A Molecular Basis for Inositol Polyphosphate Synthesis in Drosophila melanogaster

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Metabolism of inositol 1,4,5-trisphosphate (I(1,4,5)P₃) results in the production of diverse arrays of inositol polyphosphates (IPs), such as IP₄, IP₅, IP₆, and PP-IP₅. Insights into their synthesis in metazoans are reported here through molecular studies in the fruit fly, Drosophila melanogaster. Two I(1,4,5)P₃ kinase gene products are implicated in initiating catabolism of these important IP regulators. We find dmIpk2 is a nucleocytoplasmic 6-/3-kinase that converts I(1,4,5)P₃ to I(1,3,4,5,6)P₅, and harbors 5-kinase activity toward I(1,3,4,5,6)P₅ and dmIpk3 is a 3-kinase that converts I(1,4,5)P₃ to I(1,3,4,5,6)P₅. To assess their relative roles in the cellular production of IPs we utilized complementation analysis, RNA interference, and overexpression studies. Heterologous expression of dmIpk2, but not dmIpk3, in ipk2 mutant yeast recapitulates phospholipase C-dependent cellular synthesis of IP₄. Knockdown of dmIpk2 in Drosophila S2 cells and transgenic flies results in a significant reduction of IP₆ levels, whereas depletion of dmIpk3, either α or β isoforms or both, does not decrease IP₆ synthesis but instead increases its production, possibly by expanding I(1,4,5)P₃ pools. Similarly, knockdown of an I(1,4,5)P₃ 5-phosphatase results in significant increase in dmIpk2/dmIpk1-dependent IP₆ synthesis. IP₆ production depends on the I(1,3,4,5,6)P₅ 2-kinase activity of dmIpk1 and is increased in transgenic flies overexpressing dmIpk2. Our studies reveal that phospha­tase and kinase regulation of I(1,4,5)P₃ metabo­lic pools directly impinge on higher IP synthesis, and that the major route of IP₆ synthesis depends on the activities of dmIpk2 and dmIpk1, but not dmIpk3, thereby challenging the role of IP3K in the genesis of higher IP messengers.

Inositol 1,4,5-trisphosphate (I(1,4,5)P₃) is a canonical second messenger within the inositol signaling pathway that is produced in response to a wide range of extracellular stimuli and functions to release intracellular calcium through allosteric regulation of an endoplasmic reticulum localized receptor (1–3). Another important role of I(1,4,5)P₃ production in cells is to serve as a precursor for the synthesis of higher phosphorylated IPs that include inositol tetrakisphosphate (IP₄), inositol pentakisphosphate (IP₅), inositol hexakisphosphate (IP₆), and inositol diphosphates (such as PP-IP₄ and PP-IP₅) (2, 4, 5). Functional studies of these IPs provide compelling evidence for their roles as messengers that regulate mRNA export, gene expression, chromatin remodeling, DNA break repair, and vesicular trafficking (5–12). PP-IP₄ contains high energy inositol pyrophosphates that have been implicated in cellular events such as DNA metabolism, chemotaxis and environmental stress responses (13–15).

A molecular basis for the synthesis of higher IPs was first resolved in the budding yeast (Fig. 1A) (7, 8, 16). Synthesis of I(1,4,5)P₃ from PIP2 via phospholipase C (Plc1) and the subsequent action of the kinases Ipk2 (also known as Arg82) and Ipk1 produce IP₆. Ipk2 is a 6-/3-5-kinase that phosphorylates a variety of I(1,4,5)P₃, IP₄, and IP₅ substrates (8, 17, 18). Ipk1 is a 2-kinase that utilizes primarily I(1,3,4,5,6)P₅ but also has activity toward other IPs (7, 19, 20). A third kinase, Kcs1, is an inositol diphosphate synthase capable of phosphorylating IP₅ or IP₆ to generate PP-IP₄ and PP-IP₅ (10, 15, 17).

In metazoans, biochemical studies have suggested that IP₆ is synthesized from I(1,4,5)P₃ via a five-step route of several kinases and a phosphatase as shown in Fig. 1B (2, 5, 21). Two key differences between the proposed metazoan and defined yeast pathways are: 1) the initiation step in the synthesis of IP₆ occurs through an I(1,4,5)P₃ 3-kinase (IP3K) and not Ipk2, and 2) the requirement of an I(1,3,4,5,6)P₅ 5/6-kinase, orthologs of which have not yet been found in the budding yeast genome. The cloning of plant and metazoan Ipk2 (also referred to as inositol phosphate “multi-kinase”; IPMK) and Ipk1, both of which were found to possess similar activities as their yeast counterparts, has raised the possibility that synthesis of higher IP messengers in metazoans may occur similarly to that in yeast (18, 20, 22–26).

To further probe the synthesis of higher IPs in metazoans at the molecular level we initiated studies in the fruit fly, Drosophila melanogaster. Using a bioinformatics approach, we identified orthologous kinase and phosphatase genes in the Drosophila that may contribute to higher IP synthesis in metazoans. Detailed biochemical analysis was used to characterize the I(1,4,5)P₃ kinases proposed to initiate the first phosphorylation step of higher IP production, and subsequent use of RNA interference in culture cells and transgenic flies provided genetic insight.
specific knockdowns of each activity to dissect the IP synthesis pathway. We find that the synthesis of IP₃ and IP₆ in the fruit fly is dependent on Ipk2 and Ipkl, but not IPK3, 5-phosphatase or I(1,3,4)P₅/6-kinase activities. Our data provide a novel molecular basis for higher IP production in a metazoan and support a role for Ipkl2 as a key gatekeeper for activating this important signaling pathway.

MATERIALS AND METHODS

Plasmid Construction—Drosophila EST SD14726, SD19941 were obtained from Research Genetics and used as templates for amplification of dmIpkl2 and dmIP3Kß, respectively. PCR reactions were carried out using the Expand High Fidelity PCR System (Roche Applied Science). The following primers were used: dmIP3Kß: 5′-ATA TCC CGG GGA ATG CCG CGG GAC TAT GCC TAC-3′ (forward) and 5′-GCT CTT ACG TCT AGA TTA GGG GTT GCT CTC TTC-3′ (reverse), dmIpkl2: 5′-ACG TCT GGA ATT CAG ATG GCC AAC GAT CAG GAG-3′ (forward) and 5′-TGA CTT GCA GTC TCA TCG TGG GAT TAT GGA TTG-3′ (reverse). PCR products were then cut with the following enzymes and ligated in-frame with the pUNI10 Lox recombination site or into the pGEX-KG GST expression vector (Amersham Biosciences) according to the manufacturer’s instructions. Proteins fusion proteins were purified over glutathione Sepharose (Amersham Biosciences) (27). For in vivo and in vitro analysis, fusion proteins were cleaved with thrombin protease (900 ng) was then added to this reaction and incubated at 37 °C to produce I(1,3,4)[32P]P₃. The I(1,3,4)[32P]P₃ product was incubated at 37 °C for various times from 5 to 200 min. Reactions were stopped with 0.5N HCl and analyzed by HPLC or TLC.

Bacterial Expression of dmIpkl2 and dmIP3Kß—For recombinant protein expression, transformed Escherichia coli (DH5α) were grown at 37 °C to an OD₆₀₀ of 0.6 and induced with 0.1 mM isopropyl-β-D-galactopyranoside for 4 h at 0 °C. Cells were recovered by centrifugation at 4 °C, resuspended in ice-cold 50 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM dithiothreitol, Complete Mini protease inhibitor mixture (Roche Applied Science) and lysed with four passes through a cell cracker (a high shear fluid processing system for cell rupture, Microfluidics Corp.). Lysates were then cleared by centrifugation at 14,000 × g. The GST fusion proteins were purified over glutathione Sepharose (Amersham Biosciences) according to the manufacturer’s instructions. Purified proteins were injected into rabbits and antiserum was isolated according to the manufacturer’s instructions (Amersham Biosciences). The kinase kinetics was determined by precipitating enzymes and ligated in-frame with the pUNI10 Lox recombination site or into the pGEX-KG GST expression vector. Bacterial Expression of dmIpk2 and dmIP3K

In Vivo Labeling of S2 Cells and Saccharomyces cerevisiae—Partispere strong anion exchange HPLC. The kinase kinetics was calculated from measured changes in the substrate area under the curve (AUC) peak.

RNAs in S2 Cultured Cells—Drosophila ESTs SD14726, SD19941, RE1770, and GH07317 were obtained from Research Genetics and used as templates for amplification of dmIpkl2, dmIP3Kß, dm5Ptau1, and dmIpkl1, respectively. dmIP3Kß template was amplified using genomic DNA prepared from S2 cells. FTZ (dsRNA control) template was amplified from a cDNA library provided by Rick Feehan. Templates for dsRNA synthesis were made by PCR using the EST clones as templates and the following primers. dmIpkl2: 5′-TTAA'TACG ACT TCT AGAT GAGAG TGC TCTG GAGAT CTTGG CTTGG GATA CTTC3′ (reverse), dmIP3Kß: 5′-TTAA'TACG ACT TCT AGAT GAGAG TGC TCTG GAGAT CTTGG CTTGG GATA CTTC3′ (reverse), dm5Ptau1: 5′-TTAA'TACG ACT TCT AGAT GAGAG TGC TCTG GAGAT CTTGG CTTGG GATA CTTC3′ (reverse), and 5′-TTAA'TACG ACT TCT AGAT GAGAG TGC TCTG GAGAT CTTGG CTTGG GATA CTTC3′ (reverse), dsRNA control Ftz: 5′-TTAA'TACG ACT TCT AGAT GAGAG TGC TCTG GAGAT CTTGG CTTGG GATA CTTC3′ (reverse) and 5′-TTAA'TACG ACT TCT AGAT GAGAG TGC TCTG GAGAT CTTGG CTTGG GATA CTTC3′ (reverse), and 5′-TTAA'TACG ACT TCT AGAT GAGAG TGC TCTG GAGAT CTTGG CTTGG GATA CTTC3′ (reverse) and 5′-TTAA'TACG ACT TCT AGAT GAGAG TGC TCTG GAGAT CTTGG CTTGG GATA CTTC3′ (reverse). Primer sequences are conserved in the fruit fly and human to a high degree. The RNAi knockdowns were extended over a period of 15 days, dsRNA-treated cells were counted after 8 days, and again treated with dsRNA as described above.

Northern Analysis of S2 Cells—Probes for Northern analysis were made using DNA templates for dsRNA as described above. DNA templates were then incubated with ³²P-labeled dCTP and the random-primer DNA labeling kit (Amersham BioSciences) according to the manufacturer’s instructions. S2 cells were treated with dsRNA for 6 days as described above. 3 ml of cells were then recovered by centrifugation at 4 °C. Total RNA was then isolated using the Qiagen RNA pull-down system according to the manufacturer's instructions. RNA was then separated on a 2% agarose gel and transferred to hybrid nitrocellulose paper (Amersham BioSciences). The DNA probe was then hybridized in ExpressHyb hybridization solution according to the manufacturer’s instructions (Clontech).

Kinase Assays on S2 Cell Extracts—S2 cells were treated with dsRNA to deplete 5Ptau1 (3 ml) were recovered after induction and mounted on robotic stage. Pellets were resuspended in 200 μl of ice-cold 50 mM Tris, pH 7.5, 3 mM MgCl₂, 2.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, and Complete Mini protease inhibitor mixture. The cells were lysed with ten-half-second pulses, three times on a setting of 5 using a Branson sonifier and diluted to a stock concentration of 1 mg/ml. Activity assays were carried out in 20-μl volumes with 5 μg of S2 extract, 10 mM HEPES pH 7.5, 10 mM NaCl, 10 mM MgCl₂, 2 mM ATP, 0.25 units/ml creatine phosphokinase, 10 μM phosphatase, 5 μM inositol polyphosphate, and 10,000–20,000 cpm labeled inositol polyphosphate. Labeled inositol polyphosphates were [³²H][I(1,4,5)P₃, I(1,4,5,6)P₄, and I(1,3,4,5,6)P₅], respectively. Reactions were incubated at 37 °C for various times from 5 to 200 min. Reactions were stopped with the addition of 0.5 N HCl and analyzed by HPLC or TLC.

In Vivo Labeling of S2 Cells and Saccharomyces cerevisiae—S2 cells were treated with dsRNA as described above or no dsRNA as a control. On day four or thirteen, [³²H]inositol (American Radiolabeled Chemicals) was added to the media to a final concentration of 80 μCi/ml. Cells were then incubated for 2 days at 25 °C. On day 6 or 15, the cells were recovered by centrifugation and washed once in Dulbecco's phosphate-buffered saline (Invitrogen Life Technologies, Inc.). Yeast cultures were treated with 50 μg/ml Fluconazole for 2 days at 30 °C for 2 days. [³²H]inositol was added to a final concentration of 80 μCi/ml. Soluble inositol polyphosphates were harvested and analyzed by HPLC using a Partisphere SAX strong-anion exchange column as described previously (7).

Construction and Analysis of Fly Lines—Df2;L(BSC)S16 was obtained from the Bloomington stock center. Nle” was a kind gift from S. Cohen (27). For dmIpkl2 RNAi, we obtained the pWIZ vector from Richard
Carthew to construct a transgene containing inverted repeats of a 200-bp region of dmIPK2 separated by a 74-bp intron spacer (28). The following primers were used to generate the dmIPK2 region containing Xbal restriction sites on both ends: 5'-CTAGT CTAGA ACGAC TATTG CTTTG GAAAC GAGTG ATCAGG TGGAG C TCGA A AGCTT TCATC GGTGG (forward) and 5'-TGTGAGC TCTAGG AGGATG TGATTG TGTGGGA TTATG TGTGG (reverse). The PCR product was then subcloned into the pWIZ vector using AvrII and NheI sites and checked for proper orientation. For expression of FLAG-dmIPK2 we cloned the dmIPK2 coding region into pBSIISK with sequence for an N-terminal FLAG epitope inserted (a kind gift from Rick Fehon). The following primers were used to PCR dmIPK2 from the EST containing SmaI and HindIII restriction sites: 5'-CTCTCG AGTTAG GAGGT AGTACG GAGTG TGGAG AAGAC TGGCT G-3' (forward) and 5'-CTACT CTAGA TCATC ATCGG CGGAC TGGATT GATTG G-3' (reverse). The FLAG epitope and dmIPK2 were then subcloned into pUAST from AvrII and NheI sites and checked for proper orientation. Anti-FLAG M2 monoclonal antibody (Sigma) was added to the blocking buffer and incubated for one hour with agitation. Salivary glands were washed three times with PBS-T. An anti-mouse cy3 secondary antibody (Jackson ImmunoResearch) was incubated with the salivary glands for one hour. The glands were washed, stained with DAPI (Sigma), and mounted with ProLong Antifade (Molecular Probes). Glands were visualized with a Zeiss Axioscope equipped with Metamorph software.

**RESULTS**

Identification of Drosophila Genes Involved in Inositol Polyphosphate Synthesis—In this study, we utilize the tractable fly model system to study mechanisms of higher IP synthesis in metazoans. Initially, our analysis focused on two I(1,4,5)P3 kinase-dependent IP synthesis models, one derived from genetic yeast studies and one proposed from biochemical characterization of pathways metazoans (Fig. 1A). The initiation steps for each model are distinct and require I(1,4,5)P3 kinase activities encoded by separate gene products. Ipk2 functions in yeast as a dual specificity kinase that converts I(1,4,5)P3 to I(1,3,4,5,6)P5 and in metazoans IP3K phosphorylates I(1,4,5)P3 to I(1,3,4,5)P5. Of interest, the two I(1,4,5)P3 kinases are members of a small family of IP kinases that share an evolutionary ancestor and common amino acid motifs, but possess divergent substrate specificities (6, 8). The second and third steps of the pathway proposed in metazoans re-
quire inositol polyphosphate 5-phosphatase, which converts I(1,3,4,5,6)P_5 to I(1,3,4,6)P_4 and I(1,3,4,5)P_4 5/6-kinase that produces I(1,3,4,5,6)P_5 from the I(1,3,4,6)P_4 substrate. The last step for IP_6 synthesis in both yeast and metazoans requires Ipk1, an I(1,3,4,5,6)P_5 2-kinase.

In order to identify various kinase and phosphatase members of these pathways, we performed in silico searches (www.ncbi.nlm.nih.gov/BLAST/and www.flybase.net/blast/) of the Drosophila genome using hallmark motifs for each gene product. Four predicted gene products were found that harbor the IP kinase motif PXXXDXKKG: CG13688 (PcvmDvKmG), CG4026 (PcvmDvKmG), CG1630 (PcvmDcKvG), and CG10082 (PciDvKmG). Sequence analysis of CG13688 (designated here as dmIpk2) revealed that it is most similar to Ipk2 homologs from other species (Fig. 1B). dmIpk2 is a 310 amino acid polypeptide encoded by a single exon with 37% similarity and 24% identity to the human Ipk2. CG10082 (designated dmIP6K) shared the highest degree of homology with mammalian IP_6 kinases (also referred to as diphosphyryl inositol synthetases). IP6Ks use IP_5 and IP_6 as substrates to generate inositol pyrophosphate species. The putative dmIP6K open reading frame codes for an 893 amino acid protein and contains 36% similarity to human IP6K2 (GenBank™ accession number AF177145). Further studies will be required to elucidate whether this putative gene translates into a functional IP_6 kinase as it will not be discussed here. The other two PXXXDXKKG-containing proteins (CG4026 and CG1630) share 59% similarity and are highly homologous to mammalian IP3K (Fig. 1B). Three different isoforms of IP3K (A–C) are described in higher eukaryotes that differ in sequence, tissue and subcellular localization. CG4026 (dmIP3Ka, originally annotated as dmIP3K1) was previously identified as an IP3K that confers resistance to oxidative stress when overexpressed in adult flies (31). CG1630 (dmIP3Kβ, originally annotated as dmIP3K2) is most similar to the human IP3K B (45% similar and 37% identical). The putative IP3Ks do not contain calmodulin-binding sites at their N terminus as are found in mammalian I(1,4,5)P_3 3-kinases.

Type I enzymes in the 5-phosphatase family have high catalytic efficiency as D5 specific phosphatases against I(1,4,5)P_5 and I(1,3,4,5)P_5, and contain a highly conserved motif (r/n)-XP(a/w/y)/(d)P(IVG)/1(1) (30). This pattern motif was used to search Drosophila genome and identified a predicted gene product, CG31107 (designated dm5Ptae1), of 400 amino acids having 58% similarity and 43% identity to the human type I 5-phosphatase identified. Although no other putative type I 5-phosphatases were identified in the annotated genome we found that Drosophila contain open reading frames that encode two type II 5-phosphatase isozymes (CG6805 and CG9784), a synaptojanin ortholog (CG6562), and a putative Type IV family member (CG10426). Based on our analysis, we hypothesized that Drosophila is capable of generating I(1,3,4)P_3 through the activities of IP3K and 5-phosphatase. However, it does not appear that the annotated Drosophila genome possess genes products with significant sequence similarity to mammalian I(1,3,4)P_3 5/6-kinases, suggesting that the fly may not synthesize higher IPs through the proposed metazoan route. If this is the case then IP3K-dependent higher IP synthesis in Drosophila may employ a different set of enzymes than previously described.

To identify a Drosophila Ipk1, we searched the genome for putative 2-kinases containing conserved amino acid motifs including EXKPK (7, 19, 20). Using the human IP_5 2-kinase (hsIpk1) amino acid sequence, we identified a single ortholog, CG30295 (designated dmIpk1) that translated into a polypeptide sharing 34% similarity and 25% identity to hsIpk1. In summary, our genome analysis indicate that Drosophila have many of the members of both yeast and metazoan pathways required for IP_6 synthesis, with the notable exception of a I(1,3,4)P_5 5/6-kinase. We therefore postulate that flies synthesize IP_5 and IP_6 through a pathway that requires the activities of dmIpk2 and dmIpk1.

Cloning and Characterization of Drosophila I(1,4,5)P_3 Ki-nases—Of the three I(1,4,5)P_3 kinase gene products found, we tested if dmIpk2 and dmIP3Kβ translated into functional I(1,4,5)P_3-specific kinases. GST fusion proteins of each kinase were purified from E. coli and incubated with I(1,4,5)P_3 plus ATP, and the reaction products were analyzed by HPLC. dmIP3Kβ phosphorylated I(1,4,5)P_3 to IP_5 (Fig. 2, A and B); whereas dmIpk2 phosphorylated I(1,4,5)P_3 to generate IP_5 (Fig. 3, A and B). To confirm that the product of the dmIP3Kβ reaction is I(1,3,4,5)P_5, we tested it for sensitivity to purified human type I 5-phosphatase, a I(1,3,4,5)P_5 5-phosphatase. Treatment completely converted the product to I(1,3,4)P_3, demonstrating that dmIP3Kβ functions as an I(1,4,5)P_3 3-kinase (Fig. 2C). We determined the kinetic properties of dmIP3Kβ and found that the kinase has a high affinity for I(1,4,5)P_3 (K_m = 72 nm), transfers the phosphate to the D3 position at a rate of 25 nmol/min/mg and has a catalytic efficiency of 2.47 × 10^5 s^-1 M^-1 (Table 1).

To understand the mechanism by which dmIpk2 phosphorylates I(1,4,5)P_3 to generate IP_5 we characterized the inter-
mediate in the two-step reaction. Depending on the species, Ipk2 orthologs phosphorylate I(1,4,5)P₃ to IP₅ through either I(1,4,5,6)P₄ or I(1,3,4,5)P₄. In the case of yeast or plant Ipk2, phosphorylation occurs first at the D-6 and then the D-3 position (8, 17, 18, 26). In contrast, rat and human Ipk2 prefer to phosphorylate I(1,4,5)P₃ first at the D-3 and then the D-6 positions (22–24). We tested the recombinant dmIpk2 under conditions that approximate single turnover kinase reactions with trace amounts of 32P-labeled ATP and cold I(1,4,5)P₃, and found that about 60% of the product was I(1,4,5,6)P₄ and 40% was I(1,3,4,5)P₄ (data not shown). We report that dmIpk2 can phosphorylate either I(1,3,4,5)P₄ or I(1,4,5,6)P₄ to IP₅ under conditions of mass amounts of IP₄ substrate and trace ATP (Fig. 3, C–F). Under conditions of mass amounts of both I(1,4,5)P₃ (1 mM) and ATP (2 mM), and quenching prior to complete conversion (similar to the 2.5 min time point shown in Fig. 4), we found that I(1,4,5,6)P₄ was the only detectable intermediate (data not shown). A time course of substrate conversion indicated that the I(1,4,5,6)P₄ intermediate and IP₅ final product form at similar rates (Fig. 4), consistent with dmIpk2 functioning as a processive enzyme. The relative Kᵅ values of dmIpk2 toward its substrates further support this finding (Table I) as the enzyme has a higher affinity for I(1,4,5,6)P₄ (96 nM) than I(1,4,5)P₃ (444 nM). dmIpk2 phosphorylation of I(1,4,5)P₃ occurs with a higher maximal velocity than of I(1,4,5,6)P₄ (241 nmol/min/mg versus 46 nmol/min/mg) (Table I). Taken together these data suggest

**Table 1**

| Substrate | Km (nM) | Vₘₐₓ (nmol/min/mg) | kₗₐₜ/Kₘₐₓ (s⁻¹) |
|-----------|---------|------------------|-----------------|
| dmIpk2    |         |                  |                 |
| I(1,4,5)P₃| 444.0 ± 157| 241.0 ± 73.0     | 3.2 × 10⁵       |
| I(1,4,5,6)P₄| 737.0 ± 122| 89.0 ± 40.0      | 7.1 × 10⁵       |
| I(1,3,4,5)P₄| 96.0 ± 0.5  | 46.0 ± 12.0     | 2.8 × 10⁶       |
| I(1,3,4,6)P₄| 54.0 ± 16   | 36.0 ± 3.9      | 3.9 × 10⁶       |
| dmIP3Kβ    |         |                  |                 |
| I(1,4,5)P₃| 72.0 ± 9 | 25.0 ± 6.0       | 2.4 × 10⁵        |

**Fig. 3.** dmIpk2 encodes a I(1,4,5)P₃ and IP₄6-/3-/5-kinase in vitro. For each assay 1.0 μM either ³²P-labeled or ³H-labeled substrate was incubated at 37 °C with or without 100 ng of GST-tagged dmIpk2 for 25 min in a buffer containing 50 mM HEPES (pH 7.5), 50 mM NaCl, 10% glycerol, and 0.1 mg/ml BSA, 10 mM MgCl₂, 2 mM ATP. The products were separated by Partisphere strong anion exchange HPLC. Substrates used: I(1,4,5)P₃ (A and B), I(1,3,4,5)P₄ (C and D), I(1,4,5,6)P₄ (E and F), I(1,3,4,6)P₄ (G and H). The first two peaks in G and H are free ³²P and [³²P]ATP left over from the production of I(1,3,4,6)P₄. The IP₅ products were identified by comparison of elution times with known IP standards.

**Fig. 4.** Time course of dmIpk2 production of IP₅ from I(1,4,5)P₃. 1.0 μM I(1,4,5)P₃ and trace amounts of [³H]I(1,4,5)P₃ were incubated at 37 °C with 2 mM ATP/10 mM MgCl₂ and 25 ng of GST-dmIpk2 in a buffer consisting of 50 mM HEPES (pH 7.5), 50 mM NaCl, 10% glycerol, and 0.1 mg/ml BSA for the time points shown. The reaction was stopped with the addition of 10 mM NH₄PO₄ HPLC buffer, and the products were separated by Partisphere strong-anion exchange HPLC. The percent of each product was calculated from total IPs on the HPLC trace.
higher IP synthesis. We found that a pendent reaction is hypothesized as a critical step for metazoan cell produces I(1,3,4,6)P₄ does not appear to be conserved. The reported properties of the human homolog of Ipk2 for its I(1,3,4,6)P₄ were similar (Table I). This is in agreement with the experiments of control treated extract (Fig. 6 A). dmIP3K and IP3K activity over 95%. The catalytic efficiency of dmIP3K, can cooperate with an IP₅ 2-kinase to generate IP₆ in dividing cells.

**RNAi in Drosophila S2 Cells Effectively Reduces IP Kinase and Phosphatase Expression**—We next examined higher IP synthesis pathways in the cultured fly cell line, Schneider S2. Extracts prepared from cultured S2 cells were found to have both phosphatase and kinase activities toward [³H]I(1,4,5)P₃ in the presence of ATP. Specifically, we observed the formation of IP₂, IP₄, IP₅, and a small amount of IP₆ when incubated for long periods of time (data not shown). We hypothesized that endogenous dmIP3K and/or dmIP2 contribute to the I(1,4,5)P₃ phosphorylation in the cell extracts. We further postulated that dmIP5PlaseI may contribute to the I(1,4,5)P₃ phosphatase activity. Therefore, we treated the S2 cells with dsRNA to generate specific knockdowns via RNA interference (RNAi) (32). The success of the RNAi on the targeted genes was confirmed by measuring the level of mRNA degradation between control and target cells by Northern analysis (Fig. 6A). dmIP3Kβ, dmIP2, and dmIP5PlaseI mRNA levels were reduced by at least 80%, 61%, and 82% respectively. Of note, the 61% dmIP2 mRNA reduction may be an underestimate as degraded RNA interfered with our measurement. To this end, a Western blot analysis with a dmIP2-specific polyclonal antibody revealed a 90% reduction in protein levels in cells treated with dmIP2 dsRNA compared with untreated cells (Fig. 6B).

To quantify the relative contributions of dmIP3Kβ and dmIP2 toward the phosphorylation I(1,4,5)P₃, we carried out kinase reactions from dsRNA-treated S2 extracts. Competing kinase and phosphatase reactions in the assays made individual specific activity measurements difficult to determine using conventional time course assays. Therefore, we analyzed reaction products at early time points (and less than 5% substrate conversion) by HPLC under conditions that separated IP molecules ranging from IP₁ to IP₅ (for example Fig. 6C). In vitro phosphorylation of I(1,4,5)P₃ to IP₅ and IP₆ was significantly reduced in extracts made from either dmIP3Kβ- or dmIP2-depleted cells indicating 1) that each kinase was functional in extracts and 2) that the RNAi worked for both (data not shown). When dsRNA to both dmIP3Kβ and dmIP2 were simultaneously added to cells, extracts had little observable I(1,4,5)P₃ phosphorylation as compared with an equal amount of control treated extract (Fig. 6C), suggesting that dmIP2 and dmIP3Kβ comprise nearly all the I(1,4,5)P₃ kinase activities in S2 cells. Kinase activity derived from dmIP3Kβ may not be detectable in appreciable amounts in S2 cells. We further quantified the effect of dmIP2 knockdown on I(1,4,5)P₃ kinase activity (Fig. 7A) and found that RNAi treatment reduced activity over 95%.

Additionally, we tested the effects of RNAi depletion of IP₅ 2-kinase and I(1,4,5)P₃ 5-phosphatase activities. Extracts pre-
minor (Fig. 8A). The IP₃ isomer detected co-elutes with both I(2,3,4,5,6)P₅ and I(1,2,3,4,5,6)P₆, which under these conditions are not separable. We report that relative distribution of radioactivity into the Ins, IP₆, and IP₅ peaks is ~92, 3.0, and 0.8% of the total tritiated sample recovered from the soluble extract.

We tested whether S2 cellular IP₅ and IP₆ synthesis requires the activities of dmIP3Kβ or dmIpk2 by labeling cells that were RNAi-depleted for either gene. No significant changes in IP₅ or IP₆ synthesis were found in dmIP3Kβ-depleted cells after six or fifteen days of knockdown (Fig. 8, B and F). Similarly, cells treated with dsRNA for dmIP3Kα or dmIP3Kβ and dmIP3Kβ together did not affect higher IP synthesis (data not shown). In contrast, IP₅ and IP₆ levels were significantly decreased in Ipk2-depleted cells (Fig. 8, C and F). We found in repeated experiments that IP₅ levels were often reduced below the limit of detection on our HPLC apparatus, whereas IP₆ levels where decreased by an average of 75%. These data demonstrate that Ipk2 is required for IP₅ and IP₆ synthesis in Drosophila S2 cells.

Our data indicate that Ipk2 contributes to IP₆ synthesis in S2 cells by converting I(1,4,5)P₃ to I(1,3,4,5,6)P₅, which may then be phosphorylated to IP₆ by the 2-kinase dmIpk1. Analysis of steady-state radiolabeled dmIpk1-depleted S2 cells show that IP₅ levels were reduced, as measured from four independent experiments, by an average of 44% (Fig. 8, D and F). RNAi of dmIpk1 also caused levels of I(1,2,3,4,5,6)P₆ or I(2,3,4,5,6)P₆ to decrease by up to 54% suggesting that the isomer is a product of the kinase via 2-phosphorylation of I(1,4,5,6)P₄ or I(3,4,5,6)P₄. It is also possible that the D2-phosphorylated IP₅ peak arises from dephosphorylation of IP₅ at the 3- or 1-position, in which case depletion of IP₅ may also reduce this IP₅ peak by mass action. Additionally, in the Ipk1-depleted labeled cells, we observed the accumulation of a new IP₅ isomer that co-elutes with an I(1,3,4,5,6)P₅ standard. A similar build up of I(1,3,4,5,6)P₅ occurs in yeast ipk1Δ, supporting the hypothesis that Ipk1 uses I(1,3,4,5,6)P₅ as a substrate in vivo (7). These data indicate that Ipk1 contributes to IP₅ synthesis in Drosophila S2 cells by phosphorylating I(1,3,4,5,6)P₅. To further determine if IP₅ synthesis occurs through a single pathway of phosphorylation by Ipk2 and Ipk1, we simultaneously treated cells with dsRNA for both kinases. IP levels from the double-depleted cells were indistinguishable from those seen when Ipk2 was depleted alone (Fig. 8F). This suggests that Ipk2 and Ipk1 are epistatic in S2 cellular production of IP₅ and IP₆.

We also tested whether the Drosophila IP₆ synthesis pathway requires a type 1 5-phosphatase as predicted for step three in the metazoan model (Fig. 1A). This would be surprising in light of our results indicating that RNAi depletion of dmIP3Kα and dmIP3Kβ (step 1 of that model) did not disrupt IP₆ synthesis. Nonetheless, we treated cells with dsRNA targeting dm5PaseI, radiolabeled with inositol and analyzed extracts by HPLC. Interestingly, rather than a depletion, we noticed a significant increase in IP₅ and IP₆ levels as compared with control cells (Fig. 8, E and F). Likewise, co-depletion of Ipk2 and Ipk1 led to an elevation of IP₅ and IP₆ as compared with Ipk2-depleted cells alone (Fig. 8, F and G). One possible explanation for this is that depletion of Ipk2 leads to an increase in an I(1,4,5)P₃ pool that is shared with the Ipk2-dependent pathway. Similar increases in higher IPs were measured when dmIP3Kβ was depleted together with Ipk2 or Ipk1 suggesting that the kinase may also draw from the same I(1,4,5)P₃ pool (Fig. 8, F and G).

Ipk2-mediated Higher IP Production in Adult Flies—We next tested whether higher IP synthesis in the adult fly depends on...
dmIpk2 through loss and gain of function analysis in transgenic insects. Wild-type (w1118) male flies were *in vivo* labeled by feeding them a 5% sucrose solution containing [3H]inositol for 4 days. Distribution of radioactive metabolites observed by HPLC analysis of the whole fly extracts revealed an IP profile similar to S2 cells (compare Fig. 8A to Fig. 9A, top panel). To test whether the IP synthesis depends on *dmIpk2*, we examined IP levels in fly lines that were deficient in expression of the kinase. Df(2L)BCS16 is a deficiency line that fails to complement Ush and Gsc that reside on either side of *dmIpk2* at 21E2 (Kevin Cook-Bloomington stock center). Another deficiency line was obtained that is derived from a P-element excision from Nle, a neighboring gene to *dmIpk2* (Nle/H90048) (27).

We speculated that the break points of the Nle/H90048 deficiency include the *dmIpk2* open reading frame. Additionally, we generated a transgenic line that expresses an inverted repeat of *dmIpk2*-specific sequence under the Gal4 promoter for RNAi (p[WIZ-Ipk2]) (28). Western blot analysis of flies from each line revealed that *dmIpk2* levels were reduced in all three deficient lines as compared with wild type (Fig. 9B). Antigen levels of *dmIpk2* in both Df(2L)BCS16 and Nle/H90048 were reduced by 50%, consistent with them being heterozygous for *dmIpk2* (Fig. 9B).

Ubiquitous expression of the *dmIpk2* inverted repeat using an Actin-Gal4 promoter caused *dmIpk2* levels to decrease 81% (Fig. 9B).

We next *in vivo* labeled 3–6-day-old adult flies (25–35 flies)
from the dmIpk2-deficient lines along with wild-type flies (w1118). After labeling the flies for 4 days, their soluble IPs were extracted and analyzed by HPLC. In all three deficient flies, the relatively weak IP$_6$ peak observed in w1118 flies (Fig. 9A, top trace) decreased below the limit of detection suggesting a loss of synthesis (data not shown). Df(2L)BCS16 and Nle$^{38}$ flies had IP$_6$ levels that were reduced 20–30% as compared with w1118 flies whereas levels in the p[WIZ-Ipk2]/Actin-Gal4 flies were reduced by an average of 60% (Fig. 9C). The dose-dependent reduction in IP$_6$ levels observed in the fly mutants, suggests that dmIpk2 activity is an important rate-limiting step in the synthesis pathway.

Transgenic flies overexpressing epitope-tagged dmIpk2 under control of the actin promoter had a 5-fold increase in higher IP production as compared with wild-type flies (Fig. 9A, bottom trace). Interestingly, the levels of several different IP species increased in these flies, including IP$_2$ and IP$_3$ uncovering a potential catabolic component of the pathway. Collectively, our results demonstrate that regulation of higher IP production in adult flies requires a dmIpk2-dependent synthesis pathway.

The Cellular Localization of dmIpk2—Given the role and localization of yeast Ipk2 in the nucleus, we analyzed the subcellular localization of dmIpk2 in cultured cells and whole fly tissue. We observed that FLAG-tagged dmIpk2 localizes to the nucleus when expressed in Drosophila S2 cells, suggesting that Ipk2-dependent IP synthesis in the fly is nuclear (data not shown). In yeast, Ipk2 initiates a nuclear IP synthesis pathway whose products regulate events such as transcription and chromatin remodeling, raising the possibility that Ipk2 associates with active zones of chromatin DNA (7, 8, 11, 12). The Drosophila polytene chromosomes present in giant cells of the salivary glands derived from 3rd instar larvae represent a unique system to observe nuclear proteins. FLAG-tagged dmIpk2 was expressed in larvae under control of the actin promoter and immunofluorescence studies demonstrate that the majority of protein appears localized within the nucleus (Fig. 10, top panel). Closer examination of the nuclear localization and concomitant staining of the chromosomal DNA with DAPI revealed that the bulk of dmIpk2 does not appear to be bound to chromatin DNA itself, rather it appears enriched in the non-DAPI nucleosol (Fig. 10, bottom panel). The same distribution was observed for the endogenous protein using an anti-dmIpk2 antibody, indicating the FLAG-dmIpk2 localization was not a consequence of overexpression (data not shown).

These data provide new insights into a possible nuclear role for Ipk2 and its products in D. melanogaster.

**DISCUSSION**

A major finding of this work is the identification of gene products whose function is required for the genesis of higher IP synthesis in metazoans. It has been known for decades that eukaryotic cells possess an ensemble of higher phosphorylated IP messengers. Remarkably, a molecular basis for their production has only been recently determined in budding yeast but has not been clarified in higher eukaryotes. Thus, for the first
time we define the major molecular pathways used to synthesize IP\(_5\) and IP\(_6\) in the fruit fly D. melanogaster (summarized in Fig. 11). We find that dmIpk2 and dmIpk1 are the main contributors to higher IP synthesis. Our data prove an important role for dmIpk2 using both loss and gain of function experiments in cultured cells and the whole organism. In contrast, dmIP3K and dm5PtaSel appear to negatively regulate higher IP synthesis. This is highly significant as it dispels a widely proclaimed view in the literature that IP3K isoforms are required to initiate the synthesis of higher IPs from I(1,4,5)P\(_3\).

Our data also support the hypothesis that an I(1,4,5)P\(_3\) pool serves as a substrate source for three distinct metabolic routes through dmIP3K, dm5PtaSel, and dmIpk2. We find that regulation of any of these enzymes can impinge on Drosophila higher IP synthesis. One attractive explanation for why dmIP3K\(_\delta\) or dm5PtaSel depletion causes an increase in IP levels is through expansion of an I(1,4,5)P\(_3\) pool. Both of these enzymes are known to modulate I(1,4,5)P\(_3\)-stimulated calcium signaling through the phosphorylation or dephosphorylation of this second messenger (4). Depletion of either of the two enzymes could lead to increases in the I(1,4,5)P\(_3\) pool that is available for feeding into the Ipk2/Ipk1 pathway. Alternatively, separable IP synthesis pathways may exist in Drosophila that can impinge on each other when they are disrupted or down-regulated. Speed et al. (33) reported similar increases in inositol polyphosphate production in mammalian cells by depleting a human Type 1 5-phosphatase in NIH3T3 cells using antisense RNA (33). They showed that 5-phosphatase depletion causes I(1,4,5)P\(_3\), I(1,3,4,5)P\(_4\), IP\(_5\), and IP\(_6\) levels to increase. Studies of IP3K-deficient mice indicate a phenotypic role for I(1,3,4,5)P\(_4\) production, in neuronal and T lymphocyte function (34–36). Consist with our data in flies, loss of IP3K B in mice did not decrease IP\(_3\) levels (IP\(_3\) was not shown) in labeled cells (35), although one cannot exclude the possible compensation by other IP3K isoforms.

Is the Ipk2/Ipk1 pathway a common route of IP\(_3\) and IP\(_6\) synthesis in all eukaryotes? Based on our data and the evolutionary conservation of Ipk2/Ipk1 across species, the parsimonious answer is “yes”. However, some studies in the literature challenge the exclusivity of this pathway. Substrate specificity studies of human Ipk2 indicate that a catalytic preference for I(1,3,4,6)P\(_4\) 5-kinase versus I(1,4,5)P\(_3\) 6-kinase activities (22). Accordingly, human Ipk2 would act downstream of the I(1,3,4,5)P\(_3\) 5-6-kinase. Other studies of human Ipk2 show in vitro that human Ipk2 is a dual-specificity kinase that converts I(1,4,5)P\(_3\) to IP\(_5\) (23). Heterologous expression of human Ipk2 does not appear to restore IP\(_5\) synthesis in ipk2 deficient yeast (22). Thus it may be that humans, but not other metazoans, have evolved a distinct more complex pathway reminiscent of the metazoan model shown in Fig. 1A. It is also possible that different cell-types or tissues utilize more than one pathway to generate these important messengers.

It is also interesting to note that Ipk1 and Ipk2 are conserved among all eukaryotes, the I(1,3,4)P\(_3\) 5-6-kinase is not found in yeast or flies. In mammalian cells, I(1,3,4)P\(_3\) 5-6-kinase alternation through overexpression or RNAi effects tumor necrosis factor induced apoptosis possibly through alteration of higher IPs (37). In plants, Shi et al. (38) provide evidence for a role for higher IP synthesis by disruption of the maize 5-6-kinase open reading frame, which results in a 50% reduction in IP\(_6\) levels in seeds (38). Plant Ipk2 may therefore contribute to this pathway through phosphorylation of the D-5 position of I(1,3,4,5)P\(_4\) to generate I(1,3,4,5,6)P\(_6\). Since dmIpk2 also has 5-kinase activity toward I(1,3,4,6)P\(_4\) with a relative affinity that is comparable to the human and plant Ipk2 it is possible it serves a similar role in flies. Although, our sequence analysis of the Drosophila genome did not reveal a I(1,3,4)P\(_3\) 5-6-kinase gene product against this hypothesis.

Our data is consistent with a phospholipase C-dependent sequential phosphorylation of I(1,4,5)P\(_3\) to IP\(_6\). Drosophila possesses three different phospholipase C isoforms including NorpA, Plc21C, and Small wing (Sl) (39). Studies that examine these homologs may elucidate whether IP\(_3\) and IP\(_6\) are synthesized from I(1,4,5)P\(_3\) and provide an understanding of how their synthesis is regulated. Phenotypic analysis of our mutant flies and cell lines are ongoing and may expose functional roles for higher IP synthesis in eukaryotes. It is also important to note that there are phospholipase C-independent routes for IP\(_6\) synthesis reported in the literature. In the slime mold Dictyostelium, deletion of the sole phospholipase C gene does not significantly change higher IP synthesis (40). Biochemical evidence for phospholipase C-independent IP synthesis has been reported from plants and slime mold through the sequential phosphorylation of I(3)P or inositol (41–43).

It is tempting to speculate that Drosophila Ipk2 and Ipk1 comprise a nuclear inositol signaling pathway that regulates nuclear processes similar to those in budding yeast. The evolutionarily conservation of the nuclear localization of Ipk2 from a variety of species is consistent with a conserved functional role. IP\(_3\) and IP\(_6\) in yeast are implicated as messengers that mediate transcriptional events (8), possibly through the regulation of chromatin remodeling (11, 12). IP\(_3\) production is implicated in regulation at the nuclear pore and is required for the efficient export of mRNA from the nucleus (7, 44). The localization of dmIpk2 to the nucleosol and not bulk chromatin provides the first evidence indicating that the majority of Ipk2 is not physically associated with chromatin. It is possible that activation of signaling promotes the movement of a minor pool of Ipk2 to chromatin, or that local production of IP\(_4\) or IP\(_5\) in the
nucleosol activates transcriptional programs or chromatin remodeling. The generation of GFP-dmIpk2 transgenic flies will facilitate future studies aimed at examining potential dynamic changes in Ipk2 localization on polytene chromosomes during transcription.

If Ipk2 has an important role in regulating nuclear, or cytoplasmic function in Drosophila, we may observe phenotypic changes in transgenic flies. Currently, we have not observed a gross effect on development in either RNAi depleted (60% knockdown of cellular IP production) or overexpressing flies (5-fold increase in IP production). This may not be such a surprise since: 1) Ipk2 is conditionally essential in yeast; 2) several mutant Ipk2 lines with impaired but not complete loss of function in many cases appear normal; and 3) overexpression of Ipk2 in yeast has no observable phenotype. These data indicate that only a small amount of Ipk2 is required for regulation of nuclear function and that overproduction is well tolerated. Thus, our future phenotypic analysis will focus on specific tissues and/or the complete loss of function of Ipk2 through genetic mutation or deletion.

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