The Synthesis of Inositol Hexakisphosphate

CHARACTERIZATION OF HUMAN INOSITOL 1,3,4,5,6-PENTAKISPHOSPHATE 2-KINASE*

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The enzyme(s) responsible for the production of inositol hexakisphosphate (InsP₆) in vertebrate cells are unknown. In fungal cells, a 2-kinase designated Ipk1 is responsible for synthesis of InsP₆ by phosphorylation of inositol 1,3,4,5,6-pentakisphosphate (InsP₅). Based on limited conserved sequence motifs among five Ipk1 proteins from different fungal species, we have identified a human genomic DNA sequence on chromosome 9 that encodes human inositol 1,3,4,5,6-pentakisphosphate 2-kinase (InsP₅ 2-kinase). Recombinant human enzyme was produced in Sf21 cells, purified, and shown to catalyze the synthesis of InsP₆ or phytic acid in vitro. The recombinant protein converted 31 nmol of InsP₅ to InsP₆/min/mg of protein (V_max). The Michaelis-Menten constant for InsP₅ was 0.4 μM and for ATP was 21 μM. Saccharomyces cerevisiae lacking IPK1 do not produce InsP₆ and show lethality in combination with a gle1 mutant allele. Here we show that expression of the human InsP₅ 2-kinase in a yeast ipk1 null strain restored the synthesis of InsP₆ and rescued the gle1-2 ipk1-4 lethal phenotype. Northern analysis on human tissues showed expression of the human InsP₅ 2-kinase mRNA predominantly in brain, heart, placenta, and testis. The isolation of the gene responsible for InsP₅ synthesis in mammalian cells will allow for further studies of the InsP₅ signaling functions.

Cells amplify and regulate signals through the generation of a variety of second messengers. The inositol polyphosphate family of second messengers has grown in complexity with the discovery of new functions for the soluble, more highly phosphorylated inositols. The common precursor of all soluble inositol phosphates in mammalian cells is Ins(1,4,5)P₃, which is produced when phospholipase C cleaves phosphatidylinositol 4,5-bisphosphate yielding InsP₃ and diacylglycerol. InsP₃ is then metabolized to a number of more highly phosphorylated inositol species through the actions of several phosphatases and kinases; the cellular functions of these inositol polyphosphates are beginning to be elucidated (1–4). An understanding of the enzymes responsible for the production of soluble inositol polyphosphates will be critical to establishing their roles in cellular physiology.

The major inositol pentakisphosphate isomer in eukaryotic cells, inositol 1,3,4,5,6-pentakisphosphate (InsP₆), is converted to inositol hexakisphosphate (InsP₆) by phosphorylation at the D2 position of the inositol ring. A role for the product of the 2-kinase, InsP₆, or phytic acid, has been implicated in many cellular processes. InsP₆ has been shown to bind the clathrin assembly proteins AP2 and AP3 (5, 6) and to inhibit clathrin cage assembly in vitro (7, 8). InsP₆ inhibits serine and threonine protein phosphatases, which are thought to regulate L-type Ca²⁺ channels in pancreatic islet cells (9). Nonhomologous DNA and joining of double strand breaks is stimulated by InsP₆ (10) through its binding to the Ku70/80 subunits of DNA-PK (11, 12). Most recently, InsP₆ has been suggested to stimulate endocytosis, possibly by the activation of protein kinase C and inhibition of synaptotoxin (13).

The first role for InsP₆ in vivo was revealed by studies in the budding yeast Saccharomyces cerevisiae (14), in which the production of InsP₆ was shown to be required for efficient messenger RNA (mRNA) export. This is based on the results of a genetic screen for mutations that were lethal in combination with a temperature-sensitive gle1 mutant defective for the essential mRNA export factor, Gle1 (14). The synthetic lethal screen specifically identified the three gene products that together are responsible for converting phosphatidylinositol 4,5-bisphosphate to InsP₆ (14, 15). This included the previously characterized Pcl1 (16) and two inositol polyphosphate kinases, Ipkl and Ipkl2 (14, 15). Besides the genetic linkage between mutants defective in InsP₆ production and the gle1 mRNA export mutant, strains lacking the IPK1 gene alone show a marked accumulation of mRNA in their nuclei (14). This directly implicates the enzyme that produces InsP₆ in mRNA export.

Our ongoing studies have focused on testing whether the Ipkl protein and InsP₆ function are conserved across species. Recent studies have identified IPK1 genes from two other fungi, Schizosaccharomyces pombe and Candida albicans (17). Although functionally conserved, the sequence identity is limited to a few small regions with high homology. However, there is, overall, less than 24% identity in all pairwise combinations across the fungal InsP₅ 2-kinase domains. This lack of signifi-

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1 The abbreviations used are: InsP₅, inositol 1,3,4,5,6-pentakisphosphate; InsP₆, inositol hexakisphosphate; Ins(1,3,4,5)P₄, inositol 1,3,4,5-tetakisphosphate; Ins(1,3,4,6)P₄, inositol 1,3,4,6-tetakisphosphate; Ins(1,3,4,5,6)P₅, inositol 1,3,4,5,6-pentakisphosphate 2-kinase; 5-FOA, 5-fluoroorotic acid; HPLC, high performance liquid chromatography; DTT, dithiothreitol; EST, expressed sequence tag.
The gene encoding human InsP$_5$ 2-kinase was isolated by nested PCR amplification using a Marathon cDNA library (CLONTECH) as template. First-round PCR primers were chosen 90 bp upstream of the initiator methionine and 25 bp downstream from the stop codon (upstream primer: 5'-AGCTCCGGTCGCCGAGTCTTACG-3'; downstream primer 5'-AAAGACACTGCGAGGAAGAGGG-3'), and the resulting protein is a fusion to the first eight amino acids of Gle1. The resulting construct places the human InsP$_5$ 2-kinase gene behind the GLE1 promoter and the resulting protein is a fusion to the first eight amino acids of Gle1. Yeast strains were transformed using standard protocols (18) and grown on synthetic minimal media lacking leucine (19). The resulting strains were streaked onto 5-FOA plates and grown for 4 days at 23 °C.

Cloning of Human InsP$_5$ 2-Kinase—The gene encoding human InsP$_5$ 2-kinase was cloned into an E. coli vector system (pBacPAK9, Clontech) using the restriction sites EcoRI and NotI. The resulting clone was then used as a template for second-round PCR using a sense primer encoding a BamHI site followed by the sequence starting from amino acid number 2 (sense, 5'-CCGGCATCGAAGAGGGAGTTGGACGAGAATGCG-3'; antisense, 5'-AACGTTGGGACCTTGGGAACTAATGCGAATCGTTCGC-3'). PCR was performed with Taq polymerase (Fisher Scientific) using standard protocols. The PCR product was inserted into a TOPO-TA cloning vector (Invitrogen) using the manufacturer's instructions.

Analysis of Human InsP$_5$ 2-Kinase Expression in Yeast Mutant Strains—The sequence encoding the human InsP$_5$ 2-kinase was inserted into a yeast expression vector by replacing the NcoI/NotI restriction sites upstream from the yeast GLE1 promoter. The resulting expression construct was then used to transform yeast mutant strains (SWY2105) containing either an empty LEU2/CEN plasmid (pRS316) or the LEU2/CEN plasmid containing human InsP$_5$ 2-kinase were labeled with [3H]inositol along with a [32P]InsP$_6$ standard (see below) and separated at 1 ml/min using a Partisil SAX (4.5 × 126 mm) strong anion exchange HPLC column along with a [3H]InsP$_5$ standard (see below) and separated at 1 ml/min with a 0–1.7 M gradient of ammonium phosphate (pH 3.5) over a period of 20 min followed by 30 min at 1.7 M ammonium phosphate. To test complementation of the gle1-2 ipk1–4 synthetic lethal, the yeast strain SWY1793 was transformed with the appropriate LEU2 plasmids.

Purification of Human InsP$_5$ 2-Kinase from Sf21 Cells—Full-length human InsP$_5$ 2-kinase was cloned into the pBacPAK9 vector (CLONTECH) using the restriction sites EcoRI and NotI. The resulting clone was then inserted into a yeast expression vector by replacing the NotI restriction site. The resulting construct was then used to transform yeast mutant strains (SWY2105) containing either an empty LEU2/CEN plasmid (pRS316) or the LEU2/CEN plasmid containing human InsP$_5$ 2-kinase were labeled with [3H]inositol along with a [32P]InsP$_6$ standard (see below) and separated at 1 ml/min with a 0–1.7 M gradient of ammonium phosphate (pH 3.5) over a period of 20 min followed by 30 min at 1.7 M ammonium phosphate. To test complementation of the gle1-2 ipk1–4 synthetic lethal, the yeast strain SWY1793 was transformed with the appropriate LEU2 plasmids.
His- and FLAG-tagged fusion protein was expressed in Sf21 cells using the BacPAK™ baculovirus expression system (CLONTECH). Cells were infected for 3 days, pelleted, and lysed by sonication (3 H11003 10 s) in 20 ml of lysis buffer (20 mM HEPES, pH 7.6, 140 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, 40 μM iodoacetamide, 20 μM bestatin, and 40 μM leupeptin). The lysate was allowed to bind to M2 anti-FLAG-agarose beads (Sigma) for 1 h, washed with Tris-buffered saline containing 0.5 mM DTT, and eluted with FLAG peptide (0.1 mg/ml, Sigma). The resulting eluate was run on an SDS-polyacrylamide gel and stained with Coomassie Blue, revealing a single band of Mr 50,000 (not shown).

**Production of Radiolabeled Inositol Species**

[3H]InsP5 was produced starting from [3H]Ins(1,3,4,5)P4 (PerkinElmer Life Sciences). Ins(1,3,4,5)P4 was converted to Ins(1,3,4)P3 by treatment with recombinant OCRL inositol polyphosphate 5-phosphatase purified from Sf9 cells (21). Ins(1,3,4)P3 was converted to Ins(1,3,4,6)P4 by treatment with recombinant GST-Ins(1,3,4)P3 5/6-kinase purified from E. coli (22). The OCRL 5-phosphatase in the reaction converted any Ins(1,3,4,5)P4 product of 5/6-kinase back to Ins(1,3,4)P3, resulting in the production of Ins(1,3,4,6)P4 as the only product with nearly 100% conversion of the substrate. Reaction mixtures contained 2.5 μg of recombinant OCRL, 50 mM HEPES, pH 7.5, 3 mM MgCl₂, 50 μM ATP, 2.5 × 10⁶ cpm of [3H]Ins(1,3,4,5)P4, 100 μM phosphocreatine, 800 units of phosphocreatine kinase, 1 mM DTT, and 7.5 μg of GST-5/6-kinase in 1 ml. Reactions...
proceeded for 1 to 2 h until all substrate was converted to product. [3H]Ins(1,3,4,6,5,6)P6 was converted to [3H]Ins(1,3,4,5,6,6)P6 by adding 1.5 µg of recombinant Ins(1,3,4,6,5,6)P6-kinase and incubating for about 2 h until all substrate was converted to product. The identity of [3H]Ins(1,3,4,5,6,6)P6 was confirmed by its co-elution with a Partisphere SAX HPLC column with a [32P]Ins(1,3,4,5,6,6)P6 standard produced from Ins(1,3,4,5,6,6)P6 by a chick red blood cell extract (23). This [32P]InsP6 was also used as the substrate to make a [32P]InsP6 standard using partially purified InsP6 from soy bean extracts (24).

Kinetic Analysis of InsP6 2-Kinase Activity—The optimal conditions for assay of FLAG purified human InsP5 2-kinase were determined to be 50 mM HEPES (pH 7.0), 100 mM KCl, 1 mM DTT, 10 mM MgCl2, and 0.1–0.5 µg of human InsP5 2-kinase in 100 µl at 37 °C for 20–60 min. Assays were performed using [3H]InsP6 mixed with unlabeled InsP6 at a constant specific activity for each assay. The Km for InsP6 was determined at 0.5 mM ATP while varying the InsP6 concentration from 0.25 to 4 µM; the Km for ATP was determined at 10 µM InsP6 while varying the ATP from 5 to 200 µM. Reactions were performed using 0.18 µg of enzyme for 1 h and were stopped with an equal volume of 60 mM ammonium phosphate (pH 3.5) and 2 mM InsP6 to aid in recovery from a Whatman Partisphere SAX strong anion exchange column (4.6/0.8 cm) and HPLC (25).

RESULTS

Identification and Isolation of a Gene Encoding the Putative Human InsP5 2-Kinase—Based on the Ipkl protein sequence from S. cerevisiae, genes encoding InsP5 2-kinases in S. pombe and C. albicans were identified previously (17). Further use of these sequences and database searching algorithms allowed us to identify partial clones that likely encode two additional fungal InsP5 2-kinases, one each from Kluyveromyces lactis and Saccharomyces cerevisiae. The alignment of all the fungal sequences allowed the identification of a number of regions of short though significant homology throughout the sequence (designated Boxes A–D, Figs. 1 and 6) even though the overall level of conservation is limited. Using the BLAST program for short, nearly exact matches (NCBI), we found that amino acids DLK/V(DL)K(P/S)X/E/M of Box D from the fungal Ipkl1s matched a predicted gene and a number of human ESTs that all mapped to chromosome nine. A consensus from an alignment of all the ESTs was compared with the fungal Ipkl1 sequences using the Clustal method (25). The resulting align-

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post-infection, the cells were harvested and lysed, and protein was purified by affinity absorption with M2 anti-FLAG-agarose beads and elution with FLAG peptide. The Michaelis-Menten kinetic parameters were determined by following the conversion of \[^3H\]InsP5 to InsP6 at multiple concentrations of InsP5.

One strategy that we have used previously to analyze the roles of inositol polyphosphates in mammalian cells has been to deplete cells of inositol metabolites by heterologous expression of an inositol phosphate phosphatase from *Salmonella dublin* SopB (27). When SopB is overexpressed in a tetracycline-dependent system in human cells, there are rapid perturbations in cellular levels of multiple soluble inositol phosphates. In particular, total cellular InsP5 and InsP6 levels are depleted. Coincidentally, polyadenylated RNA accumulates in the nucleus, and protein synthesis is markedly inhibited, thus suggesting a conserved role for the InsP6 2-kinase of yeast and mammalian cells in mRNA export. However, this study could not distinguish the relative importance of different inositol phosphates in the mRNA export mechanism, because several inositol polyphosphates are perturbed. Isoalanization of the human InsP6 2-kinase will now allow direct gene deletion studies in which only InsP6 production is depleted. This is a goal of our future studies.

In this report, we have confirmed the functional conservation between *InsP5* 2-kinase of yeast and mammalian cells. We show that human InsP6 2-kinase can complement the deficiencies of InsP6 production in a *S. cerevisiae* ipk1 null mutant strain and the synthetic lethality of the *gle1–2 ipk1–4* mutant strain. Such broad cross-species complementation is unusual, especially when the overall protein sequence homology is so low. This suggests that enzymatic activity and InsP6 production alone is sufficient for the role of Ipkl in mRNA export. It remains possible that protein-protein interaction motifs and targeting sequences could also be conserved and remains to be discovered.

It is notable that the apparent \(V_{\text{max}}\) of human InsP6 2-kinase is quite low at 30 nmol of InsP6 formed/min/mg of protein. However, the \(V_{\text{max}}\) values for the fungal proteins are also quite low (17) as is the value for the mammalian Ins(1,3,4)P3 5/6-kinase (60 nmol of InsP6 formed/min/mg of protein) (22). The latter is the first enzyme in the pathway of synthesis of InsP6 in mammalian cells. Whether these low levels of activity result from some as yet unidentified cofactor or post-translational modification that is missing from the *in vitro* assays remains to be determined.

**DISCUSSION**

Although a role for InsP6 in mRNA export has been clearly evinced through studies in budding yeast (14), the other proposed cellular functions of InsP6 require the study of higher eukaryotes. For instance, studies of the role of InsP6 in L-type Ca\(^{2+}\) channels were performed on insulin-secreting B cells (9). In addition, although the mammalian Ku proteins were shown to be the binding partners for InsP6 in the DNA-PK complex, the yeast Ku proteins do not bind InsP6 under the same conditions (11). This suggests that the role of InsP6 in nonhomologous end joining DNA repair may be specific to mammalian cells. Whereas previous studies of the role of InsP6 in higher eukaryotes have been conducted by *in vitro* experiments or by correlating a specific function with an inositol phosphate profile, studies directed at the enzymatic source of InsP6 have been limited by the lack of a InsP6 2-kinase gene or protein. Here we have identified the first nonfungal InsP6 2-kinase.

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The human InsP$_5$ 2-kinase mRNA is found in most tissues, although to varying degrees. The abundance in the brain is consistent with its proposed function in endocytosis and its possible effect on the synaptic protein synaptotagmin (13). The mRNA level matches that of the InsP$_5$ 5/6 kinase (22), both showing robust expression in heart and brain. This is not surprising because they both act in the pathway that produces InsP$_6$. The apparent paucity of the InsP$_5$ 2-kinase in some tissues is surprising, considering the range of functions proposed for InsP$_6$. Gene deletion experiments will allow us to assess the role of InsP$_6$ in vivo.

The discovery of the putative, nonfungal InsP$_5$ 2-kinases will also aid in our understanding of the function of InsP$_6$. The alignment of the fungal, human, and the putative InsP$_5$ 2-kinases from corn, Drosophila, Anopheles, and C. elegans highlights the most conserved residues among the InsP$_5$ 2-kinases; these will provide good targets for mutagenesis to discover which residues are necessary for enzyme activity, its regulation, or possibly its interaction with other proteins. Interestingly, the cysteines in Box C are perfectly conserved among all of the sequences except for corn. ESTs of other plants (potato, rose, wheat) also lack the conserved cysteines, even though Box C itself is maintained. It will be interesting to inquire whether there is a connection between this residue substitution and the peculiarities of the plant enzymes. The discovery of a putative plant InsP$_5$ 2-kinase is also interesting because of the desire for a low phytate seed to mitigate the problems associated with high InsP$_6$ levels in seeds, i.e. pollution and malnutrition (28).

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