Disruption of the mouse inositol 1,3,4,5,6-pentakisphosphate 2-kinase gene, associated lethality, and tissue distribution of 2-kinase expression

John Verbsky*, Kory Lavine†, and Philip W. Majerus*‡

*Department of Internal Medicine, Division of Hematology, and †Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110

Contributed by Philip W. Majerus, May 3, 2005

Many functions have been suggested for inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$), including mRNA export, nonhomologous end-joining, endocytosis, and ion channel regulation. However, it remains to be demonstrated that $InsP_6$ is necessary for *in vivo* survival. We previously isolated a cDNA encoding the mammalian inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) 2-kinase (2-kinase), the enzyme that converts $InsP_5$ to $InsP_6$. We used the sequence to search the BayGenomics databases and identify an ES cell line (XA232) that has a gene trap construct embedded in the 2-kinase gene. We obtained a mouse from this line, produced heterozygotes, and confirmed that the heterozygotes contain the trapping construct and have diminished 2-kinase activity. Breeding the XA232 heterozygotes produced no homozygous offspring; thus, loss of 2-kinase is lethal in mice. Dissections of embryonic day-8.5 uteri yielded no homozygous embryos; thus, the mice die before day 8.5 postcoitum. The gene trap construct contains a β-galactosidase/neomycin reporter gene, allowing us to stain heterozygotes for β-galactosidase to determine tissue-specific expression of 2-kinase protein. 2-kinase is expressed in the hippocampus, the cortex, the Purkinje layer of the cerebellum in the brain, in cardiomyocytes, and in the testes of adult mice. At day 9.5 postcoitum, 2-kinase was expressed in the notochord, the ventricular layer of the neural tube, and the myotome of the somites. Intense staining was also seen in the yolk sac, suggesting that $InsP_6$ is necessary for yolk sac development or function. Furthermore, failure of yolk sac development or function is consistent with the early lethality of 2-kinase embryos.

embryonic lethal | gene deletion | inositol signals

Many functions of inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$) have been discovered since the time when it was thought to be merely a storage form of phosphate in plants (1). The first inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) 2-kinase (2-kinase) gene, encoding the enzyme that produces $InsP_6$, was isolated in a screen for yeast defective in mRNA export from the nucleus (2); this function was also associated with $InsP_6$ in mammalian cells (3). We cloned the 2-kinase gene from humans shortly thereafter (4). $InsP_6$ has been implicated in a number of physiological functions by *in vitro* work, including nonhomologous end-joining of double-strand DNA breaks (5), endocytosis (6, 7), and ion channel regulation (8, 9).

The many functions with which $InsP_6$ has been associated suggest that the 2-kinase may be necessary for life. This suggestion is implied in other work we have done with tissue culture lines. Using a stably transfected RNAi construct to the 2-kinase gene, we were able to deplete $InsP_6$ in HEK293 lines (unpublished data). We were only able to obtain a few clones, and even in these clones, some $InsP_6$ was retained, suggesting that a complete loss of $InsP_6$ is lethal. Other evidence for this lethality came from tetracycline-inducible expression of the inositol phosphate phosphatase SopB (3) in HEK293 cells, which breaks down $InsP_6$. These cells stopped dividing and detached from the tissue culture plate.

We now describe a disruption of the 2-kinase gene in mice to further assess its importance. Two strategies are used to generate gene deletions in mice (10): (*i*) knockout protocols, which remove part of a gene by homologous recombination, rendering the gene nonfunctional, and (*ii*) gene trapping protocols, which randomly insert a trapping construct downstream of an exon, resulting in a fusion of the gene product to the reporter protein encoded for in the construct. The trapping construct contains a gene encoding a β-galactosidase/neomycin-resistance fusion protein that will be driven by the endogenous promoter of the trapped gene. Randomly generated knockout lines can be produced by introducing the trapping construct into ES cells and selecting for resistance to neomycin. The goal is to produce knockouts of all mouse genes (11) expressed in ES cells. The presence of β-galactosidase allows the assessment of the expression level of the protein in mouse tissues; protein levels are thus correlated to the intensity of the β-galactosidase expression. A number of consortiums are currently producing ES cell lines that contain trapped exons, and they are depositing the sequences in searchable databases.

Here, we describe the mouse derived from the ES cell line XA232 from BayGenomics (which can be accessed at http://baygenomics.ucsf.edu), which has trapped exon 1 of the 2-kinase gene. We show that mouse embryonic fibroblasts (MEFs) heterozygous for the construct show decreased 2-kinase activity and that no mice homozygous for the construct are found from matings of heterozygotes. Therefore, loss of the 2-kinase is lethal. We also used β-galactosidase staining to describe areas of prominent expression in both adult and embryonic mouse tissues.

Materials and Methods

Reagents. All chemicals were reagent grade or better. Restriction endonucleases, DNA modifying enzymes, and general reagents were from Amersham Pharmacia, Fisher, Invitrogen, New England Biolabs, Sigma, and Stratagene unless stated otherwise. PCR was performed by using TaqDNA polymerase from Invitrogen. Oligonucleotide synthesis and DNA sequencing were performed by the Protein and Nucleic Acid Chemistry Laboratory, Washington University. DMEM was from Fisher, and inositol-free DMEM was produced by the Tissue Culture Support Center, Washington University.

Mice. A male chimeric mouse generated from the ES cell line XA232 was obtained from BayGenomics. The ES cell line XA232 was generated by using a gene trap protocol with the

Abbreviations: MEF, mouse embryonic fibroblast; $InsP_5$, inositol 1,3,4,5,6-pentakisphosphate; $InsP_6$, inositol 1,2,3,4,5,6-hexakisphosphate; 2-kinase, $InsP_5$ 2-kinase; E*n*, embryonic day *n*; PP-$InsP_4$, disphosphoinositol tetrakisphosphate.

‡To whom correspondence should be addressed at: Department of Internal Medicine, Washington University School of Medicine, P.O. Box 8125, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail: phil@im.wustl.edu.

© 2005 by The National Academy of Sciences of the USA

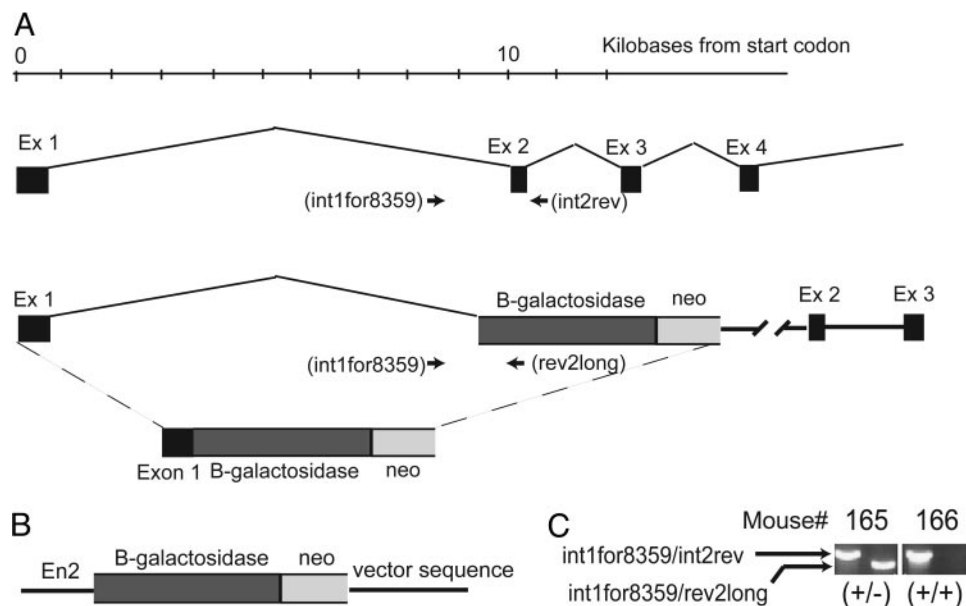

Fig. 1. The mouse 2-kinase locus on chromosome 13. (*A*) The genomic sequence on chromosome 13 corresponding to the 2-kinase locus was obtained from the National Center for Biotechnology Information, and the exons were determined by comparison to the cDNA sequence. Numbering for the region starts at codon 1 of the 2-kinase gene. The primers used for screening, the relative insertion site of the pGT0pfs construct, and the resulting fusion protein are indicated. (*B*) A map of the trapping construct, pGT0pfs. En2, engrailed 2 intron; neo, neomycin-resistance gene. (*C*) PCR genotyping on two offspring from matings of heterozygotes; the primer pairs used to generate each PCR product are indicated.

trapping construct pGT0pfs containing the intron from the engrailed 2 gene upstream of the gene encoding the β-galactosidase/neomycin-resistance fusion protein (see http://baygenomics.ucsf.edu). C57 BL6 mice are from The Jackson Laboratory.

PCR-Based Genotyping of Mice. Genotype of mice was determined by using two parallel PCRs, both using the same forward primer from intron 1, int1for8359 (5′-AGAAGCCTGAGGAGCATGTTCGAT-3′), but different reverse primers; one primes in the second intron of the 2-kinase gene, int2rev, (5′-CATTTCCCTATCCTGGGCAGCA-3′), whereas the second primes within the β-galactosidase gene of the trapping construct, rev2long (5′-GACGACAGTATCGGCCTCAGGAAGATCGCACTC-3′). PCR was carried out by using standard techniques. PCR conditions were 30 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min. DNA was prepared from 0.5 cm of clipped tail from 15-day-old mice and purified by the Puregene Genomic DNA Purification Kit (Gentra Systems) per the manufacturer's protocols. One microliter of final DNA solution was used per 50 μl of PCR. Reaction products were electrophoresed on 1% agarose gels and stained with ethidium bromide.

Generation and Maintenance of MEFs. Embryonic day (E) 12 embryos were used to generate MEFs. Embryos were dissected from the uterus of pregnant mice, separated from their yolk sac, and then homogenized with a razor blade in a p100 tissue culture plate. Homogenized embryos were aspirated 10 times in 10 ml of DMEM supplemented with 10% FBS/100 units/ml penicillin/100 μg/ml streptomycin; MEF lines were maintained in the same media. MEFs were split 1:2 by using a solution of PBS plus 0.5 mM EDTA to release cells from tissue culture plates. Genotyping was performed on the MEFs as described for mouse tails.

[^{3}H]Inositol Labeling of Heterozygote and WT MEFs. MEFs were metabolically labeled with [^{3}H]inositol (ARC, St. Louis) for 4 days. MEF lines heterozygous for the trapping construct or MEF lines from WT littermates were plated at ≈30% confluency in a solution of 80% inositol-free DMEM/20% DMEM supplemented with 10% FBS and 3 μCi/ml (1 Ci = 37 GBq) [^{3}H]inositol for 2 days, split 1:2 in the same conditions, and allowed to grow for 2 more days. The cells from two p100 tissue culture plates were lysed in methanol/0.5 M HCl (2:1) and extracted with chloroform. The aqueous phase was separated, dried, and resuspended in water. The soluble inositol phosphates were separated on a Whatman Partisphere SAX strong anion exchange HPLC column (4.6 × 125 mm) running a 20-min gradient from 0 to 1.7 M ammonium phosphate (pH 3.5), followed by a 30-min step of 1.7 M ammonium phosphate (pH 3.5). Radioactivity was measured by using an inline detector, β-RAM (IN/US Systems, Tampa, FL), and the identity of the individual inositol phosphates was assigned on the basis of elution times of $^{32}PO_4$-labeled internal standards.

β-Galactosidase Staining of Mouse Tissues and Mouse Embryos. β-Galactosidase staining was performed as described in ref. 12. Briefly, tissues were dissected in PBS and fixed in LacZ fixative (0.2% glutaraldehyde/0.1 M $MgCl_2$/5 mM EGTA in PBS) overnight at 4°C. Tissues were then washed in LacZ wash buffer (2 mM $MgCl_2$/0.01% sodium deoxycholate/1% Nonidet P-40 in PBS). After washing, tissues were sectioned on a cryotome at 12-μm thickness. Sections were then stained in X-Gal staining solution (1 mg/ml X-Gal/5 mM potassium ferrocyanide/5 mM potassium ferricyanide in LacZ wash buffer) until appropriate color development. After color development, tissue sections were washed, counterstained with hematoxylin and eosin, and mounted. Whole-mount staining was performed as above except that tissues were stained immediately after fixation and washing.

Results

ES Cell Line XA232 from a Gene Trap Screen at BayGenomics Successfully Targets the Mouse 2-Kinase. A search of the BayGenomics database (http://baygenomics.ucsf.edu) to find a potential source for a 2-kinase knockout mouse yielded one entry, XA232, which contained the sequence of the first exon of the 2-kinase gene. Sequences in this database are generated by sequencing of

Table 1. Genotypes of heterozygous matings

Stage	+/+	+/−	−/−
Adult	66	129	0
E12.5	4	5	0
E8.5	7	15	0

5′-RACE products from mRNA of ES cell lines that contain insertions of the trapping construct pGT0pfs. We obtained a chimeric male mouse generated from the XA232 ES cell line. We crossed this mouse to C57 BL6 females to generate heterozygous mice containing one copy of the potentially trapped 2-kinase gene.

The entry of the BayGenomics database indicated that the trapping construct had inserted into the first intron of the 2-kinase gene. The genomic sequence of the mouse 2-kinase gene was obtained from the National Center for Biotechnology Information. Fig. 1 depicts a map of the 2-kinase locus on mouse chromosome 13 that was generated by comparing the sequence we obtained to the cDNA sequence of the mouse 2-kinase gene. The first intron of the 2-kinase gene is 9.8 kb. We initially used a neomycin PCR to determine genotype until we developed a PCR screen for the trapping construct. We found the location of the trapping construct in the intron by screening the entire first intron of the 2-kinase gene by PCR on neomycin-positive mice, using forward primers spaced 500–1,000 bp apart and reverse primers from various locations within the trapping construct. One PCR primer pair generated a consistently positive reaction. This 1.2-kb reaction product was cloned and sequenced. The trapping construct was confirmed to have inserted ≈9 kb into intron 1 of the 2-kinase gene. The same forward primer was paired with a reverse primer located just downstream of the second exon of the 2-kinase gene (Fig. 1) to generate a 1.5-kb product. These two reactions made up the screening method used to genotype 2-kinase mice (Fig. 1*C*). Of the first 100 mice, every neomycin-positive mouse was also positive for the int1for8359/rev2long PCR product, confirming that the XA232 line has only one insertion of the pGT0pfs construct. Furthermore, we confirmed by RT-PCR on brain mRNA that the insertion results in a fusion between the β-galactosidase gene and exon 1 of the 2-kinase gene (data not shown), thus confirming the 5′-RACE results of BayGenomics.

The Insertion of the pGT0pfs Construct in the XA232 ES Cell Line Is Embryonic Lethal. The confirmed heterozygote mice were mated to generate homozygous mice. Tail clippings from 195 offspring from the heterozygous matings were screened by PCR, with none showing the 1.2-kb product alone; therefore, none was found to be homozygous for the construct (Table 1). The ratio of heterozygous mice to WT mice was 2:1, exactly as expected for a homozygous lethal mutant. When we preformed dissections of the uteri of pregnant mice on day 12 postcoitum to generate MEFs, we noticed that approximately one in four placentae did not contain embryos but did contain blood and debris, which were likely resorbed embryos. Furthermore, no homozygous mutant mice were found by PCR screening of the MEF lines produced. We looked earlier in embryogenesis and performed dissections at day 8.5 and 9.5. Even by day 8.5, all of the uteri from pregnant heterozygous mice contained resorbed embryos. The empty placentae did contain debris, suggesting that the embryos died shortly before day 8.5, but we were unable to isolate them from the surrounding placental tissue for genotyping. Genotyping of the yolk sac from day-8.5 embryos was conducted (Table 1). No embryos were found homozygous for the trapping construct, and the ratio of heterozygotes to WT embryos was again 2:1. Thus, homozygosity for the trapping construct inserted in the 2-kinase gene is lethal.

MEFs Generated from Mice Heterozygous for the Trapping Construct Show Decreased 2-Kinase Activity. We attempted to confirm that the insertion of the trapping construct had disrupted the 2-kinase gene by measuring 2-kinase activity in crude extracts of mouse brains, but we were unable to do so. Therefore, we made MEFs from day-12 embryos to label the cells with [^{3}H]inositol and compare the soluble inositol phosphate pro-

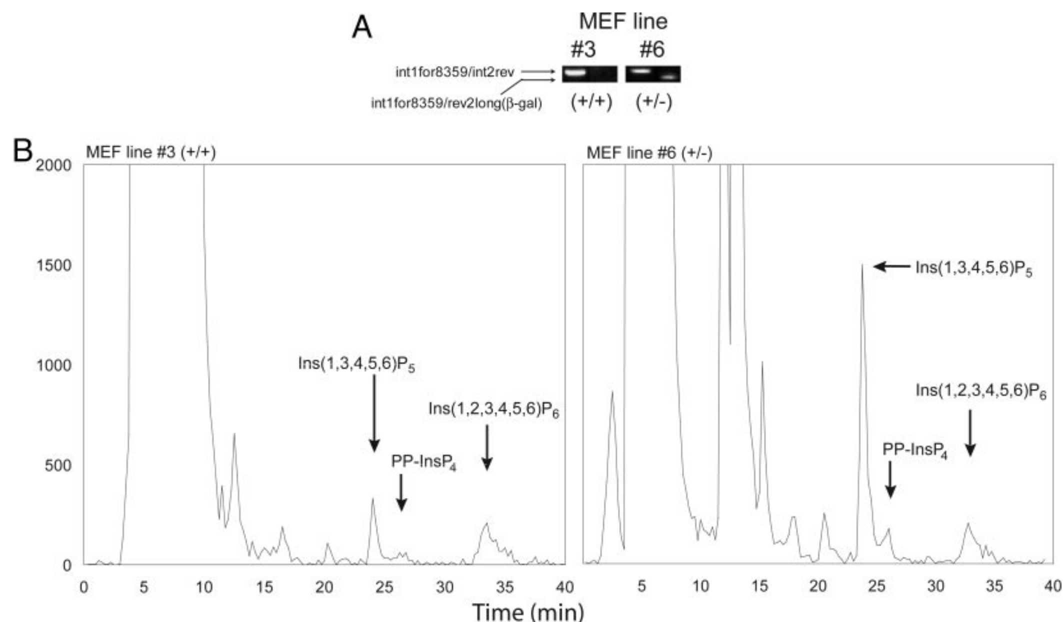

Fig. 2. [^{3}H]Inositol labeling of soluble inositol phosphates of WT or heterozygous MEFs. (*A*) Genotyping of the two MEF lines generated from E12 embryos from one litter used for [^{3}H]inositol labeling. Genotype is indicated under gel. (*B*) MEF lines were labeled for 4 days in the presence of [^{3}H]inositol, their soluble inositol phosphates were extracted, and equal counts were run on a Partisphere HPLC column. Identities of the inositol phosphates are indicated. (*Left*) MEFs from WT embryos. (*Right*) MEFs from heterozygous littermates.

files (Fig. 2). Heterozygous and WT MEFs were labeled with [^{3}H]inositol for 4 days, the soluble inositol phosphates were extracted, and equal inositol phosphate counts were loaded on a Partisphere HPLC column. Compared with WT MEFs, heterozygotes have a labeling profile consistent with decreased 2-kinase activity. $InsP_5$ and disphosphoinositol tetrakisphosphate (PP-$InsP_4$) levels are markedly elevated in heterozygous MEFs ($InsP_5$ levels are 4-fold higher, and PP-$InsP_4$ levels are 2-fold higher), whereas $InsP_6$ levels are almost the same (1,220 cpm for heterozygous mice vs. 1,400 cpm for WT). We conclude that insertion of the trapping construct in the XA232 line disrupts the activity of one copy of the 2-kinase gene, causing a delay in the conversion of $InsP_5$ to $InsP_6$ and therefore an accumulation of the substrate of 2-kinase, $InsP_5$.

Tissue-Dependent Expression of the 2-Kinase in Adults and Embryos. Gene trapping with β-galactosidase enables rapid identification of cell types and tissues that express the trapped gene. Insertion of β-galactosidase into intron 1 of the 2-kinase gene produces an exon 1–β-galactosidase fusion protein product. This product is expressed under control of the endogenous 2-kinase promoter, and, thus, β-galactosidase staining of heterozygous mice (carrying one copy of the trapped gene) offers a sensitive and accurate method to characterize the expression pattern of 2-kinase.

Northern blot analysis of adult RNAs has shown that the 2-kinase transcript is most prominently expressed in brain, heart, and testes (4). To determine which cell types express 2-kinase, heterozygous adult tissues were stained for β-galactosidase activity (Fig. 3 *A*–*D*). Staining of sections through the brain revealed specific expression throughout the hippocampus (CA1, CA2, CA3, and dentate gyrus) (Fig. 3*A*), inner layers of the cerebral cortex (Fig. 3*B*), and Purkinje cells of the cerebellum (Fig. 3*C*). Sections of the heart (Fig. 3*D*) demonstrated punctate staining in cardiomyocytes but not in interstitial cells, blood vessels, or valves. Testis staining was diffuse throughout (not shown).

Because homozygous animals presumably die during embryonic development, we examined sites of 2-kinase expression during embryogenesis to gain insight into probable causes of death (Fig. 3 *E*–*K*). Staining of E9.0 heterozygous animals uncovered expression of 2-kinase in multiple tissues. Whole-

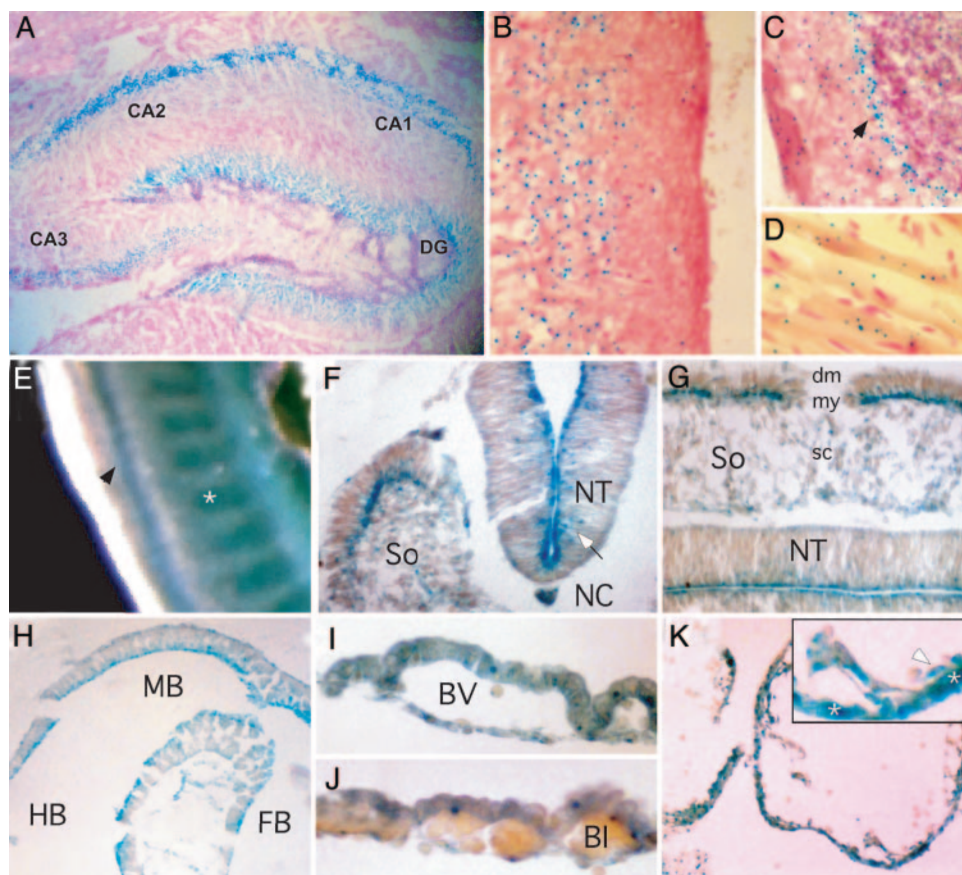

Fig. 3. 2-kinase expression in adult and embryonic mice. β-galactosidase staining of heterozygous adult and embryonic tissues was performed to characterize expression patterns of 2-kinase. (*A*–*D*) In adult tissues, prominent β-galactosidase activity was detected in the brain (*A*–*C*) and heart (*D*). Cryosections of adult brains uncovered specific staining throughout the hippocampus (*A*), inner layers of the cerebral cortex (*B*), and Purkinje cells of the cerebellum (*C*). (*D*) Cryosections through the heart uncovered β-galactosidase staining in cardiomyocytes but not in interstitial cells. (*E*–*K*) Staining of E9.0 embryos revealed expression of 2-kinase in multiple tissues. Intense staining was detected in the neural tube, notochord, embryonic brain, somites, heart, and yolk sac. (*E*) Whole-mount staining showing expression in regions corresponding to the neural tube/notochord (black arrowhead) and somites (white asterisk). (*F*) Transverse section through the trunk showing staining in the neural tube, notochord, and regions of the somite. Within the neural tube, staining is seen in the ventricular zone and in migrating neuroblasts (white arrow). (*G*) Frontal section demonstrating specific staining in the ventricular zone of the neural tube and myotome of the somite. (*H*) β-Galactosidase staining is present throughout the ventricular zone of the embryonic brain. (*I* and *J*) In the yolk sac, staining is detected in epithelial and endothelial cells but is absent from blood islands (*J*). (*K*) Sagittal section through the heart showing staining in the atrial and ventricular chambers. (*Inset*) Within the ventricle, staining is seen in the myocardium (white asterisks) and endocardium (white arrowhead). BI, blood island; BV, blood vessel; DG, dentate gyrus; dm, dermomyotome; FB, forebrain; HB, hindbrain; MB, midbrain; My, myotome; NC, notochord; NT, neural tube; sc, sclerotome; So, somite.

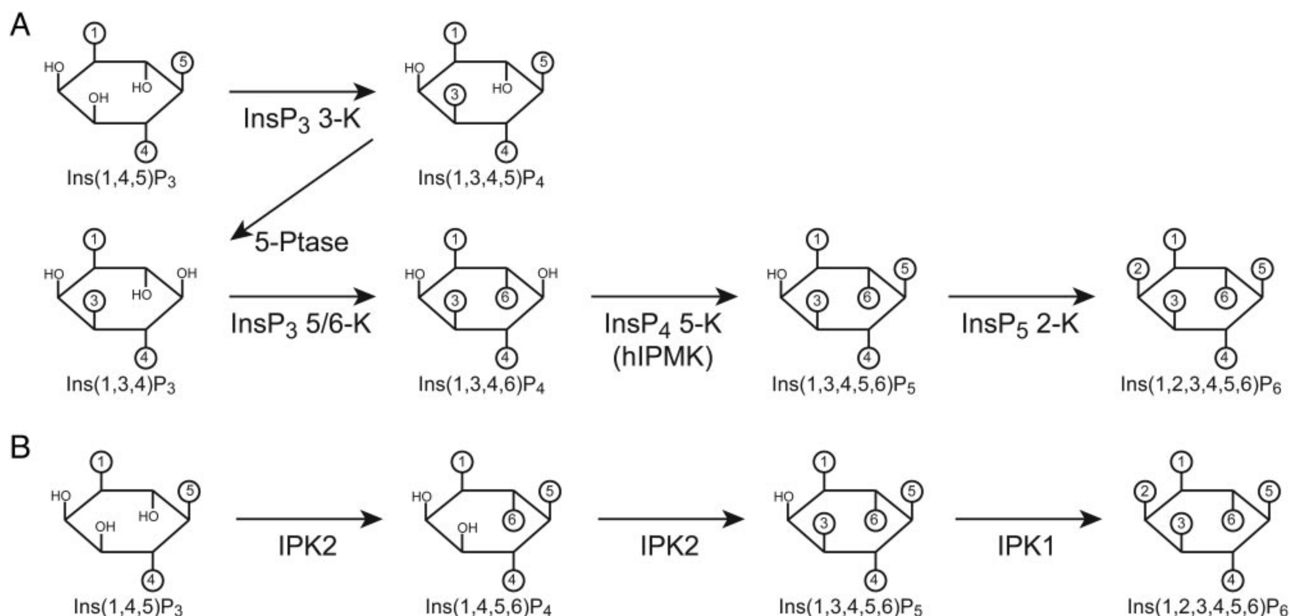

Fig. 4. Pathways for synthesis of $InsP_6$ in different organisms. (*A*) *Homo sapiens.* (*B*) *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Arabidopsis thaliana*. Adapted from ref. 13.

mount staining of E9.0 embryos demonstrated robust staining in regions corresponding to the neural tube, notochord, and somites (Fig. 3*E*). Transverse sections through the neural tube showed staining in the ventricular zone and in migrating neuroblasts (Fig. 3*F*). Intense β-galactosidase activity was also present in the notochord and specific regions of the somite. To further examine expression in the somite, frontal sections through the trunk were stained. These frontal sections contained strong staining in the myotome (future skeletal muscle) of the somite. In addition to the neural tube, staining was present in the ventricular zone and migrating neuroblasts throughout the embryonic brain.

Prominent β-galactosidase activity was also detected in the yolk sac and embryonic heart. Within the yolk sac, staining was present in both the epithelial and endothelial layers (blood vessels) but absent from the blood islands. In the heart, staining was detected in both the atrial and ventricular chambers. High magnification of the ventricular myocardium revealed staining in both the endocardium and myocardium. Other sites of expression included the cardinal vein, aorta, digestive tract, and pharyngeal arches (data not shown).

Discussion

Here, we describe the mouse line generated from the XA232 ES cell line from a BayGenomics gene trap screen. We show that the pGT0pfs construct has successfully inserted into intron 1 of the mouse 2-kinase gene on chromosome 13, where it splices to exon 1 of the 2-kinase gene and disrupts its activity; MEFs that are heterozygous for the construct show decreased 2-kinase activity, as shown by a partial block in the conversion of $InsP_5$ to $InsP_6$. Furthermore, mice homozygous for this construct die before E8.5, indicating that loss of 2-kinase is lethal in mice.

The accumulation of PP-$InsP_4$ in these lines is consistent with the result from the yeast 2-kinase deletions (2) and is thought to be derived from the excess $InsP_5$. The $InsP_6$ levels were equal in the two labelings. We have noted in studies of the human 2-kinase that cells seek to maintain $InsP_6$ levels (13), consistent with these results. Other isomers in the area of $InsP_2$ were also increased, but the columns were run without internal standards of $InsP_2$ isomers, so the identity of the isomers was not confirmed.

The eukaryotic pathway for the production of $InsP_6$ has been worked out by a number of laboratories in yeast and in tissue culture cell lines (Fig. 4) (2, 13, 14). $InsP_6$ is produced by phosphorylation of $InsP_5$ at the D2 position by 2-kinase (Fig. 4). $InsP_5$ is produced from the phosphorylation of an $InsP_4$ isomer by a single enzyme named IPK2, IPMK, or Ins(1,3,4,6) 5-kinase, depending on which laboratory cloned the gene or the organism involved. Some debate remains as to the identity of the $InsP_4$ isomer on which this protein works, although the identity of the protein itself is not in question, because these proteins are homologous. There is general agreement with respect to the substrate of 2-kinase and, thus, the lone pathway to $InsP_6$ from $InsP_5$. Because only one enzyme makes $InsP_5$ (IPK2/IPMK/5-kinase), loss of this enzyme also depletes $InsP_6$ levels (13).

The companion paper describes work by Frederick *et al.* (15) in which the researchers took a targeted approach to knocking out the IPK2 (5-kinase) gene. Because it is responsible for the production of $InsP_5$, the substrate of 2-kinase, it should share some phenotypes with the 2-kinase mouse. IPK2 (5-kinase) knockout embryos die between E8.5 and E9.5; day-8.5 embryos were able to be recovered and labeled with $[^3H]$inositol, showing that the homozygous mutant embryos are lacking $InsP_5$ and $InsP_6$. In the case of 2-kinase, mutants die before day 8.5, an interesting result considering that 2-kinase lies downstream of IPK2 (5-kinase) in the pathway for the production of $InsP_6$. In humans, the half-life of 2-kinase protein is approximately one-half that of 5-kinase protein (data not shown). Because homozygous embryos have no functional enzyme, it must be carried over from maternal stores in the egg. If there is a difference in half-lives in mice, this difference may suggest that the $InsP_6$ stores in the 2-kinase mutants may be depleted earlier than in the IPK2 knockouts. Taken together, the two knockout mice suggest that the higher inositol phosphates are required for life, although they do not necessarily pinpoint which one. Loss of 2-kinase would result in loss of $InsP_6$ and its downstream metabolites, $InsP_7$ and $InsP_8$, and the accumulation of $InsP_5$ and PP-$InsP_4$. The alteration of the levels of any of these inositol phosphates

may be responsible for the observed lethality. Future *in vivo* work would be required to define the required inositol phosphate.

The gross anatomy of the IPK2 knockout suggests that there is a defect in migration of neural crest cells. Although we did not acquire embryos from day 8.5, the strong staining in the neural precursors at this stage supports this hypothesis. Specifically, expression in the notochord is consistent with the IPK2 phenotype. The notochord is involved in neural induction and morphogenesis, and therefore a defect in its function could affect neural crest development and migration. Additional neural defects could be suggested by the β-galactosidase staining in neuroblasts. Because 2-kinase embryos die before neural tube closure, and because embryos with severe neural defects can survive until later embryonic stages, it is likely that the embryonic lethality of 2-kinase knockout embryos in not a result of defects in neurogenesis.

One pattern of expression that would suggest a cause of death in these embryos is the strong staining of the visceral endoderm of the yolk sac. This extraembryonic cell layer performs a number of functions during the period in which the 2-kinase nulls die: specifically, nutrient absorption and delivery to the developing embryo and production of factors involved in embryo development and anterior patterning (16). Efficient endocytosis and exocytosis would be required for nutrient delivery. The proposed roles of $InsP_6$ in endocytosis and exocytosis suggest that it is necessary for these functions in the visceral endoderm. This period of development is also necessary for formation of blood cells and the vasculature of the placenta and embryo. The strong expression around blood vessels and blood islands may indicate a non-cell-autonomous role of $InsP_6$ in angioblast differentiation or blood vessel formation.

The β-galactosidase staining results are also consistent with the Northern blot analysis published previously (4). Expression of 2-kinase in the somatic myotome, embryonic nervous system, and heart is supported by Northern blots demonstrating 2-kinase mRNA in adult skeletal muscle, brain, and cardiac muscle. These results not only show that our β-galactosidase staining corresponds to known sites of 2-kinase expression, but also indicate that 2-kinase expression patterns are conserved between adult tissues and their developmental progenitors.

The staining of the adult brain is significant with respect to previously reported functions of $InsP_6$. $InsP_6$ mass was reported to be highest in the hippocampus and the most variable when stimulated by electric shock, consistent with the strong staining in the hippocampus that we observe (9). Here, it may function to regulate the activity of calcium channels. The 2-kinase expression looks identical to *in situ* staining of type IV phosphatidylinositol 5 phosphatase (17), which differs in the pattern of staining from other phosphatidyl inositol 5 phosphatases (e.g., synaptojanin). The significance of this pattern of staining remains to be seen, although its does suggest some specific function for $InsP_6$ in the hippocampus.

This work was supported by National Institutes of Health Grants HL55272 and HL16634. The ES cell line was obtained from the National Heart, Lung, and Blood Institute-funded Program for Genomics Applications at BayGenomics, which is supported by Grants HL66621, HL66600, and HL66590.

1. Posternak, S. (1919) *C. R. Acad. Sci.* **169,** 138–140.
2. York, J. D., Odom, A. R., Murphy, R., Ives, E. B. & Wente, S. R. (1999) *Science* **285,** 96–100.
3. Feng, Y., Wente, S. R. & Majerus, P. W. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 875–879.
4. Verbsky, J. W., Wilson, M. P., Kisseleva, M. V., Majerus, P. W. & Wente, S. R. (2002) *J. Biol. Chem.* **277,** 31857–31862.
5. Hanakahi, L. A., Bartlet-Jones, M., Chappell, C., Pappin, D. & West, S. C. (2000) *Cell* **102,** 721–729.
6. Hoy, M., Efanov, A. M., Bertorello, A. M., Zaitsev, S. V., Olsen, H. L., Bokvist, K., Leibiger, B., Leibiger, I. B., Zwiller, J., Berggren, P. O. & Gromada, J. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 6773–6777.
7. Norris, F. A., Ungewickell, E. & Majerus, P. W. (1995) *J. Biol. Chem.* **270,** 214–217.
8. Larsson, O., Barker, C. J., Sj-oholm, A., Carlqvist, H., Michell, R. H., Bertorello, A., Nilsson, T., Honkanen, R. E., Mayr, G. W., Zwiller, J. & Berggren, P. O. (1997) *Science* **278,** 471–474.
9. Yang, S. N., Yu, J., Mayr, G. W., Hofmann, F., Larsson, O. & Berggren, P. O. (2001) *FASEB J.* **15,** 1753–1763.
10. Goldstein, J. L. (2001) *Nat. Med.* **7,** 1079–1080.
11. Austin, C. P., Battey, J. F., Bradley, A., Bucan, M., Capecchi, M., Collins, F. S., Dove, W. F., Duyk, G., Dymecki, S., Eppig, J. T., *et al.* (2004) *Nat. Genet.* **36,** 921–924.
12. Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M. & Nagy, A. (1999) *Dev. Biol.* **208,** 281–292.
13. Verbsky, J. W., Chang, S. C., Wilson, M. P., Mochizuki, Y. & Majerus, P. W. (2005) *J. Biol. Chem.* **280,** 1911–1920.
14. Fugii, M. & York, J. W. (2005) *J. Biol. Chem.* **280,** 1156–1164.
15. Frederick, J. P., Mattiske, D., Wofford, J. A., Megosh, L.C., Drake, L. Y., Chiou, S.-T., Hogan, B. L. M. & York, J. D. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 8454–8459.
16. Bielinska, M., Narita, N. & Wilson, D. B. (1999) *Int. J. Dev. Biol.* **43,** 183–205.
17. Kisseleva, M. V. (2001) Dissertation (Washington University, St. Louis).