intermediate levels in heart and testis, and lowest levels in kidney and liver. Pre-absorption with the InsP6K peptide utilized in raising the antibody abolishes the 49 kDa band (Fig. 1C)

SDS-PAGE analysis reveals two InsP6Ks bands (Fig. 1D), while Bis-Tris NuPage gel electrophoresis analysis focused on molecular weights in the InsP6K range,
discriminates three bands suggesting that there might exist three discrete InsP$_6$K enzyme proteins (Fig. 1E,F).

To search for a putative third InsP$_6$K, we examined a human genomic database (www.ncbi.nlm.nih.gov/genome/), screening with the known sequences of mInsP$_6$K$_1$ and hIP$_6$K$_2$ and located three discrete genes. mInsP$_6$K$_1$ and hInsP$_6$K$_2$ both occur on chromosome 3 at 3p21.31 and 3q11.1 regions respectively. A novel gene is located on chromosome 6 (region 6p21), which we tentatively designated hInsP$_6$K$_3$ on the basis of its substantial sequence similarity to the other InsP$_6$K genes (Fig. 3). Chromosome 6q24.1 also contains a sequence with substantial of homology to InsP$_6$K$_2$, but which lacks intron-exon organization, and so is likely a pseudogene.

We employed 3´ and 5´-RACE to clone hInsP$_6$K$_3$ from a human brain cDNA library (GeneBank accession number AF393812). The putative hInsP$_6$K$_3$ cDNA possesses 2,391 nucleotides (Fig. 2). The cDNA sequence comprises 102 nucleotide (nt) of 5´ untranslated regions rich in G/C (64%) and does not contain an in frame start or stop codon. The Open Reading Frame (ORF) is codified by 1233 nt, and the putative start codon is preceded by 5´-CCGCC-3´ region similar to the Kozak consensus sequence responsible for the translation starting site (24). The 3´ untranslated region consists of 1056 nt and terminates with a 21 nt poly(A) tail beginning 15 nt downstream of a candidate polyadenylation signal. The DNA homology with other members of the
InsP6K family in the ORF is 36-46% (Table 1). Homology is lower in the untranslated regions.

The human InsP6K3 gene defines a protein containing 410 amino acids with a molecular weight of 46,431 and a theoretical pI of 8.28. This contrasts with mInsP6K1, which contains 433 amino acids, a molecular weight of 49,215 and a theoretical pI of 6.64, while hInsP6K2 possesses 426 amino acids, a molecular weight of 49,180 and a theoretical pI of 6.52. InsP6K3 display 51 and 45 % sequence identity to InsP6K1 and 2 respectively, which possess 48 % identity to each other. Thus, hInsP6K3 appears to be a somewhat smaller protein than mInsP6K1 or hInsP6K2 and is substantially more acidic than the other proteins. The more acidic character of InsP6K3 may derive from its lower content of basic amino acids. Thus, hInsP6K3 possesses 22 lysines in contrast to 25 and 35 lysines in mInsP6K1 and hInsP6K2 respectively. hInsP6K3 possesses 24 arginines, while mInsP6K1 and hInsP6K2 possess 32 and 25 arginines respectively.

The sequence comprising the 18 amino acid peptide used to generate the antibody to InsP6K is highly conserved in the InsP3 kinases (25), IPMK (19), as well as mInsP6K1 and hInsP6K2 (17) and is identical in the three InsP6Ks (aa 210-228 in
hInsP6K3) (Fig. 3). This sequence comprises a crucial part of the catalytic site including a lysine which binds inositol phosphates which presumably account for its conservation (26). Splicing sites in InsP6K1, 2 and 3 are virtually identical (Fig. 3), indicating that the genomic structures of the three genes are essentially the same.

**hInsP6K3 Catalytic Activity:**

To assess the catalytic activity of this new member of the InsP6 kinase family we expressed a GST-hInsP6K3 construct in E. Coli (Fig. 4A). The purified protein runs as a single band with a molecular weight corresponding to the sum of GST and InsP6K3.

Incubation of the purified enzyme with [3H]InsP6 for one h at 37°C leads to a substantial reduction of the [3H]InsP6 peak in HPLC analysis and the appearance of a major peak corresponding to authentic InsP7 (Fig. 4B), establishing that InsP6K3 converts InsP6 to InsP7. We have previously showed that mInsP6K1 and hInsP6K2 can employ inositol 1,3,4,5,6-pentakisphosphate (InsP5) as a substrate (15). Incubation of InsP6K3 with [3H] InsP5 leads to a substantial reduction in the [3H] InsP5 peak in
HPLC analysis and the appearance of a prominent peak corresponding to authentic
diphosphoinositol tetrakisphosphate (PP-InsP₄) (Fig. 4C). We also observe a third,
smaller peak, which elutes identically to a peak obtained when we incubate [³H]InsP₅
with mInsP₆K₁ or hInsP₆K₂. We have previously identified this peak as bis-
diphosphoinositol trisphosphate ([PP]₂-InsP₃) (15). Thus, hInsP₆K₃, like mInsP₆K₁
and hIP₆K₂, can add two pyrophosphate groups to InsP₅. We fail to detect metabolic
activity when enzyme preparations are incubated with [³H]Ins(1,4,5)P₃ or [³H]InsP₇
(data not shown).

Analysis of hIP₆K₃ kinetic parameters reveals a Km of 0.9 µM and a Vmax 0.6
µmol/mg/min for InsP₆ and a Km of 5.5 µM and a Vmax 0.8 µmol/mg/min for
Ins(1,3,4,5,6)IP₅.

To investigate portions of hInsP₆K₃ that may be critical for catalytic activity we
performed selected mutations. Previous studies have identified a lysine in InsP₃KA
which is critical for catalytic activity, presumably because it participates in binding the
inositol phosphate substrate (26). In hInsP₆K₃ this lysine occurs at position 217.
Converting this lysine to an alanine abolishes enzymatic activity (Fig. 5).

While mInsP6K1, hInsP6K2, hInsP6K3, and IPMK are part of the same large family of inositol phosphate kinases, the sequences of IPMK and the InsP6Ks are markedly divergent. One area of amino acid identity comprises an SSLL sequence (19), which, in hInsP6K3 occurs at a.a. 334-337 (Fig. 3). Mutation of serine 335 to alanine virtually eliminates enzyme activity (Fig. 5).

**Localization of hInsP6K3:**

Northern blot analysis reveals two prominent bands at 6 and 2 kb respectively with two fainter bands at lower molecular weight (Fig. 6A). These four bands are present in all tissues examined but at varying densities. The tissue with highest density of any bands is the cerebellum in which the two largest bands are especially abundant. Moderate levels are evident in the kidney, while other tissues have lower levels. The presence of multiple putative mRNA species implies the existence of alternative splicing.

*In situ* hybridization in rat brain reveals discrete localizations for hInsP6K3 mRNA (Fig. 6B). In cerebellum highest densities occur in Purkinje cells with somewhat lower levels in granule cells. In hippocampus substantial densities occur in CA1-CA4 layers of the hippocampus as well as in the dentate gyrus. No hybridization is evident
with a sense probe.

To evaluate the disposition of hInsP6K3 protein, we conducted Western blot analysis of a virtually pure neuronal culture of embryonic (E18) rat cerebral cortex (Fig. 1E). We observe all three bands, indicating that the three enzymes are all expressed in neurons. However we cannot rule out their expression in glia as well.

To examine the intracellular localizations of hInsP6K3 protein, we transiently transfected HEK293 cells with GFP-fusion constructs of hInsP6K3, mInsP6K1, and hInsP6K2 and examined localizations by confocal microscopy (Fig. 7). We observe notable differences in the localizations of the three enzymes. The disposition of mInsP6K1 is similar to that of GFP itself with comparable amounts of staining in the nucleus and cytoplasm. By contrast, hInsP6K2 appears to be almost exclusively nuclear. hInsP6K3, on the other hand, appears to predominate in the cytoplasm with only modest nuclear levels.
DISCUSSION

In the present study we have cloned and characterized hInsP6K3, a new member of the InsP6K family. hInsP6K3 was identified by screening human genome database.

We found no other gene that would be a likely candidate for a fourth InsP6K. Our antibody to a peptide that is very highly conserved in all InsP6 kinases recognized only three bands in brain preparations. This suggests that there may exist only three InsP6Ks, though the human genome is not yet completely assembled so that we cannot fully rule out the presence of a fourth InsP6K.

The Km and Vmax values for InsP6K3 with InsP6 and Ins(1,3,4,5,6)P5 are similar to those for InsP6K1 (15, 17, 23). Thus, it is likely that the two enzymes physiologically metabolize both substrates. With InsP6K2 the Km value for Ins(1,3,4,5,6)P5 is 8µM, about 20 times its Km for InsP6, suggesting selective actions on InsP6. Vmax value for InsP6K2 using both substrates are 5-8 fold lower that for InsP6K3 and InsP6K1 (15).
In situ hybridization studies indicated a selective localization of hInsP6K3 to discrete neuronal populations in the brain with notably high densities in the Purkinje cells of the cerebellum and pyramidal cells of the hippocampus and dentate gyrus. We have observed virtually identical localizations for mInsP6K1 (27) and hInsP6K2 (EN, SHS, in preparation). The in situ hybridization studies indicate that these enzymes occur in all Purkinje cells of the cerebellum and all pyramidal cells of the hippocampus and dentate gyrus. Thus, it would appear that individual cells contain all three enzymes. Might the three enzymes manifest different functions in the same cells? Their differing intracellular localizations are consistent with this possibility. Thus, hInsP6K2 is almost exclusively nuclear, mInsP6K1 occurs at similar densities in the nucleus and cytoplasm, while hInsP6K3 is predominantly cytoplasmic.

The principal functions suggested for the inositol pyrophosphates involve nuclear events as well as the disposition of vesicular structures in the cytoplasm. A prominent nuclear role for inositol pyrophosphates is implied by our observations that these substances mediate stimulated homologous recombination (LRH, SHS, in preparation). West and associates (28) identified InsP6 as an important factor of end-joining DNA repair in mammalian nuclei mediated by DNA-dependent protein kinase (28). These authors suggested that InsP7 and InsP8 might be the physiologic mediators...
of this action. Apoptosis, which involves nuclear events, appears to be influenced by 
InsP$_6$K$_2$ (16). Thus, deletion of InsP$_6$K$_2$ diminishes interferon-β induced apoptosis in tumor cells in culture, while cells over-expressing InsP$_6$K$_2$ are more susceptible to interferon-β induced cell death (16).

InsP$_6$Ks in the cytoplasm might be involved in the regulation of vesicular dynamics ascribed to inositol pyrophosphates. InsP$_6$ (11) and InsP$_7$ (13) bind with high affinity and selectivity to a variety of clathrin-associated proteins that are participants in vesicular turnover. Deletion of yeast InsP$_6$K profoundly alters vesicular disposition in yeast (15).
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Figure 1. Analysis of InsP6K antibody.

A) Antigenic peptide used to generate InsP6Ks specific antibody. B) Western blot analysis of 20 μg rat brain homogenate employing 4-12% polyacrylamide Bis-Tris NuPage gel electrophoresis. Left lane, preimmune serum, right lane, affinity purified InsP6K antibody. C) Western blot analysis using 20 μg of rat tissues. Right panel, Pre-absorption of the antibody with 1mg/ml of the antigenic peptide. D) Western blot analysis using a long run of a Tris-glycine SDS-PAGE gel reveals two distinct bands. Western blot analysis using a long run of a Bis-Tris NuPage gel discriminates three bands in rat brain (E) as well in rat primary of cortical neuron (F). These experiment were replicated 2 times.

Figure 2. Human InsP6K3 cDNA sequence.
The putative Kozac and polyadenilation signal sequences are underlined and in bold respectively. Genebank accession number for human InsP6K3 is AF393812.

Figure 3. Multiple alignment of human InsP6K3, mouse InsP6K1 and human InsP6K2.
Regions of homology were identified using a CLUSTAL-W program \(^{(29)}\); (*) represents an identical amino acid; (.) represents a conservative amino acid change; (+) indicates the amino acid that define the aldo-keto reductase motif 3, this region partially overlaps with the region "SSLL"; (v) represents the putative splicing sites deduced from alignment of the cDNA and genomic sequences. Because the draft of the genomic sequence lacks the first part of the protein, the first splicing site for InsP6K1 cannot be identified. The 18 aa peptide used to develop InsP6Ks specific antibody is underlined. Genebank accession number for human InsP6K3 is AF393812.

Figure 4. Analysis of hInsP6K3 enzymatic activity.

A) Purity of recombinant hInsP6K3 protein. Purified, recombinant enzyme (50 ng) was loaded on a 4-12% polyacrylamide Bis-Tris NuPage gel (Novex, San Diego CA) and electrophoresed with MES-SDS running buffer. The gel was stained with Coomassie Blue. Molecular mass standards are indicated. B) Phosphorylation of \(^{[3H]}\)Ins(1,3,4,5,6)P5 and \(^{[3H]}\)InsP6 by hInsP6K3. HPLC analysis of assays containing 10 ng of recombinant enzyme incubated 120 min with \(^{[3H]}\)InsP6. C) HPLC analysis of assays
containing 10 ng of hInsP₆K3 incubated with [³H]Ins(1,3,4,5,6)P₅ for 120 min. Data are representative of three experiments with virtually identical results.

**Figure 5. Effect of point mutations on hInsP₆K3 enzymatic activity.**

Recombinant His-hInsP₆K3, His-hInsP₆K3 217 K/A and His-hInsP₆K3 335 S/A proteins (20 ng) were incubated with [³H]InsP₆ for 1h at 37⁰ and analyzed by HPLC. InsP₇ production is expressed as % of the WT gene. Activities are means of triplicates ± SD. The experiment was replicated 2 times.

**Figure 6. Expression analysis of hInsP₆K3.**

A) Total RNA (40 µg) from different mouse tissues was separated on 1% agarose/formaldehyde/MOPS gel. After transfer, the blot was hybridized with a probe for hInsP₆K3. Bottom: the gel was stained with ethidium bromide to check equivalence of loading. B) *In situ* hybridization of hInsP₆K3. Adult rat brain was probed with rat hInsP₆K3 antisense (a;b) or sense (c;d). a) In the cerebellum hInsP₆K3 message was
localized to the Purkinje cells. In the hippocampus hInsP6K3 message was detected principally in the dentate gyrus. c,d) Mouse cerebellum and hippocampus probed with sense RNA probe.

**Figure 7. Subcellular localization of InsP6Ks.**

HEK 293 cells (70% confluent) were transiently transfected using Lipofectamine 2000 with plasmids for GFP, GFP-mInsP6K1, GFP-hInsP6K2 and GFP-hInsP6K3. After 24 h, transiently transfected cells were fixed for 30 min in 4% formaldehyde in PBS and washed three times in PBS. Nucleus were stained with DAPI for 10 min. Images of fluorescent cells were obtained on a Zeiss 510 cofocal microscope.
Table 1. Sequence similarities Between the Three Mammalian InsP6Ks.

DNA homology was calculated using a Balsta program (20) comparing the DNA sequences that define the Open Reading Frame of the three proteins. Protein homology was calculated using a Blasta program (20). The percent identity was calculated considering the identical amino acids between the two proteins, whereas the percent of similarity also consider conserved amino acid substitutions.

The GeneBank accession number of the sequences are: mInsP6K1: AF177144; hInsP6K2: AF177145; hInsP6K3: AF393812.

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Figure 1
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