The Pathway for the Production of Inositol Hexakisphosphate in Human Cells*

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John W. Verbsky‡§, Shao-Chun Chang‡§, Monita P. Wilson‡, Yasuhiro Mochizuki¶, and Philip W. Majerus¶

From the ‡Department of Internal Medicine, Division of Hematology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ¶Laboratory of Molecular Cell Biology, School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Hironouchi, Hachioji, Tokyo 192-0392, Japan

The yeast and Drosophila pathways leading to the production of inositol hexakisphosphate (InsP6) have been elucidated recently. The in vivo pathway in humans has been assumed to be similar. Here we show that overexpression of Ins(1,3,4)P3 5/6-kinase in human cell lines results in an increase of inositol tetrakisphosphate (InsP5) isomers, inositol pentakisphosphate (InsP5) and InsP6, whereas its depletion by RNA interference decreases the amounts of these inositol phosphates. Expression of Ins(1,3,4,6)P4 5-phosphatase does not increase the amount of InsP6 and InsP6 whereas its depletion does block InsP6 and InsP6 production, showing that it is necessary for production of InsP6 and InsP6. Expression of Ins(1,3,4,5,6)P5 2-kinase increases the amount of InsP6 by depleting the InsP6 in the cell, and depletion of 2-kinase decreases the amount of InsP6 and causes an increase in InsP6. These results are consistent with a pathway that produces InsP6 through the sequential action of Ins(1,3,4)P3 5/6-kinase, Ins(1,3,4,6)P4 5-kinase, and Ins(1,3,4,5,6)P5 2-kinase to convert Ins(1,3,4)P4 to InsP6. Furthermore, the evidence implicates 5/6-kinase as the rate-limiting enzyme in this pathway.

Ins(1,2,3,4,5,6)P6 (InsP6) has been implicated in many cellular processes. It is required for mRNA export from the nucleus in yeast (1) and human cells (2). InsP6 binds to the clathrin assembly proteins AP2 and AP180 (3, 4) and inhibits clathrin cage assembly in vitro (5, 6). InsP6 inhibits serine and threonine protein phosphatases, which are thought to regulate L type Ca2+ channels in pancreatic islet cells (7). Nonhomologous DNA end joining of double strand breaks is stimulated by InsP6 through its binding to the Ku70/80 subunits of DNA-PK (8, 9). Most recently, InsP6 has been suggested to stimulate endonuclease, possibly by the activation of protein kinase C and inhibition of synaptojanin (10). The many roles for InsP6 necessitates an understanding of the pathway leading to its production.

InsP6 is synthesized ultimately from Ins(1,4,5)P3 (Fig. 1). The action of phospholipase C on the lipid phosphatidylinositol (4,5)-bisphosphate yields Ins(1,4,5)P3 and diacylglycerol. Ins(1,4,5)P3 can then be phosphorylated by an Ins(1,4,5)P3 3-kinase to Ins(1,3,4,5)P4 or dephosphorylated by an inositol polyphosphate 5-phosphatase to Ins(1,4,5)P4 (11); Ins(1,3,4,5)P4 can also be dephosphorylated by 5-phosphotases, yielding Ins(1,3,4)P3. When looking at the formation of the soluble inositol phosphates upon phospholipase C activation in rat pancreata cells, Menniti et al. (12) saw that in addition to the expected increase of the Ins(1,4,5)P3 isomer, Ins(1,3,4)P3 is also increased, as well as Ins(1,3,4,6)P4. They therefore argued that in vivo Ins(1,4,5)P3 is phosphorylated to Ins(1,3,4,5)P4 by an Ins(1,4,5)P3 3-kinase, dephosphorylated to Ins(1,3,4)P3 by a 5-phosphatase, and phosphorylated to Ins(1,3,4,6)P4 (Fig. 1A). The increase of Ins(1,3,4,6)P4 was also seen by Wong et al. (13) in WRK-1 cells stimulated with vasopressin. Wilson and Majerus (14) isolated a cDNA encoding the human kinase which catalyzes the conversion of Ins(1,3,4,5)P4 to InsP6 and named it Ins(1,3,4)P5 5/6-kinase because of its ability to phosphorylate both the D-5 and D-6 positions of the inositol ring. The product of 5-kinase activity of 5/6-kinase on Ins(1,3,4)P5 is the same as that of the Ins(1,4,5)P3 3-kinase, Ins(1,3,4,5)P4, and as such would lead back to Ins(1,3,4,6)P4 after the action of a 5-phosphatase. The above data would suggest that the human pathway for the production of InsP6 works through the isomerization of Ins(1,4,5)P3 to Ins(1,3,4,5)P4, and the sequential phosphorylation of Ins(1,3,4)P5 to Ins(1,3,4,6)P4 by the 5/6-kinase, of Ins(1,3,4,6)P4 to Ins(1,3,4,5)P4, and of InsP6 to InsP6 by a 2-kinase (Fig. 1A).

An alternate pathway to the one discussed above was proposed when the yeast pathway was discovered through genetic screens (Fig. 1B). In yeast, Ins(1,4,5)P3 is converted directly to InsP6 by the sequential action of two proteins: Ipk2, which produces InsP6 in a two-step phosphorylation of Ins(1,4,5)P3, first to Ins(1,4,5,6)P4 and then to InsP6; and Ipk1, which produces InsP6 and 1,15). This pathway differs from that proposed by Menniti et al. (12) in that no isomerization of Ins(1,4,5)P3 to Ins(1,3,4,5)P4 is required and in that the intermediate InsP4 isomer is not Ins(1,3,4,6)P4 but Ins(1,4,5,6)P4 (15). Deletion of IPK2 causes an increase in Ins(1,4,5)P3, whereas loss of IPK1 causes a loss of InsP6 and an accumulation of InsP6, proving that there is no other pathway to go from InsP4 to InsP6 in yeast. In addition, yeast cells do not possess a 5/6-kinase, nor...
mologs first produce Ins(1,4,5,6)P₄ from Ins(1,4,5)P₃, and then the human homolog of Ipk2, which they called Ins(1,3,4,6)P₄ 5-kinase mutant (16, 17). The homologs have been cloned and can complement the yeast Ipk2 IPK2 deletion mutant in yeasts. Interestingly, when the authors were determining the activities of the Drosophila and Arabidopsis homologs of Ipk2, they found a significant 5-kinase activity on Ins(1,3,4,6)P₄. This activity is necessary for the pathway proposed in human cells. Because the yeast pathway works through Ins(1,4,5,6)P₄, which is already phosphorylated at the D-5 position, they suggest that this activity is not relevant for the production of InsP₆ in these organisms.

A cDNA encoding the rat homolog of the yeast Ipk2 was isolated using a conserved sequence found in inositol phosphate kinases (18). These authors found that rat Ipk2 could catalyze the conversion of Ins(1,4)P₂ to Ins(1,4,5)P₃, Ins(1,4,5)P₃ to Ins(1,3,4,5)P₄, Ins(1,3,4,5)P₄ to Ins(1,3,4,5,6)P₅, and Ins(1,3,4,5,6)P₅ to the pyrophosphate PP-InsP₄. The extensive kinase activity led them to name the enzyme inositol polyphosphate multikinase (IPMK) (19). The 3- and 6-kinase activities match those of yeast Ipk2, although the rat homolog produces Ins(1,3,4,5)P₄ first, whereas InsP₅ in yeast produces Ins(1,4,5,6)P₄ first. Nonetheless it was inferred that the pathway to InsP₆ in mammals may not proceed through the Ins(1,3,4,5)P₄ isomer. Yet this work did not include any in vivo description of the activities, no other InsP₅ isomers were tested, the reactions were performed for long times with large quantities of enzyme, and no kinetic data were presented.

A cDNA encoding the human homolog of Ipk2 was described in two studies that came to different conclusions regarding its action (20, 21). Nalaskowski et al. (20) showed that the human homolog could produce Ins(1,3,4,5)P₄ from Ins(1,4,5)P₃ and Ins(1,3,4,5,6)P₅ from this InsP₅ in vitro. Its ability to produce InsP₅ from InsP₄ led them to conclude that this was the human homolog to the rat IPMK and yeast Ipk2, although the InsP₅ isomer, Ins(1,3,4,5)P₄, again differs from that in yeast, Arabidopsis, and Drosophila. Chang et al. (21) also isolated the human homolog of Ipk2, which they called Ins(1,3,4,6)P₄ 5-kinase because of its novel substrate specificity. This protein is much more active as a 5-kinase on Ins(1,3,4,6)P₄ than as a 6-kinase on Ins(1,3,4,5)P₄; its catalytic processivity (kcat) is 43 times greater for the former isomer. This suggests that it phosphorylates Ins(1,3,4,6)P₄ to InsP₅ in vivo, not Ins(1,3,4,5)P₄ to InsP₅ as suggested by the work of Saiardi et al. (19) and Nalaskowski et al. (20), neither of which tested the Ins(1,3,4,6)P₄ isomer. Second, although Chang et al. (21) found that the human homolog could act as a 3-kinase on Ins(1,4,5)P₃ in vitro (its kcat was only 1.6 times less than for Ins(1,3,4,6)P₄), it did not complement an Ipk2 deletion mutant in yeast. Therefore, in this in vivo experiment, the human 5-kinase does not convert Ins(1,4,5)P₃ to InsP₆.

This was not a surprising result considering that the human and rat proteins did not make Ins(1,4,5,6)P₄ as does yeast, but instead produced Ins(1,3,4,5)P₄ from Ins(1,4,5)P₃. Chang et al. (21) concluded that the human homolog of yeast Ipk2 and rat IPMK is primarily an Ins(1,3,4,6)P₄ 5-kinase; we will refer to this protein as such. These data suggested that the pathway in human cells does work through Ins(1,3,4,5)P₄ in vivo, but not Ins(1,4,5)P₃ by the sequential actions of 5/6-kinase to make Ins(1,3,4,6)P₄, 5-kinase to make InsP₅, and 2-kinase to make InsP₆. Here we present in vivo data that confirm this pathway in human cells.

**MATERIALS AND METHODS**

All chemicals were reagent grade or better. Restriction endonucleases, DNA-modifying enzymes, and general reagents were from Amerham Biochemicals, Roche Applied Science, Fisher, Invitrogen, New England Biolabs, Promega Corp., Sigma, and Stratagene unless stated otherwise. PCR was performed using the Pfu DNA polymerase per the protocol from Stratagene. Oligonucleotide synthesis and DNA sequencing were performed by the Protein and Nucleic Acid Chemistry Laboratory, Washington University, St. Louis, MO. Acrylamide solution, Bio-Safe Coomassie Blue stain, and the Bradford protein assay kit used for protein work were purchased from Bio-Rad. A SuperSignal West Pico kit used for detection of Western transfer blots was from Pierce. Radiolabeled inositol phosphates [³H]Ins(1,4,5)P₃, [³H]Ins(1,3,4,5), and [³H]InsP₅ were purchased from PerkinElmer Life Sciences and Amerham Biosciences.

**Strains, Plasmids, and Growth Conditions—Methods for Escherichia coli** growth and selection were described previously (22, 23). E. coli strain XL-1Blue (Stratagene) was used as the bacterial host for all plasmids unless stated otherwise. Bacterial strains were cultured in LB (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) medium supple-

![Pathway of InsP₆ Synthesis](image-url)
mented with 100 μg/ml ampicillin where appropriate and were transformed by standard methods (22, 23). All bacterial strains were grown at 37 °C.

**Cell Line Construction**—Specified tissue culture cells were treated with trypsin, washed with phosphate-buffered saline, and resuspended in 25 mM HEPES, pH 7.5, 7.2 mM KC1, 100 μg/ml bovine serum albumin, 8 mM MgCl2, 5 mM HEPES, 1 mM ATP, and proteinase inhibitors (Complete Mini EDTA-free, Roche Applied Science). Cells were lysed by two freeze-thaw cycles in an ethanol-dry ice bath, and particulate debris was removed by centrifugation at 10,000 × g in an Eppendorf centrifuge at 4 °C. The protein concentration of the clarified lysate was determined using the Bradford assay (Bio-Rad) as per the manufacturer's protocol. Samples (5 μg) were loaded onto a 10% gel for SDS-PAGE and subsequently electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). For detection of 5-kinase overexpression, anti-Myc antibody (Cell Signaling) was used, and for a loading control, anti-α-tubulin antibodies were used. The appropriate horseradish peroxidase-conjugated secondary antibody and the SuperSignal West Pico Chemiluminescent Substrate (Pierce) were used to visualize the appropriate band.

**5-Kinase Enzyme Activity Assay**—For each sample, the clarified cellular lysate was prepared as described above. Enzymatic activity was determined as described previously (21) and summarized below. Enzyme was added to 50 mM HEPES, pH 7.2, 100 mM KC1, 100 μg/ml bovine serum (Tet system approved, Clontech), 2 mM glutamine, 2 mM MgCl2, 100 units/ml penicillin G, 10 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 5 μg/ml blasticidin (Invitrogen), and 0.4 mg/ml Zeocin (Invitrogen) until single colonies were observed. Stable cell lines were maintained in medium containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Tet system approved, Clontech), 2 μg/ml glutamine, 5 μg/ml blasticidin, and 0.3 mg/ml Zeocin. These gene products were induced with 0.2 μg/ml tetracycline unless otherwise noted. Expression of the human 5-kinase was verified by Western blot analysis using monoclonal antibodies recognizing the human 5-kinase (CCL Vector) and by enzyme activity assays, whereas expression of 2-kinase was verified by Western blot analysis using monoclonal antibodies recognizing the FLAG epitope (Sigma) of 2-kinase.

**Gene Silencing of the Inositol Phosphate Kinases**—The preannealed siRNA oligonucleotides used for gene silencing of the inositol phosphate kinases were synthesized by Dharmacon RNA Technologies. The oligonucleotides used for the human 5-kinase were sense 5'-GGAUGGAGUCUCUGGAAUAdTdT-3' and antisense 5'-AAAUUGCUCCUGCAUGCCUA-CCdOdTdT-3', and for the luciferase control were sense 5'-UUAGGUCGGA-UCAGUGUAdTdT-3' and antisense 5'-UCAGAGUGAUCGCAA-GdOdTdT-3', and for 2-kinase were sense 5'-GAAGGCUCCUGAGAGA-GUdOdTdT-3' and antisense 5'-UAUUCCUGGACGCUUdcOdTdT-3'. Tissue culture cells were grown to 65–95% confluence in 12-well plates with or without tetracycline in standard media and transfected with 1.6 μg of siRNA oligonucleotide using Lipofectamine 2000 per manufacturer's protocol. After 6 h, the cells were treated with trypsin, transferred to 6-well plates, and then grown overnight in 5% CO2 at 37 °C. A second transfection with 4 μg of siRNA oligonucleotide was performed using Lipofectamine 2000, and after a 6-h incubation, the cells were transferred to 10-cm plates containing media with or without 10 μg/ml tetracycline and with or without 10 μg/ml Zeocin. Cells were harvested for Western blot analysis and HPLC of soluble inositol phosphates. Densitometry of the bands from the Western blot analysis was performed using the Kodak one-dimensional 3.5 software with the Kodak Image Station 440CF. Construction of the 5/6-kinase RNAi stable cell line was described previously (30).

**Analysis of Soluble Inositol Phosphates**—Cells were grown in complete media containing 10 μg/ml H3[32P]InsP5 (24, 25) and subjected to a 30-min step of 1.7M ammonium phosphate, pH 3.5. Radioactivity was measured using the inline detector β-RAM (IN/US System Inc.), and the identity of the individual inositol phosphates was assigned on the basis of coelution with known standards.

**RESULTS**

**Overexpression of the Inositol 5/6-Kinase Results in Elevated Levels of InosPlp, InsP3, and InsP6**—Stable HEK-293 cells expressing a tetracycline-inducible 5/6-kinase were labeled with [3H]inositol for 3–4 days, their soluble inositol phosphates extracted, and equal counts of soluble inositol phosphates were separated on an Adsorbosare SAX HPLC column. Two sets of labeling experiments were performed, one of log phase growing cells (Fig. 2, A and B) and another of confluent cells (Fig. 2, C and D). 5/6-Kinase-expressing lines were either uninduced (Fig. 2, A and C) or induced with 0.2 μg/ml tetracycline (Fig. 2, B and D). Induced cells had elevated levels of InsP3, InsP6 (7-fold and 2-fold increase in log phase and confluent growing cells, respectively), and they also showed elevated levels of InsP3 (3-fold and 5-fold, respectively) and InsP6 (1.7-fold and 2-fold, respectively). This suggests that the product of the 5/6-kinase reaction, InsP3, is phosphorylated to InsP6, which is then phosphorylated to InsP6. A large amount of InsP3, InsP4, and InsP6 is seen in labeling of confluent growing 5/6-kinase-overexpressing cell lines (Fig. 2, C and D), which is not seen consistently in labeling of log phase growing cells (Fig. 2, A and B). InsP3, InsP4, and InsP6 is thought to arise from the action of a 1-phosphatase on InsP3 (32). It therefore mirrors the rise in InsP6.
activity was sufficient to produce Ins(1,3,4,6)P4, InsP5, and InsP6. We then asked whether it was necessary for production of the higher inositol phosphates, using HeLa cells stably transfected with the 5/6-kinase RNAi construct (30). When labeled with [³H]inositol, these cells show a decrease in Ins(1,3,4,6)P4 (to 12.7% of control levels), in InsP5 (to 13%) and

FIG. 2. HPLC profiles of [³H]inositol-labeled HEK-293 cells expressing Ins(1,3,4)P₃ 5/6-kinase. A, log phase cells grown without tetracycline. B, log phase cells grown with tetracycline. C, confluent cells grown without tetracycline. D, confluent cells grown with tetracycline. All cells were labeled with [³H]inositol for 3 days, and their soluble inositol phosphates were extracted and separated by Adsorbosphere SAX HPLC. The identity of the labeled peaks was confirmed by internal standards (not shown).
in InsP₆ (to 71%). Ins(3,4,5,6)P₄ is also decreased (to 20%), consistent with a loss of its source, InsP₅. When the portions of the chromatograms corresponding to the InsP₃ isomers are lined up by the internal standards and expanded, one clearly sees an increase in Ins(1,3,4)P₃ in the 5/6-kinase RNAi cell line compared with the vector cell line (Fig. 3C). Therefore, 5/6-kinase is necessary for production of Ins(1,3,4,6)P₄ and also for InsP₅ and InsP₆.

Cells Expressing the 5-Kinase Show No Increase in InsP₅ or InsP₆ Levels—HEK-293 cells were stably transfected with the cDNA encoding 5-kinase under the regulation of a tetracycline-inducible system (T-REx, Invitrogen) as described under “Materials and Methods.” In the presence of tetracycline, a 10-fold increase in the 5-kinase enzymatic activity was observed, whereas without tetracycline, the activity was similar to that of the control HEK-293 cells stably transfected with vector DNA with or without tetracycline (Fig. 4A). Induction of 5-kinase was confirmed by the presence of Myc-tagged protein only in the presence of tetracycline in the stable cell line, whereas no recombinant 5-kinase protein was observed in vector cells (Fig. 4B) or in the uninduced stable cell line.

To determine the effect of overexpression of 5-kinase on soluble inositol phosphates in vivo, 5-kinase stable HEK-293 cells were labeled with 10 μCi/ml [³H]inositol for 3 days in the presence or absence of tetracycline. Soluble inositol phosphates were extracted and resolved by Adsorbosphere SAX HPLC. Interestingly, unlike the 5/6-kinase results, overexpression of 5-kinase does not increase the level of InsP₅, the product of the 5-kinase enzyme reaction, or InsP₆ (Fig. 4D) compared with uninduced cells (Fig. 4C). The soluble inositol phosphate pro-

![HPLC profiles of [³H]inositol-labeled HeLa cells expressing the 5/6-kinase RNAi construct. A, cell lines stably transfected with the pSuper vector alone. B, cell lines stably transfected with the pSuper vector containing an RNAi target sequence to the 5/6-kinase gene. C, expanded portions of chromatograms in A and B corresponding to the area of the InsP₃ isomers were lined up according to their internal Ins(1,4,5)P₃ standard (not shown). Lines were labeled with [³H]inositol for 3 days, and their soluble inositol phosphates were extracted and separated by Adsorbosphere SAX HPLC.](http://www.jbc.org/)

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**Fig. 3. HPLC profiles of [³H]inositol-labeled HeLa cells expressing the 5/6-kinase RNAi construct.** A, cell lines stably transfected with the pSuper vector alone. B, cell lines stably transfected with the pSuper vector containing an RNAi target sequence to the 5/6-kinase gene. C, expanded portions of chromatograms in A and B corresponding to the area of the InsP₃ isomers were lined up according to their internal Ins(1,4,5)P₃ standard (not shown). Lines were labeled with [³H]inositol for 3 days, and their soluble inositol phosphates were extracted and separated by Adsorbosphere SAX HPLC.
files are identical to those of the control vector cells with or without tetracycline (data not shown), and the lipid inositol phosphate profiles are not altered by the overexpression of 5-kinase (data not shown). These experiments were performed multiple times with the same results. Hence, increased 5-kinase activity does not alter the level of InsP5 and InsP6 in HEK-293 cells, suggesting that production of InsP5 is limited by the availability of the substrate of 5-kinase, Ins(1,3,4,6)P4.

Gene Silencing of the 5-Kinase Results in Decreased InsP5 and InsP6 Levels—Because the overexpression experiments did not produce a change in higher inositol phosphates, we performed gene silencing experiments using synthetic siRNA oligonucleotides to confirm that the 5-kinase protein was necessary for synthesis of InsP5 and InsP6 in vivo. HEK-293 cells were transfected with no siRNA oligonucleotide, siRNA oligonucleotide directed against the luciferase gene, or siRNA oligonucleotide directed against the 5-kinase gene. To determine the magnitude of gene silencing, we used the overexpressing 5-kinase HEK-293 stable cell line because we do not have antibodies that can consistently detect endogenous levels of 5-kinase. When 5-kinase stable cells induced with tetracycline were transfected with a siRNA oligonucleotide directed against the 5-kinase gene, the overexpressed 5-kinase protein is decreased significantly on Western blot analysis compared with either no siRNA oligonucleotide or siRNA oligonucleotide directed against the luciferase gene (Fig. 5A). To determine the magnitude of gene silencing, we compared the relative optical density of the 5-kinase band on the Western blot with that of the loading control AP1; we observed a ~90% decrease in the 5-kinase protein (Fig. 5B). Naive HEK-293 cells were used for

FIG. 4. Overexpression of human 5-kinase. A, activity assays for phosphorylation of Ins(1,3,4,6)P4 to InsP5 using cell extracts from induced (0.2 μg/ml tetracycline) or uninduced vector or 5-kinase overexpression lines. Each data set represents three independent assays; the error bars are the S.D. for the data set. B, Western blot analysis of 5 μg of protein from cell lysates in A. The recombinant 5-kinase was visualized by anti-Myc primary antibody. C, uninduced 5-kinase stable cells labeled with [3H]inositol for 3 days. D, induced 5-kinase stable cells labeled with [3H]inositol for 3 days. Soluble inositol phosphates were extracted from cells and separated on an Adsorbosphere SAX HPLC column, and the HPLC chromatograph is shown. The reference locations of isomers of InsP4, InsP5, and InsP6 were determined by the addition of known 32P-labeled standards. The radioactivity (cpm) of each sample was normalized to the total cell number.
labeling experiments so that we could determine the effect of silencing the endogenous 5-kinase. These cells were transfected with siRNA oligonucleotides, labeled with 10 μCi/ml [3H]inositol, the soluble inositol phosphates were extracted, and equal counts were resolved on an Adsorbosphere SAX HPLC column. In cells transfected with 5-kinase siRNA oligonucleotide, the level of InsP₅ was decreased to 29.4% and 33.1% compared with the level observed in cells mock transfected or

![Diagram](image-url)

**Fig. 5.** Gene silencing of the human 5-kinase. A, Western blot analysis of 5 μg of lysate from induced 5-kinase cells transfected with no siRNA oligonucleotide, the luciferase siRNA oligonucleotide, or 5-kinase siRNA oligonucleotide as described under “Materials and Methods.” The 5-kinase was visualized with anti-Myc antibody, and the loading control was determined by anti-AP1 antibody. B, relative densitometry of bands in A determined by Kodak Image Station 440CF. The optical density ratio of 5-kinase to AP1 was plotted on the y axis. C–E, HPLC profiles of naive HEK-293 cells transfected with no siRNA oligonucleotide (C), the luciferase siRNA oligonucleotide (D), or 5-kinase siRNA oligonucleotide (E), and labeled with [3H]inositol as described under “Materials and Methods.” Soluble inositol phosphates were extracted and separated on an Adsorbosphere SAX HPLC column, and the HPLC chromatograph is shown. The reference locations of isomers of InsP₄, InsP₅, and InsP₆ were determined by the addition of known ³²P-labeled standards. The radioactivity (cpm) of each sample was normalized to protein.
transfected with the luciferase siRNA oligonucleotide, respectively, and the level of InsP₆ was decreased to 26.8% and 28.4%, respectively (Fig. 5, C–E). Similar results were observed in 5-kinase stable cells with or without overexpression of the 5-kinase protein and in naive HeLa cells (data not shown). These findings are consistent with 5-kinase functioning in the pathway for the synthesis of InsP₅. Interestingly, HEK-293 cells transfected with the 5-kinase siRNA oligonucleotide did not accumulate Ins(1,3,4,6)P₄, the preferred substrate (21); however, in HeLa cells transfected with the 5-kinase siRNA oligonucleotide, the Ins(1,3,4,6)P₄ level increased by 70% (data not shown). Additionally, the peak representing the isomer Ins(3,4,5,6)P₄ decreased in both HEK-293 and HeLa cells transfected with 5-kinase siRNA oligonucleotide, which is expected because this isomer is derived from InsP₅.

Cells Overexpressing the 2-Kinase Produce InsP₆ by Depleting the Available InsP₅—To assess further the in vivo pathways to InsP₆, stable HEK-293 cell lines were constructed with a tetracycline-inducible 2-kinase gene and labeled with [³H]inositol for 4 days in the presence of 0.1 μg/ml tetracycline and [³H]inositol for 3 days, and their soluble inositol phosphates were extracted and separated on Partisphere SAX HPLC. The identity of the labeled inositol phosphates was confirmed by ³²P-labeled internal standards.

FIG. 6. HPLC profiles of [³H]inositol-labeled HEK-293 cells expressing the Ins(1,3,4,5,6)P₅ 2-kinase. TRex vector cells (A) or 2-kinase-expressing cells (B) were grown in the presence of 0.1 μg/ml tetracycline and [³H]inositol for 3 days, and their soluble inositol phosphates were extracted and separated on Partisphere SAX HPLC. The identity of the labeled inositol phosphates was confirmed by ³²P-labeled internal standards.

HEK-293 cells using siRNA oligonucleotides (Fig. 7B). As a control we used oligonucleotides to the luciferase gene (Fig. 7A). When these cell lines were labeled with [³H]inositol and their soluble inositol phosphates purified and separated by HPLC, there was a decrease in InsP₆ relative to the controls by about 50%, confirming that 2-kinase is necessary for production of InsP₆ in vivo. In addition, these lines had a 4-fold increase of InsP₅. Stable cell lines expressing 2-kinase RNAi and labeled with [³H]inositol showed a decrease of InsP₆ to 30% of controls (data not shown). The increase in InsP₅ and relatively smaller decrease of InsP₆ suggest that cells conserve InsP₆. During our attempts to produce stable knock-outs of 2-kinase using a RNAi expression construct, we were only able to produce a few stable cell lines, none of which had a complete depletion of InsP₅ (data not shown); we had a similar experience trying to produce stable RNAi cell lines of 5/6-kinase. Feng et al. (2) showed that depleting the higher inositol phosphates by expressing the Salmonella protein, SopB, a phosphatase that breaks down inositol phosphates, caused the cells to ball up and stop dividing. Thus it may be difficult to get a more complete silencing of 2-kinase because the effects are toxic to cells.

DISCUSSION

Here we provide in vitro evidence that the human pathway to the higher inositol phosphates proceeds through the Ins(1,3,4,5)P₄ isomer via the action of 5/6-kinase to produce Ins(1,3,4,6)P₄, 5-kinase to produce InsP₅ and 2-kinase to produce InsP₆. Expression of 5/6-kinase in human cells results in an increase of all of the isomers downstream of Ins(1,3,4)P₃ in the pathway, Ins(1,3,4,6)P₄, InsP₅, InsP₆, and Ins(3,4,5,6)P₄ from InsP₅. Silencing 5/6-kinase results in an increase of Ins(1,3,4)P₃ and a decrease of all inositol phosphates downstream of Ins(1,3,4)P₃. When we express 5-kinase in human cells, we do not detect an increase in InsP₅. Silencing 5-kinase by siRNA expression results in a decrease in InsP₅ and InsP₆. Expression of 2-kinase results in an increase in InsP₆ and...
concomitant loss of InsP₅, whereas depleting 2-kinase results in a decrease in InsP₆ and an accumulation of InsP₅. These results confirm that the pathway proposed for the production of InsP₆ in rat cells by Menniti et al. (12) is the pathway used for the production of InsP₆ in human cells.

Because overexpression of 5/6-kinase results in an increase in Ins(1,3,4,6)P₄, InsP₅, and InsP₆, whereas overexpression of 5-kinase alone does not result in an increase of InsP₅, the production of InsP₅ must be limited by the availability of substrate, Ins(1,3,4,6)P₄. This is supported by expression of 2-kinase, during which InsP₆ is produced at the expense of all of the available InsP₅, which is not replenished. Therefore, our data suggest that the rate-limiting step in this pathway is the production of Ins(1,3,4,6)P₄ by 5/6-kinase. Overexpression of Ins(1,3,4,5)P₄, 5/6-kinase showed that this protein is sufficient to cause increases in both InsP₅ and InsP₆. Silencing this gene resulted in an increase in its substrate, Ins(1,3,4)P₃, and a decrease in its product, Ins(1,3,4,6)P₄, as expected, and also a decrease in InsP₅ and InsP₆, showing that 5/6-kinase is also necessary for their production in vivo. The results from the RNAi experiments confirm that the activities described for 5-kinase and 2-kinase in vitro are likewise necessary for production of InsP₆ in vivo.

In yeast, Arabidopsis, and Drosophila, the pathway can operate directly through Ins(1,4,5)P₃ without isomerization to Ins(1,3,4)P₃, but the evidence argues against such a pathway in human cells. First, Seeds et al. (16) showed that expression of the Drosophila Ipk2 gene from the actin promoter in Drosophila resulted in a 5-fold increase in InsP₅ and InsP₆, but the expression of 5-kinase, the human homolog of Ipk2, does not alter the levels of InsP₅ and InsP₆ in human cells. Thus, in Drosophila Ipk2 is sufficient for production of InsP₅ from Ins(1,4,5)P₃, whereas in human cell lines 5-kinase is not. Also, silencing of 5/6-kinase should not affect the higher inositol phosphates if its activity were uninvolved in the pathway, but we show that silencing 5/6-kinase blocks higher inositol phosphate production. Furthermore, the expression of Drosophila Ipk2 in an ipk2-null yeast strain restores InsP₅ production, whereas expression of the human 5-kinase in yeast does not (21). Therefore, the human 5-kinase cannot convert Ins(1,4,5)P₃ to InsP₅. These data show that these organisms use different pathways.

An examination of the activities of the Ipk2 homologs on Ins(1,4,5,6)P₄ illustrates the differences between the pathways. Yeast, Arabidopsis, and Drosophila produce Ins(1,4,5,6)P₄ from Ins(1,4,5)P₃, which is then converted to InsP₅, whereas the rat IPMK and the human 5-kinase produce Ins(1,3,4,5)P₄ in vitro (Fig. 8). The work of Chang et al. (21) showed that the human 5-kinase could phosphorylate the D-3 position of Ins(1,4,5,6)P₄ when expressed in a yeast mutant strain that produces this InsP₄ isomer, but it could not produce InsP₅ from Ins(1,4,5,6)P₄ itself in a yeast ipk2 null strain (21). Therefore, the 6-kinase activity described for 5-kinase in vitro is absent in this in vivo experiment, either on Ins(1,3,4,5)P₄ or on Ins(1,4,5)P₃. Thus, although yeast convert Ins(1,4,5)P₃ to Ins(1,4,5,6)P₄, the human homolog cannot. Ins(1,4,5,6)P₄ is not a substrate for 5-phosphatases, whereas Ins(1,3,4,5)P₄ is a substrate. Subse-
quent dephosphorylation of Ins(1,3,4,5,6)P_5 would result in Ins(1,3,4,5)P_3. Therefore, the product of any activity that 5-kinase would have on Ins(1,4,5)P_3 could be converted to Ins(1,3,4,5)P_3 by a 5-phosphatase. This is analogous to the ability of 5/6-kinase to produce Ins(1,3,4,5,6)P_4 from Ins(1,3,4,5)P_3; it too will be degraded by 5-phosphatases back to Ins(1,3,4,5)P_3, which can then be made into Ins(1,3,4,5,6)P_4. This Ins(1,3,4,5,6)P_4 isomer is the committed isomer for higher inositol phosphate synthesis in human cells, as is Ins(1,4,5,6)P_4 in yeast. The only activities thus described on these two InsP_4 isomers are kinase reactions leading to InsP_5. Nonetheless, we do not see changes in the inositol phosphate profiles of labeled cells overexpressing 5-kinase, e.g., an increase in Ins(1,3,4,5,6)P_4, Ins(1,3,4,5)P_3, or the downstream products of its phosphorylation, Ins(1,3,4,6)P_4, InsP_6, or InsP_5. Thus we believe that the role of 5-kinase in the production of InsP_6 is to convert Ins(1,3,4,6)P_4 to InsP_5.

The formation of Ins(3,4,5,6)P_4 seen in these experiments is also relevant to the control of this pathway. Ins(3,4,5,6)P_4 is a potent inhibitor of 5/6-kinase (31). When the supply of InsP_5 is sufficient for InsP_6 production and the metabolic needs of the cell, Ins(3,4,5,6)P_4 builds up and inhibits 5/6-kinase. This would presumably shut down the synthesis of InsP_5 and, therefore, InsP_6. Another observation seen in these experiments is the ability of the cell to preserve InsP_6 at the expense of InsP_5. This is seen in the RNAi experiments of 5/6-kinase, and it was seen during numerous 5-kinase siRNA transfections, during which InsP_5 levels are less than InsP_6. Given the number of functions attributed to InsP_6, this is not a surprising result. When we attempted to silence 2-kinase and 5/6-kinase using generating 2-kinase and 5/6-kinase RNAi lines, whereas the control RNAi lines were abundant. None of the lines we did generate had complete depletion, suggesting that the loss of InsP_6 is toxic to cells.

We have determined the in vivo pathway for the production of InsP_6 in human cells. Although the pathway we have determined differs from the yeast, Drosophila, and Arabidopsis pathways, it does follow the pathway proposed for rat cells by Menniti et al. (12). Humans may have evolved a pathway that requires 5/6-kinase to produce InsP_6, possibly for the greater control of the pathway; in support of this, we believe that 5/6-kinase is the rate-limiting enzyme in the human pathway. It is interesting that the activity of 5-kinase on Ins(1,3,4,6)P_4, which is necessary in the human pathway, has been described for both the Drosophila and Arabidopsis Ipk2 proteins and thus seems to be conserved. What has changed is the predominance of 5/6-kinase in the human pathway. Some questions do remain. Is there a 5/6-kinase in Drosophila? Why does Arabidopsis have three copies of 5/6-kinase if they are unnecessary for production of InsP_6?
The Pathway for the Production of Inositol Hexakisphosphate in Human Cells
John W. Verbsky, Shao-Chun Chang, Monita P. Wilson, Yasuhiro Mochizuki and Philip W. Majerus

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