p60 Is an Adaptor for the Drosophila Phosphoinositide 3-Kinase, Dp110*

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The mammalian phosphoinositide 3-kinases (PI3Ks) p110α, β, and δ form heterodimers with Src homology 2 (SH2) domain-containing adaptors such as p85α or p55PIK. The two SH2 domains of these adaptors bind to phosphotyrosine residues (pY) found within the consensus sequence pYXXM. Here we show that a heterodimer of the Drosophila P13K, Dp110, with an adaptor, p60, can be purified from S2 cells with a pYXXM phosphopeptide affinity matrix. Using amino acid sequence from the gel-purified protein, the gene encoding p60 was cloned and mapped to the genomic region 21B8-C1, and the exon/intron structure was determined. p60 contains two SH2 domains and an inter-SH2 domain but lacks the SH3 and breakpoint cluster region homology (BH) domains found in mammalian p85α and β. Analysis of the sequence of p60 shows that the amino acids responsible for the SH2 domain binding specificity in mammalian p85α are conserved and predicts that the inter-SH2 domain has a coiled-coil structure. The Dp110+p60 complex was immunoprecipitated with p60-specific antisera and shown to possess both lipid and protein kinase activity. The complex was found in larvae, pupae, and adults, consistent with p60 functioning as the adaptor for Dp110 throughout the Drosophila life cycle.

Studies in vertebrates, Drosophila melanogaster and Caenorhabditis elegans, suggest that both the structure and the function of receptor tyrosine kinases (RTKs)1 are highly conserved across metazoan organisms (1). Upon stimulation with an extracellular ligand, RTKs dimerize and transphosphorylate (2). This tyrosine phosphorylation enables the recruitment of signaling molecules containing SH2 or phosphotyrosine binding (PTB) domains that recognize phosphotyrosines within specific amino acid motifs (3). In this way, the SH2 domain-containing adaptors for Class IA P13Ks (43) are recruited to tyrosine-phosphorylated RTKs and associated substrates containing the pYXXM motif (4). The recruitment of Class IA PI3Ks to activated RTKs coincides with a dramatic increase in the production of 3′ phosphorylated phosphoinositides. These 3′ phosphorylated phosphoinositides are thought to act as second messengers that affect cell growth, differentiation, membrane trafficking, and cytoskeletal organization (4).

In mammals, there are at least three Class IA P13Ks, p110α, β, and δ (5, 6, 44) that can associate with a number of adaptors. Three distinct genes encode p85α, p85β, and p55PIK, and additional adaptors are generated from alternatively spliced p85α transcripts (7-13). Each of these adaptors contains two SH2 domains, both of which selectively bind peptides containing phosphotyrosine with a methionine at the +3 position (pYXXM) (14, 15), and an inter-SH2 domain, which mediates binding to Class IA P13Ks (16). The p85α and p85β adaptors also contain an SH3 domain and a BH domain at the N terminus, whereas p55PIK and two splice variants of p85α have short N-terminal extensions. Despite this structural diversity, there has been no reported selectivity of binding between different adaptors and p110α, β, or δ (44).

We are using Drosophila to examine the role of the Class IA P13Ks genetically and to provide an in vivo system to identify downstream targets. Many molecules downstream of RTKs in Drosophila are structurally and functionally homologous to their mammalian counterparts. The best characterized example of this is the Ras/MAPK pathway downstream of the Sev, Drosophila EGF receptor and Torso RTKs (17). Drosophila possesses a Class IA P13K, Dp110 (also known as P13K_92E), which is homologous to mammalian Class IA P13Ks (18, 19). Previously, we have shown that the ectopic expression of Dp110 in larval imaginal discs affects cell growth but not cell differentiation (19).

Here we present the affinity purification of p60, the adaptor for Dp110, using immobilized phosphopeptides containing the pYXXM motif. Peptides derived from the purified protein were sequenced, and degenerate PCR and cDNA cloning were used to isolate the p60 cDNA. The structural and functional conservation of p60 with the mammalian adaptors is discussed. The Dp110+p60 complex possessed lipid and protein kinase activity and was found in Drosophila larvae, pupae, and adults and the S2 cell line. The genomic structure and location of the p60 gene has been determined to facilitate the identification and generation of genetic reagents to study the function of this PI3K adaptor in vivo.

EXPERIMENTAL PROCEDURES

Lysate Preparation and Affinity Purification—S2 cell lysates were prepared essentially as described (19) by lysis in buffer containing 1% Triton X-100 and the protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 18 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 5 mM benzamidine. Detergent lysates of Oregon R third instar larvae, pupae, and adult flies were prepared in the same buffer, but which additionally contained 5 μM diisopropylfluorophosphate and 15 μM N-α-tosyl-L-lysine chloromethyl ketone, by homogenization with a poltron (2 ×

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1 The abbreviations used are: RTK, receptor tyrosine kinase; P13K, phosphoinositide 3-kinase; pY, phosphotyrosine; SH2 and SH3, Src homology 2 and 3, respectively; BH, breakpoint cluster region homology; Class IA, the class of P13Ks that associate with RTKs via SH2 domain-containing adaptors; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PLCγ, phospholipase Cγ, contig, group of overlapping clones.

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10 s, on ice). The homogenate was clarified by centrifugation at 20,000 × g for 1 h and then at 20,000 × g for 30 min. Affinity purification using phosphopeptide coupled to Actigel (Stoegene) beads was performed as described (19).

**Peptide Microsequencing and cDNA Cloning**—Proteins affinity purified from approximately 10^9 S2 cells were resolved by SDS-PAGE and stained with Coomassie Blue R250. Each band was excised and digested with lysyl endopeptidase C (Wako Chemicals), and the resulting peptides were resolved by passage through an AX-300 pre-column and then a C18 (150 × 1 mm) column (Relasiel). The peptides were sequenced at the 500 fmol level using an ABI Procise system. Peptides 6 and 9 (see Fig. 2A) were used to design the degenerate primers, CA/AGGA/AG/C/T/T/TTCT/C/T/CA/C/T/TA/CA/CT/TT/C/CA/GCTTG (and C/G/A/T/A/C/AC/CT/ T/C/G/A/T/A/A/G)/C/T/TG/C/T/TG, respectively. Poly(A) mRNA was prepared from S2 cells using oligo(dT)-cellulose (Stratagene) and used to synthesize first strand cDNA with Moloney murine leukemia virus reverse transcriptase (Pharmacia Biotech Inc.). PCR reactions were performed with cDNA as a template and 0.05 units/μl Taq, 1.5 mM MgCl₂, 0.2 mM dNTP, and 1 μl each primer in a total volume of 50 μl. 40 cycles of amplification (94°C for 30 s, 50 °C for 30 s, and 72°C for 60 s) were performed. The 200-base pair product obtained was cloned into pGEM-T (Promega), sequenced, and found to encode peptides 7 and 8 (see Fig. 2A). This fragment was used to screen a AgT1 eye imaginal disc cDNA library (A. Cowan), and four positive clones were isolated that appeared to be identical by restriction mapping and by sequencing of the 5′ and 3′ ends. One clone was digested with EcoRI, and the other two resulting fragments were subcloned into pBluescript SKII (Stratagene). PCR and direct sequencing of the original lambda clone showed the two fragments to be adjacent. Each fragment was sequenced in both directions with T3, T7, and p60-specific primers on an ABI 373 automated DNA sequencer.

**Immunological Methods**—Peptides corresponding to the N terminus (GGGMQPSPLHYSTMHPQ, CGGSVLDPNEDLRRM) and the C terminus (CGGGYWKNPLQVMQIQLQEE, CGGSLEAQAAAPFSNFPSTSQ) of p60, including a CGG coupling linker at the N terminus, were coupled to maleimide-activated keyhole limpet hemocyanin as directed (Pierce). Antibodies were raised in rabbits against pools of N-terminal (c606N) and C-terminal antigens (c606C). Immunoblotting was performed using p60 (1:1000), or p60N (1:1000), or p606C (1:2000) and developed with enhanced chemiluminescence as directed (Amer sham Life Science). Immunoprecipitation was performed by incubating the lysate with a 1:200 dilution of antisera (6 μl) for 1 h at 4 °C and then adding protein A-Sepharose (Pharmacia) beads and incubating for an additional 30 min. The beads were washed in the same manner as the peptide-coupled beads (19).

**Kinase Assays**—For the lipid and protein kinase assays, the beads were washed three times in lysis buffer and twice in 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM EGTA. Lipid kinase assays were performed essentially as described (20) in 60 μl of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM EGTA, 2.5 mM MgCl₂, 100 μM ATP containing 2.5 μCi [γ-32P]ATP and 200 μM sonicated phosphatidylinositol (Sigma). The reaction was incubated for 30 min at room temperature and terminated with acidified chloroform, and the lipid was extracted and resolved by thin layer chromatography with chloroform/methanol/4M ammonium hydroxide (45:35:10). Protein kinase assays were performed in 30 μl of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM EGTA, 2.5 mM MgCl₂, 100 μM ATP containing 2.5 μCi [γ-32P]ATP for 30 min at room temperature and resolved by SDS-PAGE on 7.5% polyacrylamide gels.

**RESULTS**

**Affinity Purification of the Dp110-p60 Complex from Drosophila S2 Cells**—Phosphopeptides, containing one or both of the pYXXM motifs found at positions 740 and 751 of the human PDGFrβ receptor, bind selectively to the adaptors for Class I, PI3Ks (14). When coupled to agarose beads, these phosphopeptides can be used to affinity purify heterodimeric complexes containing the adaptors bound to Class I, PI3Ks (9, 21). We investigated whether this approach could be used to identify an adaptor for Dp110, the Drosophila Class I, PI3K. Three peptides of approximately 145, 120, and 60 kDa were affinity purified from Drosophila S2 cells, using the tyrosine phosphorylated peptide GGYMDMSKDESVDypypVML (pY751) coupled to agarose beads (Fig. 1). The same proteins were purified when the peptide was phosphorylated on tyrosine 740 or on both tyrosines 740 and 751, but they were not recovered with beads lacking peptide (data not shown). The affinity purified complex possessed lipid and protein kinase activities, and immunoblotting with αDp110 antisera showed that the 120-kDa protein was Dp110 (see below). We washed the complex at high stringency to determine which of the remaining bands was the adaptor for Dp110. Washing with lysis buffer containing increasing concentrations of sodium chloride removed the majority of the 145-kDa band, suggesting that p60 was the adaptor (Fig. 1). A large scale affinity purification was performed, and peptides derived from p60, p120, and p145 were sequenced (see “Experimental Procedures”). Three of the peptide sequences obtained from p120 confirmed that it was Dp110 (LMANYTGL, EQYQVYISTFN, and LHVL). Two peptides sequenced from p145, FMEXIYTDVR and FXNXCGYIL, revealed that this protein was Drosophila phospholipase Cγ (PLCγ1D) (22). Interestingly, human PLCγ can be affinity purified from human cell lines using the same pYXXM phosphopeptide, and the C-terminal SH2 domain of mammalian PLCγ can interact with pYXXM motifs (14) though it binds preferentially to phosphotyrosines in other sequence contexts (15). Nine peptide sequences were obtained from p60 and used to design degenerate PCR primers. PCR amplification from first strand cDNA derived from S2 cell mRNA generated a 200-base pair fragment that was used to isolate p60 cDNAs from an eye imaginal disc cDNA library (see “Experimental Procedures”). These cDNAs contained an open reading frame that could encode a protein with a predicted size of 57.5 kDa, which contains all nine of the peptide sequences recovered from p60 (Fig. 2A).

**Sequence Analysis of p60**—Like the other identified adaptors for Class I, PI3Ks, the predicted amino acid sequence of p60 includes two SH2 domains and an inter-SH2 domain. However, the SH3 and BH domains in p85α and β, the N-terminal extensions in p55PIK and the p56α isoforms, and the proline-rich SH3 domain-binding motifs (23) found in all mammalian adaptors are absent in p60 (Fig. 2B). p60 has a short N terminus (similar in size to the N terminus of p50α, a recently isolated splice variant of p85α (12), and a unique C terminus of 70 amino acids that shows no significant similarity to other proteins. When the amino acid sequences of the core SH2-inter-SH2–SH2 region of p60, p85α, p85β, and p55PIK are compared, p60 shows an equal degree of similarity to all three mammalian adaptors (Fig. 2A, data not shown).

The N-terminal and C-terminal SH2 domains are the most conserved regions of p60 and are 58% and 48% identical to the
respective domains of bovine p85α. The three-dimensional structures of the N-terminal and C-terminal SH2 domains of p85α in complex with pYXXM phosphopeptides have been determined by x-ray crystallography and nuclear magnetic resonance (24, 25). These SH2 domain structures identify the amino acids responsible for the pYXXM binding specificity. These amino acids, including the phenylalanine of the beta strand E and the leucines of the loop between the alpha helix B and the beta strand G, are conserved in p60. Together, these three amino acids, shown in Fig. 2A, define the hydrophobic pocket that allows the specific binding of methionine three residues C-terminal to the phosphotyrosine (26).

The inter-SH2 domain of mammalian p85α mediates binding to the Class IA PI3K, p110α. Modelling studies of this inter-SH2 domain predict a two- or four-helix antiparallel coiled-coil structure similar to the solved crystal structure of the inter-SH2 domain of ZAP-70 (16, 27). The inter-SH2 domain of p60 is approximately 20% identical to the corresponding region of the mammalian adaptors. Despite this low homology, this region of p60 is likely to form a similar structure since it contains the leucine-rich heptad repeats characteristic of coiled-coil alpha helical bundles (see “Results”).

The proline-rich motif found in the mammalian adaptors is indicated as P2. B, comparison of the domain structure of p60 with mammalian adaptors for Class IA PI3Ks. The relative positions of the SH3, BH, SH2, and inter-SH2 domains are shown for p85α/β. The proline rich motifs are indicated as P1 and P2. The C-terminal extension of p60 is shaded. C, genomic structure and localization of the gene encoding p60. The EcoRI restriction sites (E) are from the previously described map of the region (31). The distal breakpoint of Df(2L)al lies within the 3-kilobase EcoRI restriction fragment. The direction of transcription is from distal to proximal. The exons shown account for all the nucleotides within the cDNA clone. The first exon is bases 1–481, the second exon is bases 482–1385, and the third exon is bases 1369–3218. The initiation codon is at position 481, and the stop codon at position 1999. The coding region of the exons is shown in black.

3 M. J. Zvelebil, personal communication.
p60 is significantly homologous to coiled-coil regions of proteins that form stable heterodimers (data not shown).

Genomic Location and Structure of the p60 Gene—A BLASTN (28) data base search with the p60 nucleotide sequence identified a sequence tagged site (STS, Dm0574) from the Berkeley Drosophila Genome Project that is identical to the 3′-untranslated region of the p60 cDNA and has been mapped to the genomic region, 21B6-C2 (30). To further characterize the genomic structure of the p60 gene, we performed Southern analysis of a λ-phacontig of the region (kindly provided by M. Noll). This analysis determined the position and orientation of the p60 gene with respect to the published EcoRI restriction map (31). Furthermore, the exon/intron structure of the gene was determined by PCR, subcloning, and sequence analysis of the genomic clones (Fig. 2C). The gene encoding p60 has three exons and probably overlaps the breakpoint of the deficiency D82Lal at 21B8-C1.

Expression of p60 at Different Stages of the Drosophila Life Cycle—We initially purified p60 from S2 cells, an embryonically derived cell line. We next sought to characterize the expression of p60 at different stages of the Drosophila life cycle. The Dp110-p60 complex was affinity purified from Triton X-100 lysates of third instar larvae, pupae, and adult flies using pYXXM phosphopeptide beads (Fig. 3A). The complex is present in all stages examined although we consistently recovered lower levels from larvae than from the other stages. Immunoblotting with antisera against N- and C-terminal sequences of p60N and p60C (Fig. 3B). An additional 55-kDa band can be seen that is immunoreactive with preimmune sera (A) or transferred to nylon membranes and immunoblotted with aDp110, op60N and op60C (B).

DISCUSSION

p60 has been affinity purified from Drosophila with a pYXXM phosphopeptide previously used to purify mammalian adaptors for Class IA PI3Ks. Analysis of the p60 amino acid sequence and the lipid and protein kinase activity of the Dp110-p60 complex indicates that p60 is both structurally and functionally homologous to the mammalian adaptors for Class IA PI3Ks. Since p60 is the most divergent member of the family identified to date, its sequence provides an insight into the evolution of the structure and function of these molecules. Notably, the residues responsible for the SH2 domain binding specificity are conserved, and the prediction of a coiled-coil structure for the inter-SH2 domains of the adaptor subunits for Class IA PI3Ks is supported.

Since we have shown that the pYXXM phosphopeptide can be used to purify adaptors in complex with Class IA PI3Ks from mammals and Drosophila, affinity purification with this phosphopeptide might also be used to isolate homologous PI3K complexes from many species. Interestingly, the SH2 domain of the Drosophila signaling molecule Drk also binds to the same phosphotyrosine motif recognized by its mammalian homologue Grb2 (33). Thus, affinity purification with peptides containing specific phosphotyrosine motifs might be used to isolate SH2 domain-containing proteins from various organisms.

Putative YXXM docking sites for the Dp110-p60 complex are found in the RTK Dret, the RTK substrate, Dos, and the Drosophila homologue of the insulin receptor, INR (34–36). However, it remains to be shown, either biochemically or genetically, whether these motifs are used in vivo. INR contains three YXXM motifs and is able to bind the N-terminal SH2 domain of mammalian p85a when phosphorylated (36). Class IA PI3Ks associate with the mammalian insulin receptor via multiple pYXXM motifs in its substrate IRS-1 and are thought to mediate many of the effects of insulin stimulation (37). Consistent with an analogous role for Class IA PI3Ks downstream of the Drosophila INR, certain mutations in inr and the ectopic ex-
pression of dominant negative Dp110 both affect imaginal disc cell growth (19, 38). It must be noted that mammalian adaptors for Class I α PI3Ks can also bind to the pYXXV motif in the Met receptor though with a lower affinity than for pYXXM motifs (39). Therefore, it is possible that p60 might recognize phosphotyrosine binding sites other than pYXXM.

It is likely that p60 is the only adaptor for Class I α PI3Ks present in *Drosophila*. However, we cannot rule out the possibility that additional adaptors exist. Together with Dp110, p60 and a mode of regulation that might be related to the presence of the pYXXM motif in the Met receptor (39), suggests that these motifs are involved in a more recently evolved PI3K.

Drosophila expression maps. We thank Thomas Twardzik, Thomas Raabe, and Martin Hansen, and Justin Hsuan for peptide microsequencing and Marina Courtneidge, S. A., and Garfallo, R. (1996) *Endocrinology* 137, 846–856

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