A family of phosphoinositide 3-kinases in *Drosophila* identifies a new mediator of signal transduction

Lindsay K. MacDougall*, Jan Domin* and Michael D. Waterfield*†

*Ludwig Institute for Cancer Research, University College Branch, 91 Riding House Street, London W1P 8BT, UK.†Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK.

Background: Mammalian phosphoinositide 3-kinases (PI 3-kinases) are involved in receptor-mediated signal transduction and have been implicated in processes such as transformation and mitogenesis through their role in elevating cellular phosphatidylinositol (3,4,5)-trisphosphate. Additionally, a PI 3-kinase activity which generates phosphatidylinositol 3-phosphate has been shown to be required for protein trafficking in yeast.

Results: We have identified a family of three distinct PI 3-kinases in *Drosophila*, using an approach based on the polymerase chain reaction to amplify a region corresponding to the conserved catalytic domain of PI 3-kinases. One of these family members, PI3K_92D, is closely related to the prototypical PI 3-kinase, p110α; PI3K_59F is homologous to Vps34p, whereas the third, PI3K_68D, is a novel PI 3-kinase which is widely expressed throughout the *Drosophila* life cycle. The *PI3K_68D* cDNA encodes a protein of 210 kDa, which lacks sequences implicated in linking p110 PI 3-kinases to p85 adaptor proteins, but contains an amino-terminal proline-rich sequence, which could bind to SH3 domains, and a carboxy-terminal C2 domain. Biochemical analyses demonstrate that PI3K_68D has a novel substrate specificity *in vitro*, restricted to phosphatidylinositol and phosphatidylinositol 4-phosphate, and is unable to phosphorylate phosphatidylinositol (4,5)-bisphosphate, the implied *in vivo* substrate for p110.

Conclusions: A family of PI 3-kinases in *Drosophila*, including a novel class represented by PI3K_68D, is described. PI3K_68D has the potential to bind to signalling molecules containing SH3 domains, lacks p85-adaptor-binding sequences, has a Ca^{2+}-independent phospholipid-binding domain and displays a restricted *in vitro* substrate specificity, so it could define a novel signal transduction pathway.

Current Biology 1995, **5**:1404–1415

Background

Mammalian phosphoinositide 3-kinases (PI 3-kinases) were initially identified in association with viral oncoproteins and growth-factor receptors and have been shown to be an essential component of the signal transduction processes involved in transformation and mitogenesis. PI 3-kinases associated with these receptors phosphorylate the D-3 position of the inositol ring of the phosphoinositides phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI4-P) and phosphatidylinositol (4,5)-bisphosphate (PI(4,5)-P_2) *in vitro*, to generate the novel phospholipids PI3-P, PI(3,4)-P_2 and PI(3,4,5)-P_3. *In vivo*, the rapid and transient elevation in the cellular levels of the membrane phospholipids PI(3,4)-P_2 and PI(3,4,5)-P_3 in response to ligand stimulation has identified these products as potential signalling molecules (reviewed in [1,2]). Because these 3-phosphorylated lipids are not substrates for any of the known phospholipase C enzymes [3] it has been proposed that they, themselves, act as second messengers. Target molecules and more distant downstream interactions of PI 3-kinases have not been clearly delineated, although certain protein serine/threonine kinases have been implicated: Akt activity is elevated in response to platelet-derived growth factor (PDGF), apparently in a PI 3-kinase-dependent manner [4]; in addition, it has been shown that certain isoforms of protein kinase C can be activated by PI(3,4)-P_2 and PI(3,4,5)-P_3 *in vitro* [5].

It is evident that PI 3-kinase activity is associated with cell-surface receptors involved in triggering diverse cellular responses. These include metabolic processes (such as glucose transport), inflammatory responses (such as superoxide production), membrane ruffling and cellular motility ([6–9]; reviewed in [1]). Additionally, stimulation of PI 3-kinase activity may occur through a direct interaction with the GTP-bound form of Ras [10].

Three forms of PI 3-kinase have been identified and characterized at the molecular level in mammalian cells. The prototype PI 3-kinase linked to receptor tyrosine kinases is composed of a regulatory p85 subunit and a catalytic p110 subunit [11–15]. Ligand stimulation of receptor tyrosine kinases results in the autophosphorylation of specific tyrosine residues; these phosphotyrosines then act as docking sites for the SH2 domains of p85, allowing translocation of the associated catalytic activity to lipid substrates at the membrane. A number of distinct p85 [12,16] and p110 subunits (p110α [15] and p110β [17]) have been identified, but no differences in function have been ascribed to them (reviewed in [1]).

A G-protein-activated PI 3-kinase seems to be a distinct form of the enzyme. Stephens *et al.* have partially purified a PI 3-kinase that is immunologically distinct from p110α, does not couple to p85 and is activated by $G_{\beta\gamma}$ subunits in response to the stimulation of G-protein-linked receptors [18]. Stoyanov *et al.*, have reported the

Correspondence to: Michael D. Waterfield. E-mail address: mikew@ludwig.ucl.ac.uk

molecular structure of a PI 3-kinase of this type, termed PI3Kγ [19]. The recombinant enzyme is activated *in vitro* by both G_{α} and $G_{\beta\gamma}$ subunits. The substrate specificity and sequence homology in the catalytic domain indicate that PI3Kγ is a member of the p110 family.

The third form of PI 3-kinase, Vps34p, was originally identified as part of the molecular complex responsible for the control of intracellular protein trafficking in yeast [20,21]. Biochemical characterization identified Vps34p as a PI 3-kinase with a substrate specificity restricted to PI. In higher eukaryotes, analogous sorting mechanisms exist for the active diversion of lysosomal enzymes from the default secretory pathway (reviewed in [22]). The partial purification of a bovine PI-specific PI 3-kinase [23] and the cloning of human [24], soybean [25], *Arabidopsis* [26] and *Dictyostelium* [27] Vps34p homologues has been described. The human enzyme, termed PtdIns 3-kinase, interacts with a human homologue of the yeast protein Vps15p, a protein which in yeast seems to constitutively localize Vps34p to the Golgi membrane and may play a role in activating the lipid kinase [28]. Thus, like the heterodimeric p110–p85 PI 3-kinase, the Vps34p–Vps15p holoenzyme forms an evolutionarily conserved complex of regulatory and catalytic subunits that are responsible for its distinct subcellular localization and substrate specificities.

A number of other proteins have been identified which have sequence homology to the kinase domain of PI 3-kinases but have not been shown to phosphorylate the D-3 position of phosphoinositides. These can be divided into three families, some of which may be protein, rather than lipid, kinases: Tor-related proteins, homologues of the protein encoded by the *ataxia–telangiectasia mutated* (*ATM*) gene and the PI 4-kinases (reviewed in [29,30]). The Tor proteins [31,32] and their mammalian homologues have, like PI 3-kinases, been implicated in mitogenic signalling. The Tor proteins are required for the transition from G1 to the S phase of the cell cycle and their sensitivity to the immunosuppressant rapamycin, which also promotes G1 arrest, has led to the identification of p70 S6 kinase and cyclin-dependent kinases as downstream components in this pathway (reviewed by Downward in [33]). The ATM-related proteins are also involved in cell-cycle progression (reviewed by Zakian in [30]). These proteins are involved in the recognition of damaged DNA and include ATM itself, Rad3 and the DNA-dependent protein kinase. The final group consists of two distinct groups of PI 4-kinases [34–36]. Surprisingly, PI 5-kinase displays very limited sequence similarity to members of these three families, despite its activity, which is restricted to the phosphoinositide PI4-P [37].

The molecular characterization of a number of PI 3-kinases has explained the nature of their association with transducing elements lying upstream of the enzyme and provided clues to differences in substrate specificity. However, the downstream components of signalling pathways involving PI 3-kinases have not been clearly defined, despite the availability of recombinant proteins. Therefore, one of the approaches we have taken is to use *Drosophila* as a genetically manipulatable organism in which to delineate the signal transduction pathways involving PI 3-kinases. As a first step towards this goal we have undertaken the molecular and biochemical characterization of *Drosophila* PI 3-kinases. Here, we report the identification of a family of *Drosophila* PI 3-kinases. We also describe a novel class of PI 3-kinase that is characterized by a distinct substrate specificity and domain structure, indicating that this novel enzyme may be involved in a signal transduction pathway distinct from that of the prototype p110 PI 3-kinases.

Results

Identification of a family of PI 3-kinases in *Drosophila*

As a first step towards identifying a *Drosophila* PI 3-kinase, we used an approach based on the polymerase chain reaction (PCR) and designed degenerate primers based on regions of strong amino-acid homology found within the putative lipid-kinase domain of mammalian p110α and yeast Vps34p. These regions contain a number of conserved amino acids that have been identified in protein kinases as key residues for ATP binding and catalysis and which are therefore likely to be conserved in phosphotransferases — specifically DxxxxN (single-letter amino-acid code, where x indicates any amino acid), within the catalytic loop of subdomain VIB, and an aspartic acid residue in subdomain VII which chelates Mg^{2+} (reviewed in [29]). Using PCR and these primers, a single product of the expected size (approximately 400 base pairs (bp)) was amplified from mRNA isolated from different stages of the *Drosophila* life cycle. The product was subcloned and sequenced. This analysis revealed three distinct sequences; two of these were most similar to mammalian p110 (50 and 55 % identity at the amino-acid level to p110α), whereas the other, which was more closely related to Vps34p (56 % identical to the yeast protein), had a conserved 11 amino-acid deletion and a 4 amino-acid insertion when aligned with the amino-acid sequence of p110α (Fig. 1a).

We next performed *in situ* hybridization analysis, which revealed that the individual fragments localized to unique and separate regions of *Drosophila* polytene chromosomes, clearly demonstrating that each corresponded to a distinct gene (Fig. 1b). We have thus termed the isolated clones *PI3K_59F*, *PI3K_68D* and *PI3K_92D*. The phylogenetic relationship between these three *Drosophila* sequences and the corresponding core catalytic region of the established PI 3-kinases is depicted in Figure 1c. This underlines the similarity between *Drosophila* PI3K_59F and *Saccharomyces cerevisiae* Vps34p. Furthermore it demonstrates that the p110 family can be divided into two subfamilies. One of these consists of the established p110 proteins (p110α, β and PI3Kγ), as well as the *Drosophila* PI3K_92D sequence, whereas the other contains only the novel PI3K_68D sequence. We have subsequently

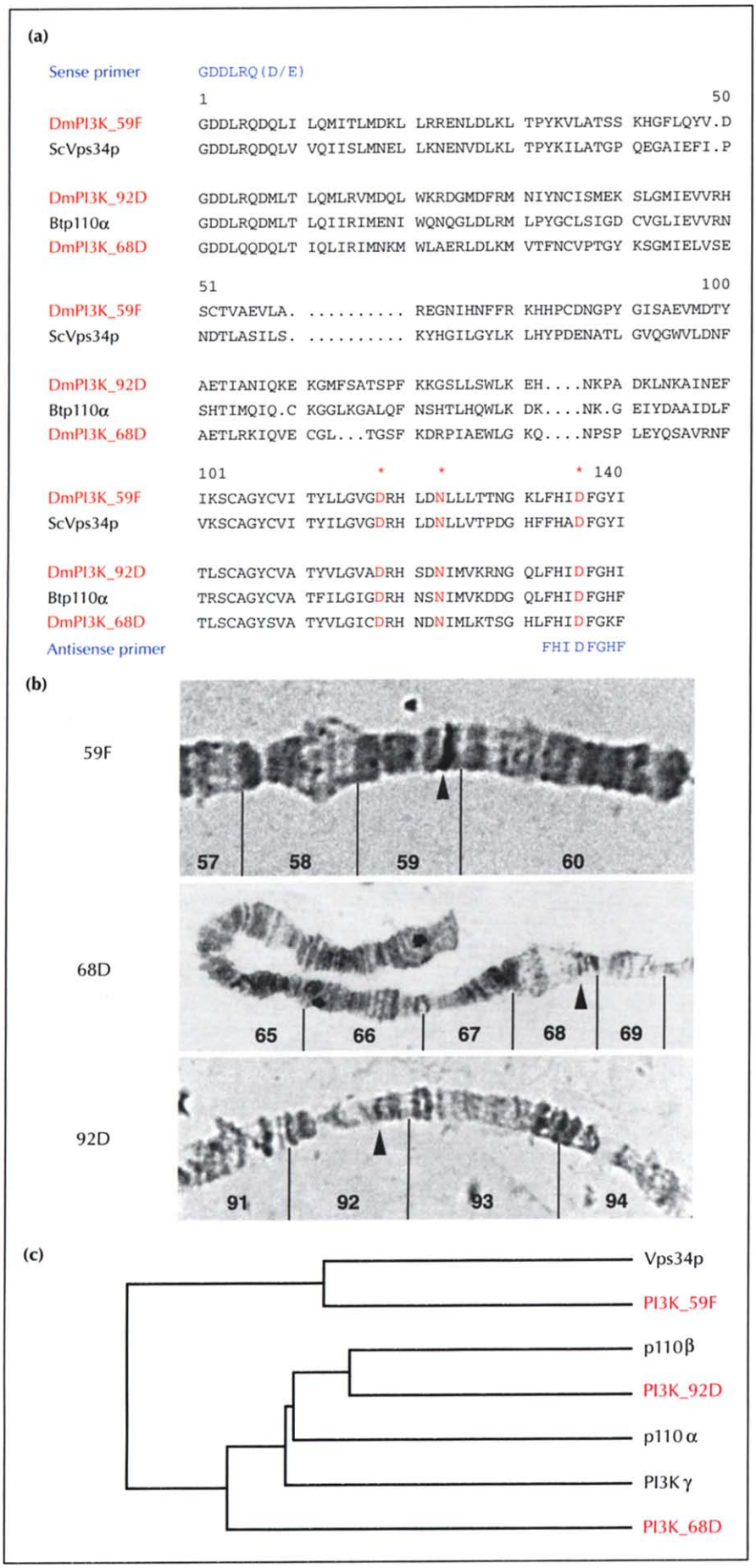

Fig. 1. Comparison of the catalytic domains of mammalian p110α, yeast Vps34p and a family of *Drosophila* PI 3-kinases. **(a)** Comparison of the deduced amino-acid sequence of the region amplified by PCR. Individual clones were sequenced using SP6 and T7 primers and aligned using the PILEUP programme [62]. The sequences are corrected for errors induced by the *Taq* DNA polymerase. The D and N of the sequence DxxxxN within the catalytic loop of subdomain VIB and an aspartic acid in subdomain VII which chelates Mg^{2+} are indicated in red. The sense and antisense primers used to amplify this region are shown in blue. **(b)** Chromosomal localization of *Drosophila* PI 3-kinases. Sequences corresponding to each class of PI 3-kinase were excised from the plasmids, individually labelled and hybridized to *Drosophila* polytene chromosomes as described in Materials and methods. The numbers refer to cytological divisions of the *Drosophila* genome. The arrow heads indicate the position of the biotinylated probes. **(c)** Dendrogram to show the evolutionary relationship between PI 3-kinase family members. The dendrogram was generated using the PILEUP programme with the *Drosophila* sequences shown in (a) and those of p110α (Btp110α; [15]), p110β (Hsp110β; [17]), PI3Kγ (HsPI3Kγ; [19]) and Vps34p (ScVps34p; [20]).

identified full-length cDNAs corresponding to each of the PCR fragments (see below) and established that PI3K_59F is the homologue of Vps34p (C. Linassier, L.K.M., and M.D.W., unpublished observations) and that PI3K_92D is closely related to the p110α/β family (S.J. Leevers, L.K.M., and M.D.W., unpublished observations). The identification of two human homologues of PI3K_68D (S. Volinia, F. Pages, J.D. and M.D.W., unpublished observations) demonstrates that this *Drosophila* PI 3-kinase is the prototype of a distinct family and not a species-specific isoform.

Analysis of the sequence of PI3K_68D

The PCR product which localized to position 68D on the left arm of the third chromosome in *in situ* hybridization experiments, was used to screen *Drosophila* embryo cDNA libraries. Several partial, and one full-length, cDNA clones were obtained, the latter containing multiple in-frame stop codons 5′ to the initiating methionine codon. The complete open reading frame of *PI3K_68D* encodes a novel protein with a predicted molecular mass of 210 kDa (1876 amino acids), as shown in Figure 2a. Two regions of strong sequence homology to other PI 3-kinases are apparent (Fig. 2b). Homology region 1, the putative catalytic domain (amino-acid residues 1326–1587 towards the carboxyl terminus of PI3K_68D), represents the region of greatest sequence identity to other PI 3-kinases, whereas homology region 2 (residues 1042–1187), or the 'PIK domain', is a region of unknown function, apparently unique to lipid kinases and Tor2 [34]. Beyond these regions, however, homology with other PI 3-kinases is low. Notably, the region of mammalian p110 (residues 20–108 of p110α, [38]) required for its interaction with the p85 regulatory subunit, is absent, as is sequence homology with the amino terminus of Vps34p, via which this protein is assumed to interact with its regulatory subunit, Vps15p. However, the region of PI3K_68D corresponding to the amino

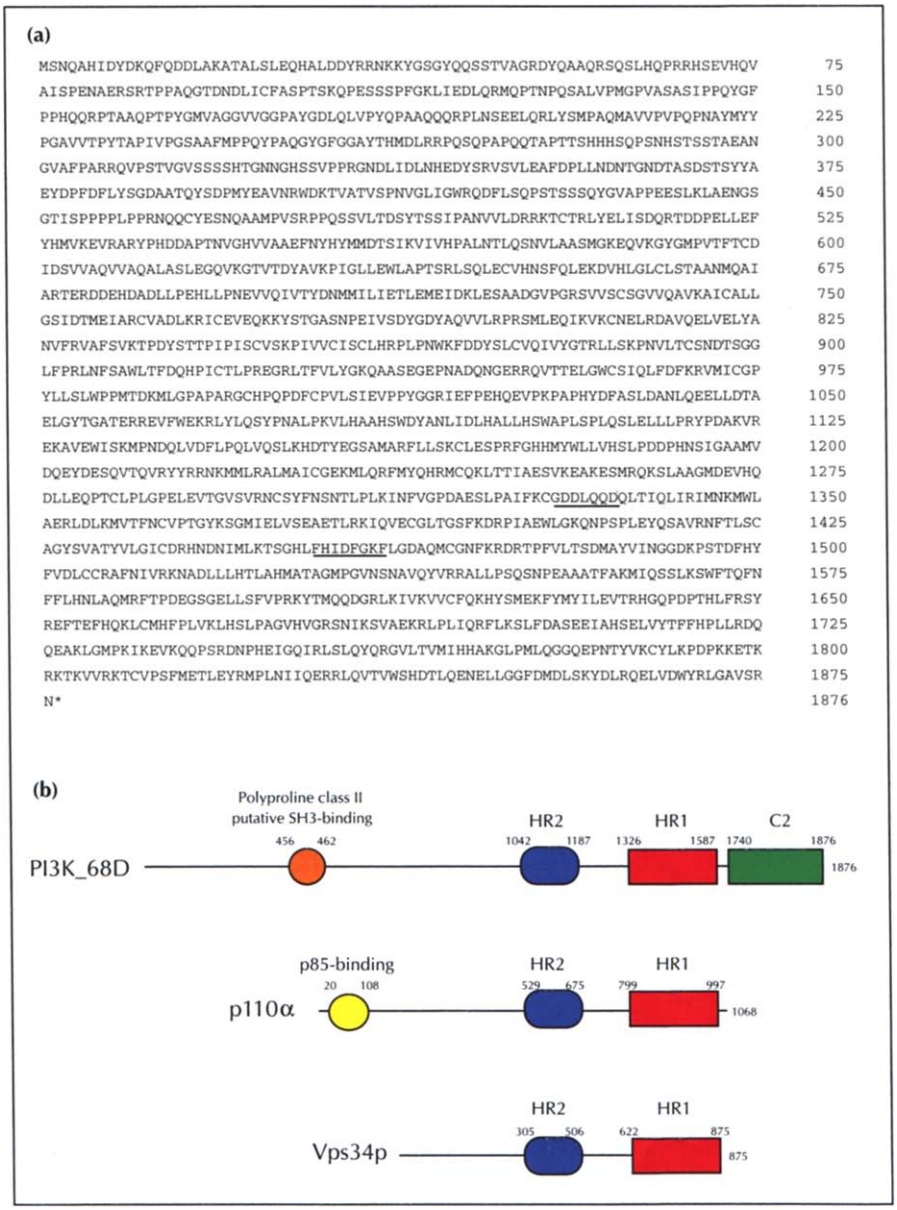

Fig. 2. Sequence analysis and domain structure of PI3K_68D. **(a)** Amino-acid sequence of PI3K_68D: the locations of the PCR primers used to amplify the core catalytic region are underlined. **(b)** Regions of sequence homology in PI3K_68D. Homology region 1 (HR1) represents the catalytic domain, homology region 2 represents the region unique to lipid kinases, known as the PIK domain. For details see text and [50]. Numbers above modules refer to the amino acids predicted to form the boundary of each domain.

terminus of p110α includes a type II class of polyproline motif [39] (PPPLPPR, residues 456–462, within a proline-rich region), indicating that PI3K_68D has the potential to interact with SH3 domains (Fig. 2).

Further novel sequences which may specify functions unique to this class of PI 3-kinase include an amino-terminal extension of 444 amino acids (compared to p110α) of unknown function and a carboxy-terminal extension of ~230 amino acids. The carboxy-terminal extension includes a region (amino-acids 1740–1876) with strong homology to a domain (the C2 domain) originally identified as a Ca^{2+}- and acidic-phospholipid-binding module [40]. The C2 domain in PI3K_68D shows 29% amino-acid identity in an 138 amino-acid overlap with the neuronal protein, synaptotagmin I (see Fig. 4b).

Biochemical characterization of recombinant and endogenous PI3K_68D activity

In order to characterize PI3K_68D biochemically, we constructed a recombinant baculovirus encoding a fusion protein comprised of the amino-acid sequence of glutathione-S-transferase (GST) linked to that of the PI3K_68D protein. After infection of Sf9 cells, the fusion protein was purified by glutathione–Sepharose affinity chromatography and eluted with glutathione. Analysis by SDS–PAGE revealed that a protein of ~236 kDa corresponded to the fusion protein (data not shown). The enzymatic activity and substrate specificity *in vitro* was investigated and compared to that of mammalian p110α, also expressed as a GST-fusion protein using the baculovirus system. GST–PI3K_68D displayed Mg^{2+}-dependent PI 3-kinase activity towards both PI,

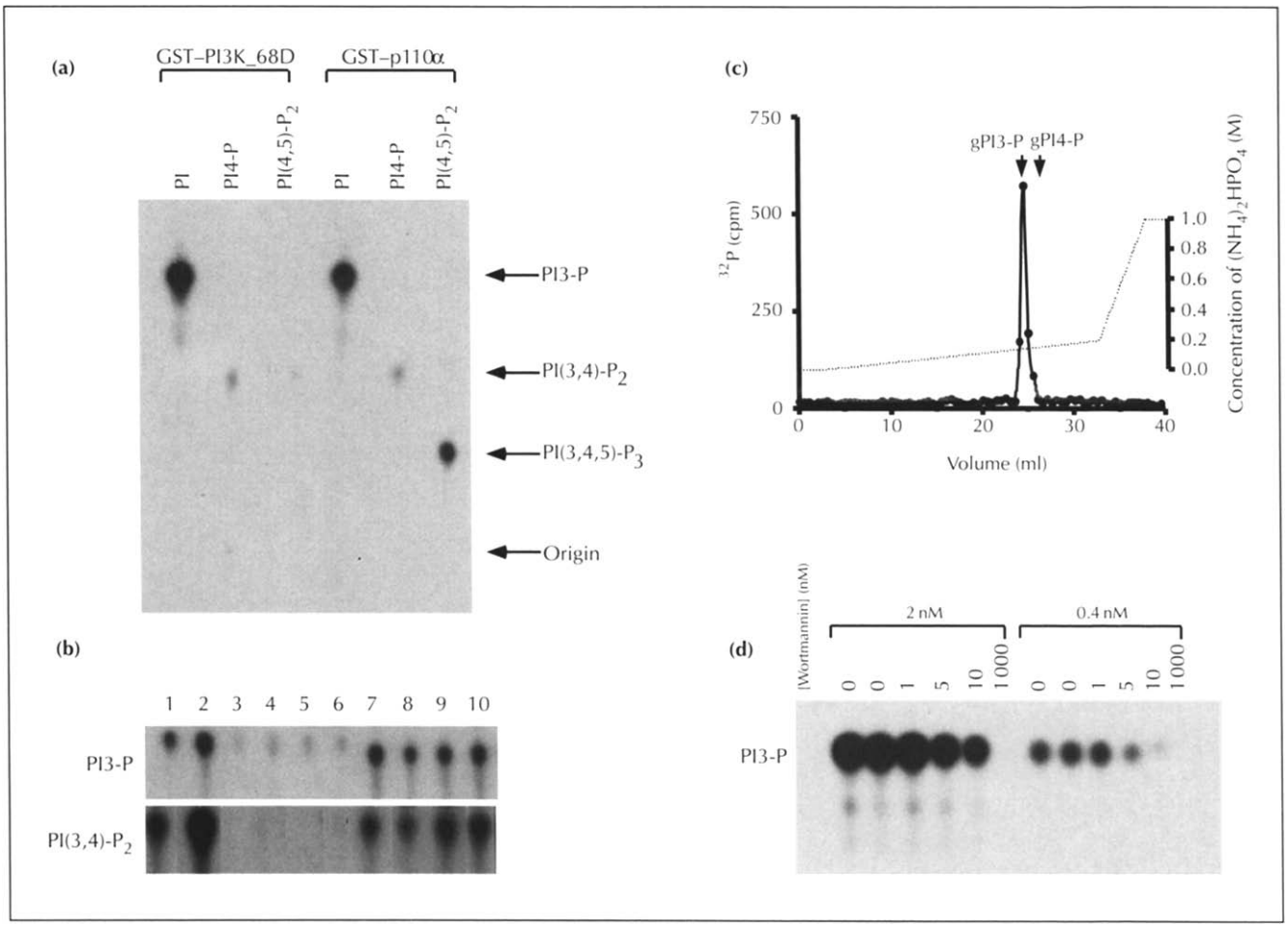

Fig. 3. Biochemical characterization of recombinant and endogenous PI3K_68D activity. The extracted lipids were resolved either by thin layer chromatography (TLC), and detected by **(a)** autoradiography or **(b** and **d)** phosphorimaging, or by **(c)** HPLC. (a) *In vitro* substrate specificity of recombinant GST–PI3K_68D and GST–p110α. PI 3-kinase assays were performed as described in Materials and methods, using PI, PI4-P and PI(4,5)-P_2 as substrates. The specific radioactivity of the Mg^{2+}–ATP was five-fold higher for GST–PI3K_68D with PI4-P and PI(4,5)-P_2 as substrates. (b) Activity of endogenous PI3K_68D towards PI and PI4-P: PI 3-kinase assays were performed on immunoprecipitates as described in Materials and methods. Lanes 1 and 2, recombinant GST–p110α and GST–PI3K_68D, respectively. Lanes 3–10, extracts immunoprecipitated with preimmune serum (lanes 3–6) or antiserum directed against PI3K_68D (lanes 7–10). Extracts were prepared from larvae (lanes 3 and 7), pupae (lanes 4 and 8), adults (lanes 5 and 9) and S2 cells (lanes 5 and 10). Flies were Oregon R strain. (c) HPLC analysis of the deacylated products of PI phosphorylation by recombinant GST–PI3K_68D. The dotted line represents the $(NH_4)_2HPO_4$ gradient; gPI3-P and gPI4-P denote deacylated PI3-P and PI4-P, respectively. (d) Inhibition of PI 3-kinase activity by wortmannin: inhibition of the activity of GST–PI3K_68D (at 2 nM and 0.4 nM) towards PI was assayed using the concentrations of wortmannin indicated. A similar level of inhibition was observed when PI4-P was used as a substrate.

with a similar specific activity to that of GST–p110α (~ 2 nmol min^{-1} mg^{-1}), and towards PI4-P, with an approximately 20-fold lower specific activity compared to GST–p110α. In contrast, GST–PI3K_68D did not phosphorylate PI(4,5)-P_2 (Fig. 3a).

To overcome problems of lipid presentation *in vitro*, PI(4,5)-P_2 was presented in a number of different lipid vehicles and in the presence of detergents which stimulate the activity of GST–p110α towards PI(4,5)-P_2 relative to PI (results not shown). In order to investigate whether an endogenous factor required for PI(4,5)-P_2 phosphorylation was lacking in the recombinant enzyme preparation, similar assays were performed using PI3K_68D immunoprecipitated from *Drosophila* S2 cells (an embryonic cell line) and from wild-type (Oregon R) flies at different developmental stages using an antibody directed against the carboxyl terminus of PI3K_68D. The endogenous kinase, which was detected in immunoprecipitates from larval, pupal and adult extracts, and from S2 cells, displayed activity towards both PI and PI4-P, demonstrating that PI3K_68D is widely expressed throughout the *Drosophila* life cycle (Fig. 3b). Consistent with the data obtained with the recombinant enzyme, endogenous PI3K_68D was unable to phosphorylate PI(4,5)-P_2 (data not shown). We cannot exclude the possibility that under specific conditions *in vivo*, there is a stimulation of activity towards PI(4,5)-P_2, but as recombinant mammalian p110α does display this activity in parallel experiments *in vitro* we think this unlikely.

In order to formally confirm that PI3K_68D phosphorylates phosphatidylinositol at the 3-position, as implied from its close sequence homology to p110α in the kinase domain, we performed anion-exchange high-pressure liquid chromatography (HPLC). The deacylated products of PI phosphorylation yielded a single peak of radioactive glycerophosphate. This represented deacylated PI 3-monophosphate, as determined by its coelution with the product of GST–p110α using PI as a substrate. There was no detectable production of PI4-P by GST–PI3K_68D (Fig. 3c). These results confirm that PI3K_68D specifically phosphorylates the D-3 position of the inositol ring.

The antifungal compound wortmannin is a potent inhibitor of PI 3-kinases [41], particularly those of the p110 class, and has been used extensively in tissue culture studies to investigate the role of the classical p110–p85 PI 3-kinase in intracellular signalling. The activity of PI3K_68D towards both PI and PI4-P was inhibited by wortmannin with a similar IC_{50} (5 nM) to that of GST–p110α (2 nM, when assayed in parallel) (Fig. 3d and Table 1), indicating that, on the basis of its sensitivity to this reagent, it may contribute to some of the cellular processes previously attributed to p110α. As PI3K_68D and PI3Kγ are also inhibited by wortmannin, this inhibitor cannot be used to implicate a specific PI 3-kinase in intracellular signalling. Indeed, some classes of PI 4-kinase are reported to be sensitive to wortmannin [42], as has the more distantly related protein kinase, DNA-dependent protein kinase, although with a considerably higher IC_{50} value [43].

Table 1: A comparison of the properties of the novel PI 3-kinase, PI3K_68D, with p110α and Vps34p.

	PI3K_68D *Drosophila*	p110α bovine	Vps34p *S. cerevisiae*
Mr (calculated)	210 kDa	124 kDa	95 kDa
Cation requirement	Mg^{2+}	Mg^{2+}	Mn^{2+}/Mg^{2+}
In vitro substrate specificity	PI, PI4-P	PI, PI4-P PI(4,5)-P_2	PI only
IC_{50} for inhibition by wortmannin	5 nM	2–5 nM	3 μM
Regulatory subunit	?	p85	Vps15p

Values for p110α are from [15,23,24] and this study. Values for Vps34p are from [20,70]. Note that a recombinant human Vps34p has a sensitivity to wortmannin similar to that of p110α [24].

Analysis of the C2 domain

It is now recognized that conserved protein modules (such as SH2 and SH3 domains) are the basis of many of the protein–protein interactions which are a feature of signal transduction pathways. The C2 domain, together with a putative PH domain in PI3Kγ [19], are among the first such protein modules, previously identified in other signalling molecules, to be found in PI 3-kinase catalytic subunits. The C2 domain was originally identified in the lipid-dependent enzyme PKC [40], and subsequently in cytosolic phospholipase A_2 [44], where it functions to permit Ca^{2+}, acting as a second messenger, to activate the enzyme by translocating it to its lipid substrates at the membrane. As PI 3-kinases must also access lipid substrates, we investigated whether the isolated C2 domain from PI3K_68D could bind to synthetic phospholipids in a Ca^{2+}-dependent manner.

In order to test this possibility we expressed a GST-fusion protein containing the C2 domain of PI3K_68D, GST–PI3K_68D(C2), in bacteria and used this in a sedimentation assay to analyse binding to phospholipids in the presence or absence of Ca^{2+}. GST–PI3K_68D(C2) bound weakly to acidic phospholipids. However, this association was not affected by EGTA or EDTA, demonstrating that it was not cation-dependent (Fig. 4a). As a control, we performed similar experiments with GST constructs containing C2 domains derived from synaptotagmin. Synaptotagmin is a neuronal protein that is implicated in the Ca^{2+}-triggered exocytosis of synaptic vesicles and contains tandem C2 domains [45]. A GST-fusion protein containing the first C2 domain alone (Fig. 4a), or both C2 domains of synaptotagmin (data not shown), bound to membranes in a Ca^{2+}-dependent manner, consistent with previous results [46]. However, the second C2 domain of synaptotagmin, like the C2 domain of PI3K_68D, bound to acidic phospholipid-containing

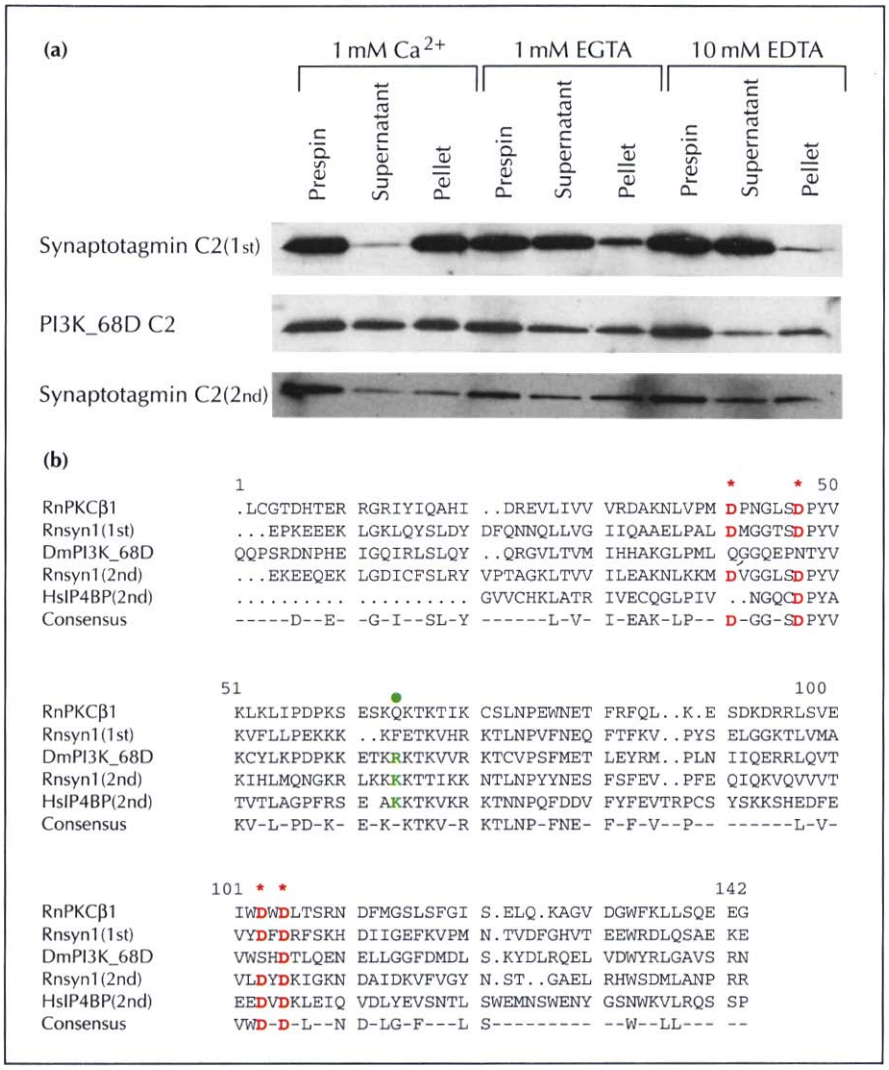

Fig. 4. Analysis of the C2 domain of PI3K_68D. **(a)** The C2 domain of PI3K_68D binds weakly to phospholipids independently of Ca^{2+}: GST-fusion proteins corresponding to the C2 domains of PI3K_68D and synaptotagmin were prepared and assayed for lipid binding as described in Materials and methods. Fusion proteins were detected using antibodies directed against GST and visualised with enhanced chemiluminescence (ECL) and autoradiography. **(b)** Alignment of Ca^{2+}-dependent and Ca^{2+}-independent/IP4-binding C2 domains. Sequences corresponding to the C2 domains from PI3K_68D (residues 1740–1876), rat PKCβ1 (RnPKCβ1, accession number P04410; residues 150–283), rat synaptotagmin I (RnsynI, accession number P21707; first C2 domain, residues 135–268; second C2 domain, residues 265–399) and $GAP1^{IP4BP}$ (HsIP4BP, accession number X89399; residues 140–258) were aligned using the PILEUP programme. Asterisks denote the position of the asparagine residues implicated in Ca^{2+}-binding based on crystallographic studies of the first C2 domain of synaptotagmin; analogous residues in aligned proteins are shown in red. Note that these four residues are conserved in the Ca^{2+}-independent second C2 domain of synaptotagmin. The circle corresponds to the lysine, identified in synaptotagmin II, as being involved in IP4 binding [48]. Corresponding residues in aligned proteins are shown in green. In PI3K_68D this residue corresponds to an arginine (conserving the positive charge) whereas in the first C2 domain of synaptotagmin, which is unable to bind to IP4, a hydrophobic residue (phenylalanine) is present at this position.

vesicles in a manner largely independent of Ca^{2+} (Fig. 4a and [47,48]). Thus, PI3K_68D contains a Ca^{2+}-independent type of C2 domain. The weak lipid binding demonstrated by the GST-fusion protein containing the C2 domain of PI3K_68D, but not by GST alone, indicates that the domain could be involved in membrane binding, which would presumably be mediated by the highly charged stretch of amino acids found between residues 1788–1808, in which 10 out of 21 amino acids are positively charged (Fig. 2). Polybasic regions have been shown to contribute to membrane binding in certain forms of Ras. In the Ras family, membrane association requires two components, one of which is farnesylation, the other is either the presence of a polybasic region or palmitoylation [49].

Discussion

A family of PI 3-kinases in *Drosophila* includes a novel class of enzyme

Previous work has suggested that a number of distinct PI 3-kinase enzymes are involved in pathways mediating several aspects of cell physiology. These include the heterodimeric PI 3-kinases (p110α and β, which are recruited and activated through the p85 regulatory subunit by tyrosine kinases), PI3Kγ (a G-protein-activated PI 3-kinase), and the PI 3-kinase encoded by the *VPS34* gene. PI 3-kinases p110α and β and PI3Kγ are able to use PI, PI4-P, and PI(4,5)-P_2 as substrates *in vitro*, although only the higher phosphorylated lipid products (PI(3,4)-P_2 and PI(3,4,5)-P_3) are elevated in response to ligand stimulation (reviewed in [1]). In contrast, Vps34p seems to be restricted to using PI as a substrate and may be sensitive to intracellular changes rather than extracellular signals. We have identified a family of PI 3-kinases in *Drosophila* on the basis of sequence homologies with the putative catalytic domain of established PI 3-kinases. Two of the members exhibit strong homologies to the prototype PI 3-kinases, p110α and Vps34p, whereas the third represents a novel class of enzyme.

The three PI 3-kinases identified in *Drosophila* are more closely related to each other than to PI 4-kinases or the structurally unrelated PI 5-kinases, or to the two groups of 'PI 3-kinase-related' proteins typified by Tor2 and ATM (reviewed in [50]). This result was not unexpected because the primers used in our PCR-based cloning

strategy were biased towards regions of homology in p110α with Vps34p. In contrast, a recent report describing a PI kinase gene family in *Dictyostelium* used primers based on sequence homology with p110α, Vps34p and a yeast PI 4-kinase. This approach identified a putative PI 4-kinase and four putative PI 3-kinases, on the basis of sequence homology in the catalytic domain [27]. One of these was most similar to Vps34p, whereas the other three contained a cluster of p110-like sequences more closely related to each other than to distinct members of the p110 family of PI 3-kinases (p110α/β and PI3Kγ) or to PI3K_68D.

Potential functions for modules towards the amino and carboxyl termini of PI3K_68D

PI3K_68D lacks the region present in p110α/β that associates with p85 [38] but contains two regions, not found to date in other PI 3-kinases, which may specify interactions with other molecules and allow access to lipid substrates at the membrane. The first of these is a polyproline motif towards the amino terminus (Fig. 2). The type II class of polyproline motif in PI3K_68D is similar to the polyproline motifs identified in the Ras guanine nucleotide exchange protein Sos, and may serve an analogous role. In Sos, this motif has been shown to be the binding site for the amino-terminal SH3 domain of the Drk adaptor protein (the *Drosophila* homologue of mammalian Grb2) and thus links Sos to upstream signals in a signal-transducing complex at the plasma membrane [51]. We are currently investigating potential interactions between the polyproline motif of PI3K_68D and the Drk adaptor and other SH3-containing proteins.

A second domain novel to PI 3-kinases that is present in PI3K_68D is the carboxy-terminal C2 domain (Fig. 2), which we have shown not to function in a Ca^{2+}-dependent manner in phospholipid binding (Fig. 4a). Crystallographic studies have revealed the three-dimensional structure of the first C2 domain of synaptotagmin and have shown that the Ca^{2+}-binding site is a bipartite structure formed by two remote segments of the polypeptide chain, with four aspartyl residues (D^{178}, D^{230} and, to a lesser extent, D^{232} and D^{172}) implicated in Ca^{2+}-binding [52]. Only one of these residues (equivalent to D^{230}) is conserved in PI3K_68D (D^{1840}). PI3K_68D is, therefore, unlikely to function in the same manner as the first C2 domain of synaptotagmin, with respect to Ca^{2+}-binding, consistent with our observations (Fig. 4).

Previous studies have indicated that C2 domains may mediate a variety of interactions. In addition to the prototypical function of Ca^{2+}-dependent phospholipid binding, Ca^{2+}-independent interactions with proteins and/or inositol polyphosphates have been demonstrated. The structural basis for these functional differences is not readily apparent. Molecules such as synaptotagmin [45], rabphilin [53] and certain Ras-GAPs (see, for example, [54]) contain tandem C2 domains, only one of which confers Ca^{2+}-dependent phospholipid binding. Typically, both C2 domains are highly conserved in terms of their amino-acid sequence (see Fig. 4b). The second (or Ca^{2+}-independent) C2 domain of synaptotagmin has also been reported to function both as an inositol polyphosphate receptor, with a preference for the highly phosphorylated inositols IP4 (inositol (1,3,4,5)-tetrakisphosphate) and IP6 (inositol (1,2,3,4,5,6)-hexakisphosphate) [48], and as a region of protein–protein interaction with AP2, implicating it in the process of clathrin-mediated endocytosis [55].

Similar interactions have been reported for the C2 domains of several other proteins and a function for IP4 binding has been demonstrated for a mammalian Ras-GAP that contains two C2 domains. Cullen *et al.* reported that the binding of IP4 to Ras-GAP relieved the inhibition of the GTPase activity towards Ras that occurred in the presence of artificial lipids that mimicked the composition of the plasma membrane [54]. The C2 domain of PKC has also been proposed to bind to intracellular proteins, termed RACKS (receptors for activated C kinases), which participate in the release of the enzyme from pseudosubstrate inhibition [56]. The Ca^{2+}-independent C2 domain of PI3K_68D may therefore represent a protein–protein interaction site. At present we are addressing this question using a 'pull-down' assay with a GST–PI3K_68D(C2) fusion protein and *Drosophila* cell lysates.

A potential new mediator of signalling by 3-phosphorylated lipids defined by novel biochemical properties

The distinctive biochemical properties of PI3K_68D *in vitro*, specifically its inability to phosphorylate PI(4,5)-P_2, indicate that this enzyme may signal through PI(3,4)-P_2 *in vivo*. Although we have not yet determined the mechanism of PI3K_68D activation, it is conceivable that this PI 3-kinase may contribute to, or be responsible for, the elevations in PI(3,4)-P_2 that have been observed in ligand-stimulated cells. This *in vitro* substrate specificity distinguishes PI3K_68D from the Vps34p family of PI 3-kinases, members of which are restricted to using PI as a substrate, and from the p110α/β/PI3Kγ family of proteins, which are able to phosphorylate PI(4,5)-P_2 *in vitro* and have been proposed to signal through PI(3,4,5)-P_3 *in vivo* (reviewed in [1]). Although the specific activity of PI3K_68D towards PI4-P *in vitro* was lower than that towards PI, the results suggest that this enzyme may signal through a distinct lipid, PI(3,4)-P_2, which, unlike PI3-P, is elevated in response to extracellular signals. Immunostaining of cellularized embryos localizes PI3K_68D at or close to the plasma membrane (L.K.M., E. Hafen, M.D.W., unpublished observations), indicating that this enzyme, like p110α/β/PI3Kγ, may respond to extracellular signals and act at the plasma membrane. In contrast, yeast Vps34p is located at intracellular membranes (probably the Golgi), consistent with the role of this kinase in protein sorting and trafficking [28]. The p110 class of enzyme shares with PI3K_68D the ability to phosphorylate PI *in vitro* with a higher specific activity than when it phosphorylates PI4-P (Fig. 3a). However, quantitative analyses of relative activities *in vitro* are difficult to assess because of the problems in mimicking the

in vivo presentation of lipids (discussed in [1]), and ligand stimulation of cell-surface receptors does not seem to elevate the levels of PI3-P in cells. This suggests that PI may not be accessible despite its higher bulk concentration in the plasma membrane relative to other phosphoinositides. This interpretation is supported by recent studies on the requirement of a PI transfer protein for a PI 4-kinase activity at the plasma membrane [57].

A potential role for PI(3,4)-P_2 in intracellular signalling?

Studies on the elevation of D-3 phosphorylated lipids in ligand-stimulated cells have demonstrated differences in the kinetics of production of PI(3,4)-P_2 and PI(3,4,5)-P_3. Both of these phosphoinositides accumulate and are subsequently degraded rapidly in response to ligand stimulation. Studies of G-protein-activated and tyrosine kinase receptors have shown that a more rapid stimulation of PI(3,4,5)-P_3, with a much slower and sustained increase in the level of PI(3,4)-P_2, occurs. As a result of these studies, which were interpreted on the basis of the existence of a single or a limited number of closely related PI 3-kinase activities, it has been assumed that PI(3,4,5)-P_3 is the mediator of ligand-stimulated responses and that PI(3,4)-P_2 is formed as a result of the action of a potent PI(3,4,5)-P_3 5′-phosphatase, acting to terminate the PI(3,4,5)-P_3 signal [1]. In certain cell systems PI(3,4)-P_2 is the major lipid produced in response to extracellular signals (see, for example, [58–60]), and studies of specific PKC isoforms have demonstrated that both of the higher phosphorylated lipids PI(3,4)-P_2 and PI(3,4,5)-P_3 may stimulate their activities *in vitro* [5].

The finding that PI3K_68D is not able to produce PI(3,4,5)-P_3 *in vitro* now allows a reinterpretation of the role of PI(3,4)-P_2 in cell signalling. PI(3,4)-P_2 may thus be formed by two routes involving PI 3-kinases: the direct phosphorylation of PI4-P by PI3K_68D, and possibly other p110 PI 3-kinases, or the indirect action of a PI(3,4,5)-P_3 5′-phosphatase. The differences in substrate specificity observed between PI3K_68D and p110α are underlined by the differences in primary amino-acid sequence within the catalytic domain (Fig. 1a,c). PI3K_68D, therefore, forms a novel class of PI 3-kinases distinct from an increasing number of mammalian p110-related molecules, including p110α and β and PI3Kγ, which can phosphorylate PI(4,5)-P_2, and from Vps34p, which is restricted to using PI as a substrate. Thus, each member of the family of *Drosophila* PI 3-kinases identified in this study seems to represent a distinct subgroup of PI 3-kinases. It is possible that, in response to specific activation of the PI3K_68D class of enzymes, PI(3,4)-P_2 alone may be produced in a particular cellular context that allows a novel signal transduction cascade.

Conclusions

Using a PCR-based approach and degenerate primers, we have identified three cDNAs from *Drosophila*, each of which defines a distinct subgroup of PI 3-kinases. One is homologous to the yeast Vps34p, which is involved in protein trafficking, and a second is related to PI 3-kinases that are linked to receptor protein tyrosine kinases. The third cDNA defines a novel PI 3-kinase, termed PI3K_68D, which is characterized biochemically by a distinct substrate specificity and structurally by a carboxy-terminal C2 domain and an amino-terminal polyproline motif. These structural motifs indicate that PI3K_68D may display novel regulatory interactions, particularly as it lacks the site for interaction with the p85 adaptor protein. *In vitro*, PI3K_68D displayed a substrate specificity that is distinct from the p110-related molecules (p110α/β/PI3Kγ) and from Vps34p, indicating that it may target distinct downstream molecules and thus define a novel signal transduction pathway.

Materials and methods

General molecular biology techniques were performed as described by Sambrook [61]. cDNA probes were labelled by random priming using α-[^{32}P]dCTP. Oligonucleotides were labelled with γ-[^{32}P]ATP and polynucleotide kinase. DNA sequencing was performed using the *Taq* DyeDeoxyTerminator Cycle Sequencing Kit (Applied Biosystems) with T3, T7, SP6 or PI3K_68D-specific primers and analysed on an Applied Biosystems 373A automated DNA sequencer. Data was analysed using the University of Wisconsin Genetics Computer Group software package [62].

Cloning of PI 3-kinase homologues

Poly A^+ RNA was isolated from *Drosophila* larvae, pupae and adults using oligo(dT) cellulose (Pharmacia) and used to synthesise first-strand cDNA with AMV reverse transcriptase (Pharmacia). PCR reactions were carried out using *Taq* DNA polymerase (Promega), each of the first-strand cDNA templates, 4 mM Mg^{2+} and 1 μM primers. The nucleotide sequences of the primers, followed by the amino-acid sequence that they represent (in parenthesis) are as follows: sense, GGNGA T/C GA T/C T/C T A/G CGNCA A/G GA (GDDLRQD/E); and antisense, A/G AA A/G TGICC A/G AA A/G TC A/G/T AT A/G TG A/G AA (FHIDFGHF). The conditions were 35 cycles at 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 30 sec followed by a final extension at 72 °C for 7 min. Individual clones were sequenced following subcloning into the pGEM-T vector (Pharmacia) using SP6 and T7 primers.

In situ *hybridization*

Polytene chromosome preparations obtained from the salivary glands of female *Drosophila melanogaster* (Oregon R) third instar larvae were hybridized with biotin-labelled probes as described in [63]. Probes were generated using the PCR fragments corresponding to the kinase domain of each of the *Drosophila* PI 3-kinases identified. The localization of PI3K_68D was confirmed with a 5.4 kb cDNA probe.

Isolation of the PI3K_68D cDNA

The PCR fragment, localizing to position 68D, was excised from the pGEM-T vector and used to screen a high coverage reference library of individual *Drosophila* embryo cDNAs (provided by J. Hoheisel and H. Lerach [64]). A single positive cDNA clone (cDNA1:69F7) was obtained corresponding to a 3.2 kb cDNA, which on sequencing was found to contain

about 2 kb of open reading frame and no in-frame stop codon at the 5′ end. To extend the sequence, probes corresponding to the 5′ end of this clone were used to screen an embryonic library in lambda ZAP II (Stratagene) provided by M. Noll. Three clones were obtained and sequenced on both strands following subcloning of restriction fragments into pBluescript KS^+. Gaps in the sequence were filled using *PI3K_68D*-specific oligonucleotide primers. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X92892.

Plasmid constructs

Constructs were verified by restriction site and sequence analysis. All PCR-amplified sequences were confirmed by sequencing. To facilitate subcloning, a shuttle cassette was created from the 7.2 kb PI3K_68D cDNA, incorporating *Bam*HI sites at the 5′ end, immediately prior to the initiating ATG codon, and at the 3′ end, after the stop codon. The procedure for creating this cassette is outlined below.

5′ *Bam*HI site: using the oligonucleotides GCGCGCGGC-CGCGGATCCATGTCAAATCAAGCGCATATCGACT-ACG (sense) and GAGGGCATGCTGCTCTAGACTCAGG (antisense) we amplified a 1.2 kb fragment. The product was cut with *Not*I and *Xba*I and the released fragment cloned into the parent cDNA (modified pSK_68D).

Mutation of the internal *Bam*HI site (nucleotide 5379 in the open reading frame): a *Sal*I fragment corresponding to nucleotides 3422–5597 was subcloned into M13 in the forward orientation and the single-stranded template mutated using the oligonucleotide GGTCTCCTTTTTGGGGTCCGGTTTT-AGG and the Sculptor IVM kit (Amersham), the product was then recloned into the modified pSK_68D plasmid. This mutation did not alter the amino-acid sequence of the encoded protein.

3′ *Bam*HI site: A PCR fragment was amplified using oligonucleotides CCCAGTCCTCTCGAGTACC (sense) and GC-GCGGTACCGGATCCTCCCTAGGATCTGGTCAGT-TCCTGG (antisense) and the mutated pSK_68D template was digested with *Sse*8387i and *Kpn*I and religated into *pSK_68D* modified as above.

Production of vectors encoding GST-fusion proteins: to produce the vector encoding GST fused to PI3K_68D, pAcGEX-PI3K_68D, the shuttle cassette was released from pBluescript SK^+ by digestion with *Bam*HI and subcloned into the *Bam*HI site of pAcGEX2T [65]. The vector encoding the C2 domain of PI3K_68D fused to GST, pGEX-PI3K_68D(C2), was produced as follows: a PCR fragment corresponding by sequence homology to the C2 domain of PKC, with added restriction sites *Xba*I and *Xho*I, was amplified by using oligonucleotides GCGCTCTAGACCTGCTGCGCGATCAGCAGGAAGC and GCGCCTCGAGTCCCTAGGATCTGGTCAGTTCC-TGG, and digested with *Xba*I and *Xho*I for insertion into the same sites in pGEX-KG [66].

Cell culture and transfections

Sf9 cells were cultured in IPL4-1 containing 20 % yeastolate, 10 % lipid extract and 10 % FCS as described [67]. Plasmid DNA was cotransfected with BaculoGold DNA (Pharmingen) into Sf9 insect cells using Lipofectin (Gibco). Recombinant plaques were isolated and characterized by established methods [67].

Expression of recombinant GST-fusion proteins in Sf9 cells and bacteria

Typically, Sf9 cells, at a density of 10^6 cells ml^{-1}, were infected with baculoviruses for ~ 65 h. Cells were then harvested, lysed in PBS-T (1 × PBS, 1 % (w/v) Triton X-100, 2 mM EDTA) containing 0.1 % β-mercaptoethanol and the following protease inhibitors: 5 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride and 100 mU ml^{-1} aprotinin, Sigma) and the extract incubated with glutathione–Sepharose beads for 2 h at 4 °C with rotation. GST-tagged proteins bound to the beads were recovered by centrifugation and, after repeated washing with PBS-T then Hepes buffer (50 mM Hepes, 150 mM NaCl (pH 8) and 1 mM DTT), eluted into Hepes buffer containing 25 mM glutathione. The released protein was dialyzed against Hepes buffer containing 50 % glycerol and stored at −20 °C. Expression of GST-fusion proteins in bacteria was essentially as described [66]; fusion proteins were eluted with glutathione as described for recombinant GST–PI 3-kinases.

Assay of PI 3-kinase activity

Standard PI 3-kinase assays were performed essentially as described in [6], in a volume of 50 μl containing 20 mM Hepes–NaOH (pH 7.4), 100 mM NaCl, 0.1 mM EGTA, 2.5 mM $MgCl_2$, 0.1 % β-mercaptoethanol, 100 μM ATP (plus 0.2 μCi γ-[^{32}P]ATP) and 200 μM phosphoinositide (PI, PI4-P or PI(4,5)-P_2 from Sigma or Lipid Products). For assays with PI(4,5)-P_2, 0.7 % cholate (w/v) was included. After preincubation with lipid for 20 min on ice and 5 min at 30 °C, reactions were initiated by the addition of Mg^{2+}–ATP. Assays were terminated with acidified chloroform:methanol, the lipid products extracted and resolved by TLC. For standard assays using PI as substrate, the solvent used was chloroform:methanol:4 M ammonium hydroxide (45:35:10). For separation of PI3-P, PI(3,4)-P_2 and PI(3,4,5)-P_3 the solvent used was propan-1-ol:2 M acetic acid (65:35) [11]. Radioactivity was analysed by autoradiography or quantified using a Phosphorimager (Molecular Dynamics). Protein concentrations were estimated by Coomassie staining of SDS–polyacrylamide gels and by using the Pierce BCA protein detection reagent, with bovine serum albumin (BSA) as the standard. Assays were linear with respect to time and enzyme concentration.

HPLC characterization of PI3K_68D reaction products

Assays of GST–PI3K_68D were performed as above, extracted with acidified chloroform:methanol, then dried and deacylated as previously described [68]. HPLC analysis of glycerophosphoinositols was performed using two Partisphere SAX columns (Whatman) connected in series and eluted using a linear gradient of $(NH_4)_2HPO_4$ (pH 3.8) in water at 0.5 ml min^{-1}. Tritiated, deacylated PI4-P (Amersham) was used as an internal standard in all column runs and each fraction was analysed in the absence and presence of scintillant. A PI3-P standard was prepared by phosphorylating PI *in vitro*, with baculovirus-expressed p110α, followed by deacylation as described above.

Immunological methods

Standard procedures for immunization and serum preparation were as described in Harlow and Lane [69]. Antibodies were raised in rabbits against the C2 domain of PI3K_68D released from glutathione–Sepharose beads by thrombin treatment. For immunoprecipitations, *Drosophila* larvae, pupae and adults (10 mg ml^{-1} lysis buffer) or *Drosophila* S2 cells (10^7 cells per ml) were lysed in PBS-T and clarified by centrifugation. Extracts were incubated with a 1:50 dilution of anti-68DC2 and 20 μl of 50 % (v/v) protein A–Sepharose beads for 2 h at 4 °C. After

extensive washing in PBS-T, immunoprecipitates were washed with assay buffer and analysed for PI 3-kinase activity as described above.

Assay of lipid binding

Liposomes containing 40 % phosphatidylserine and 60 % phosphatidylcholine were prepared in 20 mM Hepes–NaOH (pH 7.4), 150 mM NaCl by sonication and collected by centrifugation. GST-fusion proteins (2 pmol) were incubated with phospholipid (1 mg ml^{-1}) in 20 mM Hepes–NaOH (pH 7.4), 150 mM NaCl, 0.1 mg ml^{-1} BSA, 1 mM benzamidine, 1 mM DTT and either 1 mM Ca^{2+} or 1 mM EGTA or 10 mM EDTA for 60 min at 4 °C in a volume of 1.6 ml. Liposomes were collected by centrifugation (45 min at 40 000 rpm in a Beckman SW60). Pellets were resuspended to the original volume in the binding buffer containing 0.2 % (w/v) Triton X-100. Samples consisting of equal volumes of prespin, supernatant and resuspended pellet were resolved on 15 % SDS gels, transferred to nitrocellulose and developed with a polyclonal antibody directed against GST (provided by George Panayotou) using ECL (Amersham).

Acknowledgements: We would like to thank D. Mann for help with in situ hybridizations; M. Noll, T. Raabe, Ernst Hafen, J. Hoheisel and H. Lehrach for provision and assistance with cDNA libraries; T. Südhof for plasmids encoding synaptotagmin C2 GST-fusion proteins; B. Vanhaesebroeck for baculovirus encoding GST-p110α; G.Panayotou for antibody to GST; Wei-Peng Lee and R. Marshall for technical assistance; and D. Mann, S. Leevers and M. Zvèlebil for constructive criticism of the manuscript.

References

1. Stephens LR, Jackson TR, Hawkins PT: **Agonist-stimulated synthesis of phosphatidylinositol (3,4,5)-trisphosphate: a new intracellular signalling system?** *Biochim Biophys Acta* 1993, **1179:**27–75.
2. Kapeller R, Cantley LC: **Phosphatidylinositol 3-kinase.** *BioEssays* 1994, 16:565–576.
3. Serunian LA, Haber MT, Fukui T, Kim JW, Rhee SG, Lowenstein E, *et al.*: **Polyphosphoinositides produced by phosphatidylinositol 3-kinase are poor substrates for phospholipases C from rat liver and bovine brain.** *J Biol Chem* 1989, **264:**17809–17815.
4. Burgering BMT, Coffer PJ: **Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction.** *Nature* 1995, **376:**599–602.
5. Toker A, Meyer M, Reddy KK, Falck JR, Aneja R, Aneja S, *et al.*: **Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3.** *J Biol Chem* 1994, **269:**32358–32367.
6. Whitman M, Kaplan DR, Schaffhausen B, Cantley LC, Roberts, TM: **Association of phosphatidylinositol 3-kinase activity with polyoma middle-T competent for transformation.** *Nature* 1985, **315:**239–242.
7. Hara K, Yonezawa K, Sakaue H, Ando A, Kotani K, Kitamura T, *et al.*: **1-phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells.** *Proc Natl Acad Sci USA* 1994, **91:**7415–7419.
8. Valius M, Kazlauskas A: **Phospholipase C-γ1 and phosphatidylinositol 3-kinase are the downstream mediators of the PDGF receptor's mitogenic signal.** *Cell* 1993, **73:**321–334.
9. Wennstrom S, Hawkins P, Cooke F, Hara K, Yonezawa K, Kasuga M: **Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling.** *Curr Biol* 1994, **4:**385–393.
10. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry M, *et al.*: **Phosphatidylinositol-3-OH kinase as a direct target of ras.** *Nature* 1994, **370:**527–532.
11. Morgan SJ, Smith AD, Parker PJ: **Purification and characterization of bovine brain type I phosphatidylinositol kinase.** *Eur J Biochem* 1990, **191:**761–767.
12. Otsu M, Hiles I, Gout I, Fry MJ, Ruiz-Larrea F, Panayotou G, *et al.*: **Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60c-src complexes, and PI 3-kinase.** *Cell* 1991, **65:**91–104.
13. Skolnik EY, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, *et al.*: **Cloning of PI 3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases.** *Cell* 1991, **65:**83–90.
14. Escobedo J, Navankasattusas S, Kavanaugh WM, Milfay D, Fried VA, Williams LT: **cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI 3-kinase to the PDGF beta-receptor.** *Cell* 1991, **65:**75–82.
15. Hiles I, Otsu M, Volinia S, Fry MJ, Gout I, Dhand R, *et al.*: **Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit.** *Cell* 1992, **70:**419–429.
16. Pons S, Asano T, Glasheen E, Miralpeix M, Zhang Y, Fisher TL, *et al.*: **The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase.** *Mol Cell Biol* 1995, **15:**4453–4465.
17. Hu P, Mondino A, Skolnik EY, Schlessinger J: **Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85.** *Mol Cell Biol* 1993, **13:**7677–7688.
18. Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweiss PC, Hawkins PT: **A novel phosphoinositide-3 kinase activity in myeloid-derived cells is activated by G protein βγ subunits.** *Cell* 1994, **77:**83–93.
19. Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D, *et al.*: **Cloning and characterization of a G-protein-activated human phosphoinositide-3 kinase.** *Science* 1995, **269:**690–693.
20. Herman PK, Emr SD: **Characterization of Vps34p, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae*.** *Mol Cell Biol* 1990, **10:**6742–6754.
21. Schu PV, Takegawa K, Fry MJ, Stack JH, Waterfield MD, Emr SD: **Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting.** *Science* 1993, **260:**88–91.
22. Chapman RE: **Vacuolar sorting: tracking down an elusive receptor.** *Curr Biol* 1994, **4:**1019–1022.
23. Stephens L, Cooke FT, Walters R, Jackson T, Volinia S, Gout I, *et al.*: **Characterization of a phosphatidylinositol-specific phosphoinositide 3-kinase from mammalian cells.** *Curr Biol* 1994, **4:**203–214.
24. Volinia S, Dhand R, Vanhaesebroeck B, MacDougall LK, Stein R, Zvèlebil MJ, *et al.*: **A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p–Vps15p protein sorting system.** *EMBO J* 1995, **14:**3339–3348.
25. Hong Z, Verma DPS: **A phosphatidylinositol 3-kinase is induced during soybean nodule organogenesis and is associated with membrane proliferation.** *Proc Natl Acad Sci USA* 1994, **91:**9617–9621.
26. Welters P, Takegawa K, Emr SD, Chrispeels MJ: **AtVps34, a phosphatidylinositol 3-kinase of *Arabidopsis thaliana*, is an essential protein with homology to a calcium-dependent lipid binding domain.** *Proc Natl Acad Sci USA* 1994, **91:**11398–11402.
27. Zhou K, Takegawa K, Emr SD, Firtel RA: **A phosphatidylinositol (PI) kinase gene family in *Dictyostelium discoideum*: biological roles of putative mammalian p110 and yeast Vps34p PI 3-kinase homologs during growth and development.** *Mol Cell Biol* 1995, **15:**5645–5656.
28. Stack JH, Herman PK, Schu PV, Emr SD: **A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole.** *EMBO J* 1993, **12:**2195–2204.
29. Hunter T: **When is a lipid kinase not a lipid kinase? When it is a protein kinase.** *Cell* 1995, **83:**1–4.
30. Zakian VA: **ATM-related genes: what do they tell us about the functions of the human gene?** *Cell* 1995, **82:**685–687.
31. Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, Hall MN: **Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression.** *Cell* 1993, **73:**585–596.
32. Cafferkey RT, Young PR, McLaughlin MM, Bergsma DJ, Koltin Y, Sathe GM, *et al.*: **Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and Vps34 abrogate rapamycin cytotoxicity.** *Mol Cell Biol* 1993, **13:**6012–6023.
33. Downward J: **Regulating S6 kinase.** *Nature* 1994, **371:**378–379.
34. Flanagan CA, Schnieders EA, Emerick AW, Kunisawa R, Admon A, Thorner J: **Phosphatidylinositol 4-kinase: gene structure and requirement for yeast cell viability.** *Science* 1993, **262:**1444–1448.
35. Yoshida S, Ohya Y, Goebl M, Nakano A, Anraku Y: **A novel gene, STT4, encodes a phosphatidylinositol 4-kinase in the PKC1 protein kinase pathway of *Saccharomyces cerevisiae*.** *J Biol Chem* 1994, **269:**1166–1172.
36. Wong K, Cantley LC: **Cloning and characterisation of a human phosphatidylinositol 4-kinase.** *J Biol Chem* 1994, **269:**28878–28884.
37. Boronenkov IV, Anderson RA: **The sequence of phosphatidylinositol-4-phosphate 5-kinase defines a novel family of lipid kinases.** *J Biol Chem* 1995, **270:**2881–2884.
38. Dhand R, Hara K, Hiles I, Bax B, Gout I, Panayotou G, *et al.*: **PI 3-kinase: structural and functional analysis of intersubunit interactions.** *EMBO J* 1994, **13:**511–521.

39. Feng S, Chen JK, Yu H, Simon JA, Schreiber SL: **Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3–ligand interactions.** *Science* 1994, **266:** 1241–1247.
40. Kaibuchi K, Fukumoto Y, Oku N, Takai Y, Arai K-I, Muramatsu M-A: **Molecular genetic analysis of the regulatory and catalytic domains of protein kinase C.** *J Biol Chem* 1989, **264:**13489–13496.
41. Arcaro A, Wymann MP: **Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses.** *Biochem J* 1993, **296:**297–301.
42. Nakanishi S, Catt KJ, Balla T: **A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids.** *Proc Natl Acad Sci USA* 1995, **92:** 5317–5321.
43. Hartley KO, Gell D, Smith GCM, Zhang H, Divecha N, Connelly MA, *et al.*: **DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product.** *Cell* 1995, **82:**849–856.
44. Clark JD, Lin L-L, Kriz RW, Ramesha CS, Sultzman LA, Lin AY: **A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP.** *Cell* 1991, **65:**1043–1051.
45. Perin MS, Fried VA, Mignery GA, Jahn R, Südhof TC: **Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C.** *Nature* 1990, **345:**260–263.
46. Davletov BA, Südhof TC: **A single C2 domain from synaptotagmin I is sufficient for high affinity Ca^{2+}/phospholipid binding.** *J Biol Chem* 1993, **268:**26386–26390.
47. Damer CK, Creutz CE: **Synergistic membrane interactions of the two C2 domains of synaptotagmin.** *J Biol Chem* 1994, **269:** 31115–31123.
48. Fukuda M, Aruga J, Niinobe M, Aimoto S, Mikoshiba K: **Inositol-1,3,4,5-tetrakisphosphate binding to C2B domain of IP4BP/synaptotagmin II.** *J Biol Chem* 1994, **269:**29206–29211.
49. Hancock JF, Cadwallader K, Paterson H, Marshall CJ: **A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins.** *EMBO J* 1991, **13:**4033–4039.
50. Zvèlebil MJ, MacDougall LK, Leevers SJ, Volinia S, Vanhaesebroeck B, Gout I, *et al.*: **Structural and functional diversity of phosphoinositide 3-kinases.** *Proc R Soc Lond [Biol]* 1995, in press.
51. Raabe T, Olivier JP, Dickson B, Liu X, Gish GD, Pawson T, *et al.*: **Biochemical and genetic analysis of the Drk SH2/SH3 adaptor protein of Drosophila.** *EMBO J* 1995, **14:**2509–2518.
52. Sutton RB, Davletov BA, Berghuis AM, Südhof TC, Sprang SR: **Structure of the first C2 domain of synaptotagmin I: a novel Ca^{2+}/phospholipid-binding fold.** *Cell* 1995, **80:**929–938.
53. Shirataki H, Kaibuchi K, Sakoda T, Kishida S, Yamaguchi T, Wada K: **Rabphilin-3A, a putative target for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin.** *Mol Cell Biol* 1993, **13:**2061–2068.
54. Cullen PJ, Hsuan JJ, Truong O, Letcher A, Jackson TR, Dawson AP, *et al.*: **Identification of a specific Ins(1,3,4,5)P4-binding protein as a member of the GAP1 family.** *Nature* 1995, **376:**527–529.
55. Zhang JZ, Davletov BA, Südhof TC, Anderson RGW: **Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling.** *Cell* 1994, **78:**751–760.
56. Ron D, Chen C-H, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D: **Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins.** *Proc Natl Acad Sci USA* 1994, **91:**839–843.
57. Kaufmann-Zeh A, Thomas GMH, Ball A, Prosser S, Cunningham E, Cockroft S, *et al.*: **Requirement for a phosphatidylinositol transfer protein in epidermal growth factor signalling.** *Science* 1995, **268:**188–1190.
58. Kapeller R, Chen KS, Yoakim M, Schaffhausen BS, Backer J, White MF, *et al.*: **Mutations in the juxtamembrane region of the insulin receptor impair activation of phosphatidylinositol 3-kinase by insulin.** *Mol Endocrinol* 1991, **5:**769–777.
59. Pignataro OP, Ascoli M: **Epidermal growth factor increases the labelling of phosphatidylinositol 3,4-bisphosphate in MA-10 Leydig tumour cells.** *J Biol Chem* 1990, **265:**1718–1723.
60. Sultan C, Plantavid M, Bachelot C, Grondin P, Breton M, Mauco G, *et al.*: **Involvement of platelet glycoprotein IIb-IIIa (αIIb-β3 Integrin) in thrombin-induced synthesis of phosphatidylinositol 3',4'-bisphosphate.** *J Biol Chem* 1991, **266:**23554–23557.
61. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual,* 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1989.
62. Devereux J, Haeberli P, Smithies O: **A comprehensive set of sequence analysis programs for the VAX.** *Nucleic Acids Res* 1984, **12:**387–395.
63. Dombrádi V, Mann DJ, Saunders RDC, Cohen PTW: **Cloning of the fourth functional gene for protein phosphatase 1 in *Drosophila melanogaster* from its chromosomal location.** *Eur J Biochem* 1993, **212:**177–183.
64. Hoheisel JD, Lennon GG, Zehetner G, Lehrach H: **Use of high coverage reference libraries of *Drosophila melanogaster* for relational data analysis.** *J Mol Biol* 1991, **220:**903–914.
65. Davies AH, Jowett JBM, Jones IA: **Recombinant baculovirus vectors expressing glutathione-S-transferase fusion proteins.** *Biotechnology* 1993, **11:**933–936.
66. Guan KL, Dixon JE: **Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase.** *Anal Biochem* 1991, **192:**262—267.
67. Summers MD, Smith HE: **A manual of methods for baculovirus vectors and insect cell culture procedures.** *Tex Exp Station Bull* 1987, **1555:**1–56.
68. Clarke NG, Dawson RM: **Alkaline O to N-transacylation.** *Biochem J* 1981, **195:**301–309.
69. Harlow E, Lane D: *Antibodies: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1988.
70. Stack JH, Emr SD: **Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities.** *J Biol Chem* 1994, **269:**31552–31562.

Received: **24 October 1995**.
Accepted: **3 November 1995.**