Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin

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The generation of phosphatidylinositide 3-phosphates has been observed in a variety of cellular responses. The enzymes that mediate synthesis are the phosphoinositide 3-kinases (PI3-Ks) that form a family of structurally diverse enzymes with distinct substrate specificities. In this paper, we describe the cloning of a novel human PI3-K, namely PI3-K-C2α, which contains a C-terminal C2 domain. This enzyme can be assigned to the class II PI3-Ks, which was defined by characterization of the *Drosophila* 68D enzyme and includes the recently described murine enzymes m-cpk and p170. Despite the overall similarity in the amino acid sequence of the murine and human enzymes, which suggests that they are encoded by closely related genes, these molecules show marked sequence heterogeneity at their N-termini. Biochemical analysis of recombinant PI3-K-C2α demonstrates a restricted lipid substrate specificity. As reported for other members of this class, the enzyme only phosphorylates PtdIns and PtdIns4*P* when the lipids are presented alone. However, when lipids were presented together with phosphatidylserine acting as a carrier, phosphorylation of PtdIns(4,5)P_2 was also observed. The catalytic activity of PI3-K-C2α is refractory to concentrations of wortmannin and LY294002 which inhibit the PI3-K activity of other family members. The comparative insensitivity of PI3-K-C2α to these inhibitors suggests that their use should be re-evaluated in the study of PI3-Ks.

INTRODUCTION

There is now compelling evidence that phosphoinositide 3-kinases (PI3-Ks) and their phosphoinositide (PI) products, which are phosphorylated at the D3 position of the inositol ring, are involved in numerous, apparently unrelated, regulatory events in cell physiology and pathophysiology. These range from the mediation of growth-factor-stimulated mitogenesis and transformation [1,2] to the regulation of apoptosis [3] and differentiation [4]. The enzyme has also been implicated in a number of other events, such as the regulation of glucose transport through effects on Glut4 translocation [5,6], the regulation of cell motility, membrane ruffling and cytoskeletal rearrangements [7]. In addition, there is good evidence that protein trafficking in yeast and mammalian cells is dependent upon the action of a PI3-K [8,9]. Initially it was unclear how sufficient regulatory control could be exerted within the cell to allow PI3-Ks to serve such distinct functions. It is now evident that restricted substrate specificity, diversity in function related to protein structure and selective expression of different PI3-Ks can all contribute to achieve regulatory control.

Diversity in function can occur as a result of variation of PI3-K substrate specificity, which determines the range of lipid products generated and gives selectivity in downstream events. Early studies showed that three distinct PI 3-phosphates are produced in cells, namely PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [10]. Subsequently it has been shown that in some cases the responses are associated with the selective production of individual PIs. For example, during mitogenic stimulation, there is a rapid accumulation of PtdIns(3,4,5)P_3, followed by a delayed increase in PtdIns(3,4)P_2 [11,12]; whereas PtdIns3*P* remains at the levels that are observed in quiescent cells, reflecting a possible role in the maintenance of housekeeping functions.

Additionally, differential control of PI3-K activation can be generated through the presence of either an intrinsic regulatory domain or an extrinsic adaptor subunit. This was first observed following the characterization of a bovine PI3-K that was shown to be a heterodimer of an 85 kDa (p85) and a 110 kDa protein (p110) [13]. The cloning, expression and biochemical characterization of p85 showed that it serves as an adaptor, mediating the selective association of this enzyme with phosphorylated tyrosine residues via its two src homology 2 (SH2) domains [14]. At least two major forms of this adaptor exist [14], as well as truncated forms [15–17] that lack the N-terminal SH3 and BCR (breakpoint cluster region) domains, and represent the products of different PI3-K genes or alternative splicing. The p110 protein contains the catalytic domain of the enzyme and upon ligand stimulation translocates as a p85–p110 complex from the cytosol to membranes where the enzyme is activated [18,19]. Using reverse-transcriptase (RT)-PCR with oligonucleotides corresponding to selected sequences in the catalytic domain of p110α, a number of novel PI3-Ks have been identified from species as diverse as yeast, *Drosophila* and man. The members of this enzyme family can now be subdivided into three main classes, based of their structural similarity and *in vitro* substrate specificity [20,21].

Class IA includes p110α, together with a number of highly conserved homologues including the human p110β [22] and p110δ [23]. All of these catalytic subunits of PI3-K exist in a dimeric complex together with a p85 adaptor subunit and can

Abbreviations used: HR, homology region; PI, phosphoinositide; PI3-K, phosphoinositide 3-kinase; PtdSer, phosphatidylserine; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase.

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associate *in vitro* with an N-terminally truncated form of p85 [15]. Class IB contains p110γ, which differs from the other p110s, as this enzyme does not interact with a p85-like adaptor [24], and can be activated by G$\beta\gamma$ subunits *in vitro* [25,26]. Although all members of this class share the same lipid substrate specificity, and are structurally closely related, they have adopted different mechanisms of receptor recruitment and enzyme activation.

The class II members include the recently described PI3-K from *Drosophila* termed PI3-K_68D [21] or cpk [27] and those from the mouse termed m-cpk [27] and p170 [28]. The class II enzymes have extensions to their amino acid sequence at both their N- and C-termini in comparison with class I enzymes. All class II enzymes contain a C-terminal C2 domain [29], which may confer phospholipid or Ca^{2+} regulation to this class of PI3-Ks. Furthermore, where examined, the lipid substrate specificity displayed by members of this class has been shown to be restricted to PtdIns and PtdIns4*P*. Although the amino acid sequences of the *Drosophila* and mouse enzymes are closely related in their kinase domains, significant differences are observed at their N-termini, suggesting that this region may serve to link adaptor proteins and perhaps to regulate catalytic activity.

The third class of the family of PI3-Ks are structurally related to the product of the *Saccharomyces cerevisiae* gene Vps34 [8]. Originally Vps34 was identified amongst a group of 40 genes, which are implicated in vesicle-mediated membrane trafficking in yeast; the Vps34 gene product is involved in sorting vacuolar/lysosomal hydrolases and in regulating endocytosis [30]. Vps34p is a PI3-K with a substrate specificity strictly restricted to PtdIns. *In vivo*, Vps34p associates with a Ser/Thr kinase termed Vps15p and this interaction is thought to regulate Vps34p enzyme activity [31]. The human homologue of Vps34p is also a PtdIns-specific 3-kinase that associates with a Vps15p homologue termed p150 [32,33]. This suggests that a protein-sorting complex that is dependent on phospholipid signalling is conserved from yeast to man.

A number of proteins have been described that contain regions of amino acid sequence similar to that conserved in all known active PI3-Ks. These include the yeast TOR (Targets Of Rapamycin) proteins [34] and their mammalian homologues [35,36], which are involved in regulation of the cell cycle, perhaps as G1 sensors, as well as other proteins that are concerned with the monitoring of DNA or telomere damage [37,38]. The substrates of these potential PI3-Ks have not been identified, except in the case of the DNA-dependent protein kinase that has serine kinase activity [39]. Some PI3-K family members also have an intrinsic protein kinase activity [37]. For example, p110α is able to phosphorylate Ser-608 of the p85 subunit *in vitro*, thereby down-regulating its lipid kinase activity [40,41]. Similarly, Vps34p is able to autophosphorylate on serine residues [42]. The physiological relevance of these observations is as yet unclear.

The study of the role of PI3-Ks in cell physiology has been aided by the use of the inhibitors wortmannin and LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one]. Wortmannin was first characterized as an inhibitor that is effective at micromolar concentrations of myosin light-chain kinase but was later shown to inhibit mammalian PI3-Ks at nanomolar concentrations [43,44].

In the present study, we report the cloning and expression of the first human class II PI3-K, named PI3-K-C2α. The enzyme contains a C-terminal C2 domain and utilizes PtdIns and PtdIns4*P* as lipid substrate *in vitro*. Furthermore, when lipids are presented together with phosphatidylserine (PtdSer), PI3-K-C2α can also phosphorylate PtdIns(4,5)P_2. In contrast with the *Drosophila* homologue and all other mammalian PI3-Ks, PI3-K-C2α is significantly refractory to inhibition by both wortmannin and LY294002. These results demonstrate that wortmannin and LY294002 should not be regarded as universal inhibitors of PI3-K activity.

MATERIALS AND METHODS

Identification of PI3-K-C2α cDNA

Poly(A)$^+$ RNA was isolated from U937 cells using oligo(dT)–cellulose (Stratagene) and used to synthesize first-strand cDNA with avian myeloblastosis virus RT (Pharmacia). PCR reactions were then performed using Taq DNA polymerase (Life Technologies), first-strand cDNA, 4 mM Mg^{2+} and 1 μM primers. The nucleotide sequences of the primers used were: sense GGNGA T/C GA T/C T/C T A/G CGNCA A/G GA (GDDLRQD/E); antisense A/G AA A/G TGICC A/G AA A/G TC A/G/T AT A/G TG A/G TG A/G AA (FHIDFGHF). To facilitate subsequent cloning, an *Eco*RI restriction site was incorporated into the 5′-end of each primer. The conditions used were 30 cycles at 94 °C for 30 s, at 56 °C for 30 s and at 72 °C for 30 s. After sub-cloning into a Bluescript SK vector, individual clones were selected and sequenced (ABI).

Isolation of PI3-K-C2α cDNA

The PCR fragment was excised from the vector, labelled with [^{32}P]dCTP using random primers (Amersham) and used to screen a λ Zap U937 cDNA library (Stratagene). Approx. 3×10^6 plaques were plated and transferred on to Hybond-N filters (Amersham). These were hybridized with the probe for 16 h at 65 °C in 0.5 M sodium phosphate (pH 7.2), 7 % (w/v) SDS and 1 mM EDTA. After this time, the filters were washed twice with 0.5 × SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS for 20 min at 60 °C and positive clones identified by autoradiography. After three rounds of screening, clones were plaque purified and the cDNA insert in Bluescript SK was rescued using ExAssist helper phage (Stratagene). The nucleotide sequence of the longest clone was determined using both Taq dye deoxy terminator cycle sequencing (ABI) and dye deoxy chain termination using T7 DNA polymerase and [α-^{35}S]thio-dATP (Lark Sequencing Technologies).

The 5′-end of the cDNA was obtained by rapid amplification of cDNA ends (RACE). cDNA was synthesized (Life Technologies) from poly(A)$^+$ mRNA obtained from U937 cells using primers complementary to the original cDNA sequence (CTCTTCCTCATGGTCTAATACCTCCAC and TATCTCCAAATCAGTCCTTGCTTTCCC). After sequencing, this fragment was ligated on to the parent cDNA in a pBK-cytomegalovirus vector (Stratagene) to produce the full-length PI3-K-C2α cDNA.

Northern-blot analysis

Multiple human tissue poly(A)$^+$ mRNA blots (Clontech) were hybridized with a 1.46 kb cDNA fragment corresponding to nucleotides 789–2249 of the PI3-K-C2α coding sequence. The probe was labelled using [α-^{32}P]dCTP and random primers (Amersham). Hybridizations were performed in Express Hyb (Clontech) at 65 °C for 16 h. The blots were washed twice with 2 × SSC/0.05 % SDS at room temperature and twice in 1 × SSC/0.1 % SDS at 50 °C. Bands were visualized by autoradiography.

Plasmid constructs and expression of recombinant protein in Sf9 cells

For the expression of recombinant PI3-K-C2α in insect cells, an N-terminal Glu-tagged construct was prepared by RT-PCR

from pBK-cytomegalovirus-PI3-K-C2α. The forward primer contained a *Sma*I site, a Kozak consensus sequence, sequence encoding the epitope tag (EFMPME), a short linker (PGG) and the first seven residues of PI3-K-C2α; the reverse primer encompassed the unique *Nhe*I site [45]. The *Sma*I/*Nhe*I-digested product was ligated into a *Sma*I/*Nhe*I-digested PVL1393 vector containing the full-length PI3-K-C2α cDNA. Recombinant DNA (2 μg) was transfected into Sf9 cells with 0.25 μg of BaculoGold DNA (Pharmingen) using Lipofectin (Gibco). Infections and amplifications (6×10^5 cells/ml) of the viral stocks were performed for 48–60 h or 96 h respectively. After protein expression, cells were harvested and lysed in 10 mM Tris/HCl (pH 7.6)/5 mM EDTA/50 mM NaCl/30 mM sodium pyrophosphate/50 mM NaF/100 μM Na_3VO_4/1 % (v/v) Triton X-100/1 mM PMSF (lysis buffer). The extracts were incubated with anti-(Glu-tag) sera and anti-mouse IgG (whole molecule) agarose for 2–4 h at 4 °C. Bound proteins were recovered by centrifugation and the pellets washed quickly twice with lysis buffer.

Assay of PI3-K activity

Typically, PI3-K assays were performed in a total volume of 50 μl containing 20 mM Tris (pH 7.5), 100 mM NaCl, 0.5 mM EGTA and 200 μM PI. After preincubating sonicated lipid with sample for 10 min, the reactions were initiated upon the addition of 4 mM $MgCl_2$ and 40 μM ATP (2 μCi of [γ-^{32}P]ATP). To determine K_m and V_{max} values, experiments were performed by varying either the lipid concentration or the ATP concentration in the absence or presence of PtdSer (2 mg/ml) acting as a lipid carrier [46]. Assays were incubated at 30 °C for 30 min and terminated with acidified chloroform/methanol. The extracted lipid products were analysed by TLC using silica-gel-60 plates and chloroform/methanol/4 M ammonium hydroxide (9:7:2, by vol.) for assays examining phosphorylation of PtdIns. When separation of PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 was required, propan-1-ol/2 M acetic acid (13:7, v/v) was used as the solvent system. Phosphorylated lipids were visualized by autoradiography and quantification was performed by scintillation counting. Assays were linear with respect to time and enzyme addition.

Characterization of PI3-K-C2α reaction products by HPLC

Phosphorylation of PtdIns was performed using recombinant protein as described above. The reaction products were extracted in acidified chloroform/methanol and deacylated with methylamine [47]. HPLC analysis of glycerophosphoinositols was performed using a Partisphere SAX column (Whatman International) and eluted using a linear gradient of 1 M $(NH_4)_2HPO_4$ (pH 3.8) against water at 1 ml min^{-1}. Radioactive peaks were detected using an in-line detector (Reeve Analytical).

RESULTS

Cloning of PI3-K-C2α

Degenerate primers and mRNA from the human cell line U937 were used to produce a partial cDNA by RT-PCR. One product of these reactions was found to be a novel cDNA and this was used to screen a λ Zap cDNA library made from U937 cells. Twenty-seven positive clones selected from 3×10^6 plaques were obtained following tertiary screening. A 6.2 kb clone was sequenced and found to contain an open reading frame of 4618 nucleotides that encoded a putative catalytic domain. Since no 5′-Kozak consensus sequence was identified [48], RACE PCR was performed using cDNA from U937 cells. A 643-nucleotide extension to the original cDNA was generated. This cDNA fragment contained an ATG sequence that was in-frame with the longest open reading frame of the parent clone together with an upstream stop codon. The RACE product was then ligated on to the parent clone to produce a composite cDNA of 5.1 kb that could encode a protein of 1686 amino acid residues that would have a predicted molecular mass of 190 kDa (Figure 1).

The protein that we have termed PI3-K-C2α, has an amino acid sequence which, when optimally aligned, displays an overall sequence identity of 32.5 % with the sequences of *Drosophila* PI3-K_68D/cpk [21,27] and 90.2 % and 90.8 % with the murine proteins m-cpk [27] and p170 [28] respectively (Figure 2, upper panel). When compared with p110α and PtdIns 3-kinase, sequence identity falls to only 45 % and 35 % respectively. Two homology regions (HRs) define areas that have the greatest sequence similarity to other PI3-Ks. The putative catalytic domain (HR1) is defined by residues 1137–1398 of the PI3-K-C2α protein. Within this region, the amino acid sequence of PI3-K-C2α is 62 % identical with that of PI3-K_68D/cpk, 97 % with p170 and 99.6 % with m-cpk (Figure 2, lower panel). Amino acid residues 871–1020 of PI3-K-C2α define a region termed the PIK domain (HR2), which is also present in other lipid kinases and TOR2 [49]. The function of this domain is unknown.

The amino acid sequence of the C-terminal region of PI3-K-C2α (residues 1549–1686) shows strong identity with a domain originally identified as a Ca^{2+}- and phospholipid-binding module in protein kinase C, termed the C2 domain [29]. In this region, the PI3-K-C2α amino acid sequence is 96 % identical with that of the C2 domains identified in m-cpk and p170, 39 % identical with the C2 domain of PI3-K_68D/cpk and 32 % and 24 % identical with the C2A and C2B domains of synaptotagmin respectively (Figure 2, lower panel).

At the N-terminal region, large insertions are necessary in the PI3-K-Cα amino acid sequence before optimal alignment is obtained with *Drosophila* PI3-K_68D/cpk. It is perhaps significant that the type II polyproline motif PPLPPR, identified in PI3-K_68D and cpk (residues 456–462), is absent in PI3-K-C2α. Within this region, however, numerous proline residues are evident. The N-terminus of PI3-K-C2α lacks any clearly delineated regulatory domain. Interestingly, alignment of the two murine amino acid sequences with that of PI3-K-C2α shows that the p170 protein lacks the first 176 amino residues of the longer murine m-cpk sequence. The m-cpk protein, however, has a 28 amino acid residue deletion in this region (residues 275–301 of the PI3-K-C2α sequence), which is absent in both p170 and PI3-K-C2α (Figure 2, middle panel). These sequence differences require further investigation.

Tissue expression of PI3-K-C2α

Northern-blot analysis carried out using poly(A)$^+$ RNA isolated from human tissue revealed that the cDNA encoding human PI3-K-C2α hybridized to an 8 kb species in RNA from a wide variety of tissues (Figure 3). Highest levels of expression were found in the heart, placenta and ovary. The mRNA was undetectable only in the kidney. The size of the mRNA is consistent with the length of the full-length cDNA. In testes, an additional smaller transcript of approx. 6.5 kb was present and may have arisen from differential splicing.

Characterization of lipid substrate specificity

To allow biochemical analysis of PI3-K-C2α, a recombinant baculovirus was produced. The construct generated encoded the full-length PI3-K-C2α protein containing a Glu epitope tag

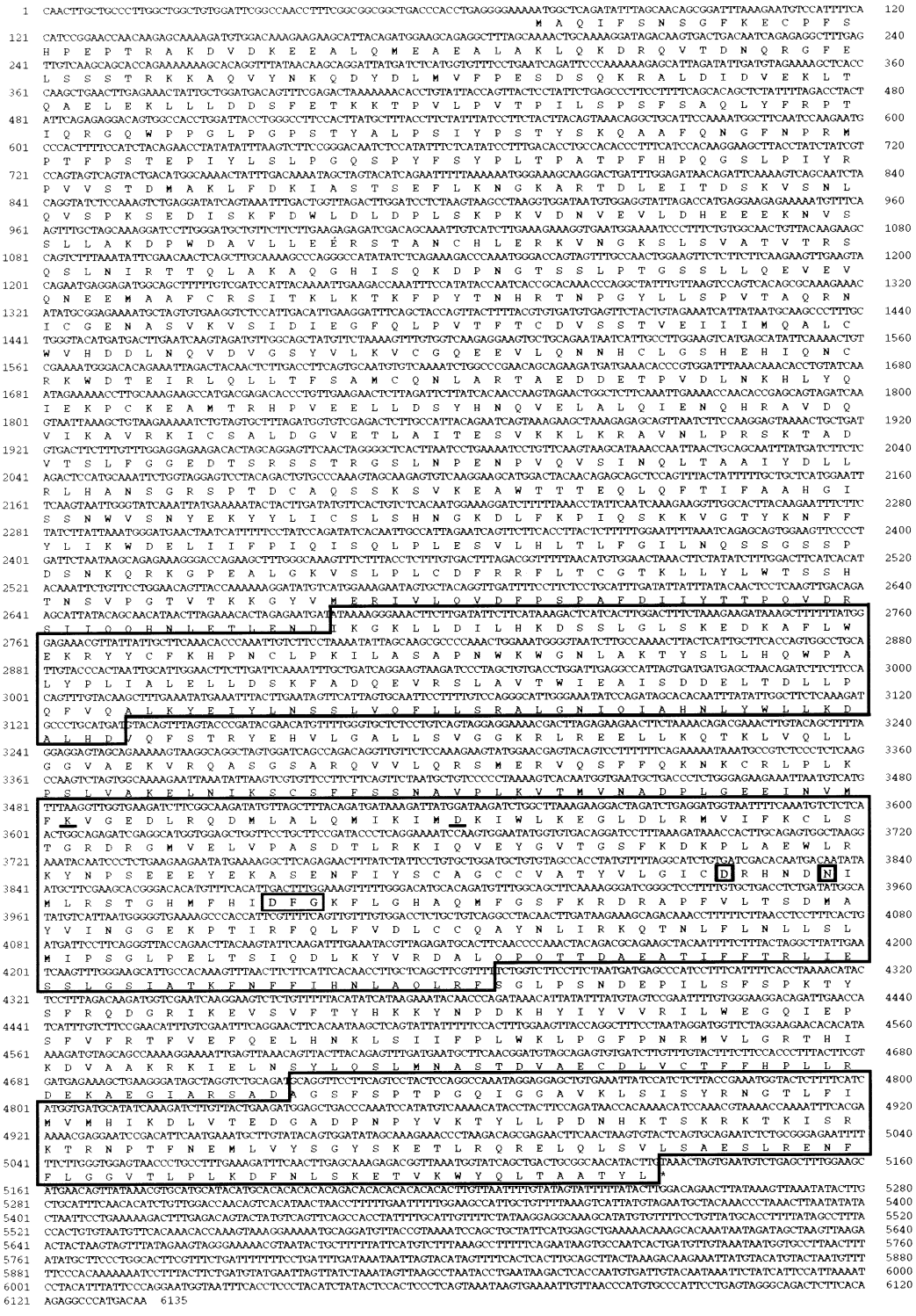

Figure 1 Predicted protein sequence of human PI3-K-C2α

The predicted protein sequence from the longest open reading frame of the PI3-K-C2α cDNA is shown. Regions of sequence similarity to domains identified in other PI3-Ks are highlighted. Residues 871–1020 represent the HR2 (PIK domain), residues 1137–1398 delineate the catalytic domain (HR1) and residues 1549–1686 represent the C2 domain. The residues K1138 and D1157 underlined represent those proposed to be involved in ATP binding, whereas those boxed D1250, N1255 and DFG1268–1270 represent the putative catalytic/substrate binding site [20].

(EFMPME) followed by a short linker (PGG) at the N-terminus. Sf9 cells were infected with the virus and after 2 days the expressed protein was isolated by immunoprecipitation using a monoclonal anti-(Glu-tag) antibody. The enzymic activity and substrate specificity were investigated and compared with that of Glu-tagged p110α co-expressed with p85α in the same system. In

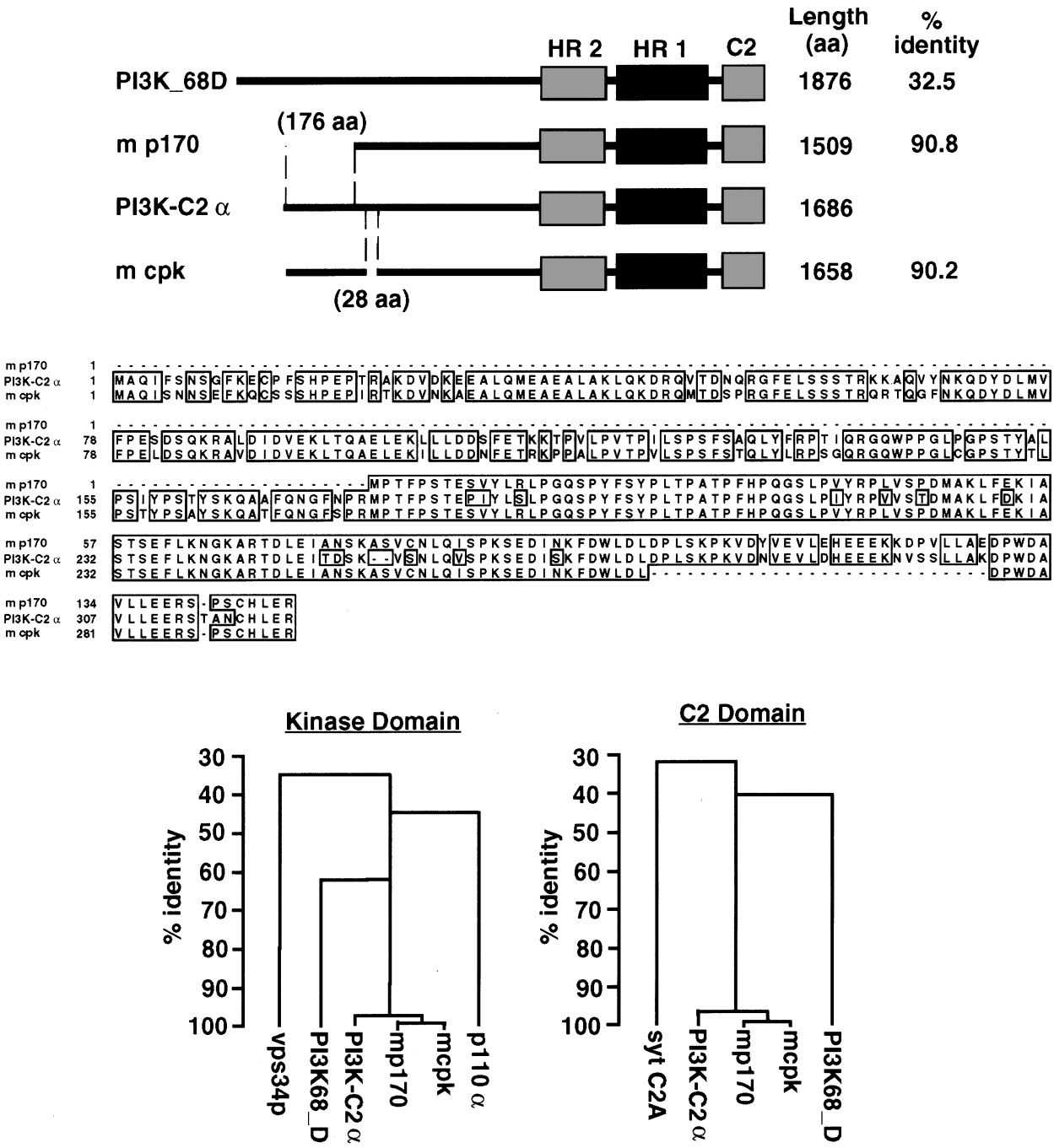

Figure 2 Comparison of PI3-K-C2α with the other class II PI3-Ks

(Upper panel) The domain structures of PI3-K_68D, PI3-K-C2α, p170 and m-cpk are shown schematically and the percentage amino acid (aa) identity relative to PI3-K-C2α is indicated for comparison. (Middle panel) A more detailed alignment of the N-terminal sequences from the two murine clones and PI3-K-C2α. Identical residues are boxed. (Lower panel) The percentage sequence identity within the catalytic (HR1) domain and the C2 domain is represented by dendrograms.

the presence of Mg^{2+} p110α was able to utilize PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 to produce PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively (Figure 4, upper panel, lanes 7–9). In contrast, PI3-K-C2α was only able to phosphorylate PtdIns and PtdIns4*P* when the lipids were presented alone (lanes 1 and 2). The products seen using PtdIns(4,5)P_2 under these conditions resulted from contamination of the preparation by PtdIns4*P* and possibly the generation of lysoPtdIns(3,4)P_2 (lane 3). Presenting the lipids together with PtdSer to act as a carrier, resulted in the phosphorylation of PtdIns(4,5)P_2 (lane 6). The degree of PtdIns(4,5)P_2 phosphorylation by PI3-K-C2α under these conditions precluded any attempt at quantitative analysis. Like p110α, PI3-K-C2α lipid kinase activity was inactive in the presence of Mn^{2+} (results not shown). This cation dependence contrasts with that of the human PtdIns 3-kinase, which shows a preference for Mn^{2+} [32].

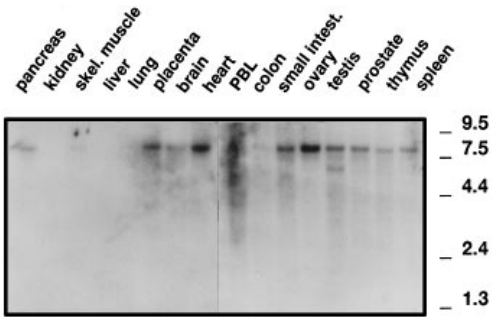

Figure 3 Tissue expression of human PI3-K-C2α

Northern blots of human poly(A)$^+$ RNA were hybridized with PI3-K-C2α cDNA (nucleotides 789–2249). Equivalent amounts of RNA (2 μg/lane) from each of the tissues indicated were loaded. PBL, peripheral blood leucocytes. Hybridizations were performed at 65 °C by the Quickhyb protocol (Stratagene). The position of RNA molecular mass markers is indicated (kb).

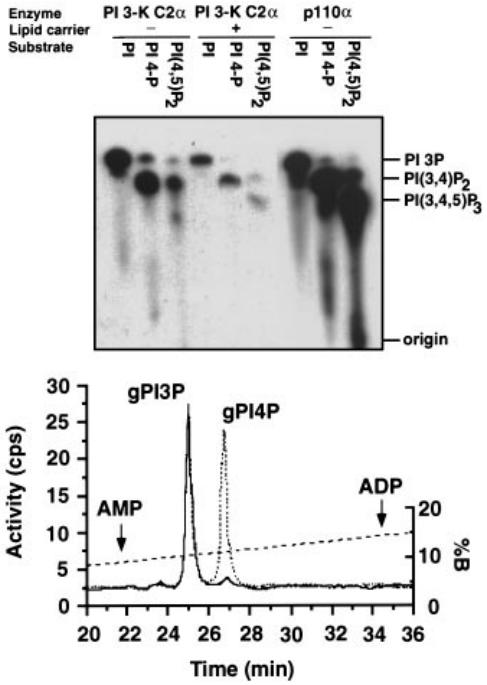

Figure 4 Analysis of PI3-K-C2α lipid kinase activity

(Upper panel) Glu-tagged PI3-K-C2α was expressed in Sf9 cells. This was used to determine the lipid substrate specificity of PI3-K-C2α by presenting either PtdIns (PI), PtdIns4*P* (PI 4-P) or PtdIns(4,5)P_2 [PI(4,5)P_2] in the absence (−) or presence (+) of PtdSer (carrier). Assays were performed in the presence of Mg^{2+} (4 mM). The results obtained using recombinant p85α/p110α are shown for comparison. The lipid products were extracted, analysed by TLC and visualized by autoradiography. (Lower panel) To determine the site of phosphorylation on the inositol ring, the product of a PI3-K-C2α lipid kinase assay using PtdIns as substrate was analysed by HPLC as described in the Materials and methods section. The elution positions of glyceroPtdIns3*P* (gPI3P) and glyceroPtdIns4*P* (gPI4P) are illustrated by the dotted line. The elution positions of AMP and ADP, which serve as internal controls standards, are indicated by arrows. cps, counts per second. B, 1M $(NH_4)_2HPO_4$ (pH 3.8).

Table 1 shows the K_m and V_{max} values for PI3-K-C2α using PtdIns, PtdIns4*P* and ATP in the absence and presence of PtdSer. PtdSer was used because without it, PtdIns4*P* produced non-linear enzyme kinetics. For comparative purposes, data were generated with PtdIns in both the absence and presence of PtdSer. The K_m of PI3-K-C2α for PtdIns, presented either with or without PtdSer, and for PtdIns4*P* were similar. Likewise, the K_m of PI3-K-C2α for ATP was not significantly influenced by the lipid substrate nor its presentation. The V_{max} values obtained using PtdIns and PtdIns4*P* were also similar, although in the presence of PtdSer there was a 5-fold decrease in the rate of PtdIns phosphorylation (Table 1). When the ATP concentration was varied, a similar dependence of V_{max} on lipid substrate was observed. Comparing the V_{max} values for ATP, little difference was observed between PtdIns and PtdIns4*P* in the presence of PtdSer. The introduction of PtdSer, however, produced an 8-fold decrease in the V_{max} for ATP using PtdIns. This effect of PtdSer on the rate of lipid phosphorylation can be observed in Figure 4 (compare lanes 1 and 2 with 4 and 5).

To confirm that PI3-K-C2α phosphorylated PtdIns at the D-3 position on the inositol ring, anion-exchange HPLC was performed on the deacylated reaction products (Figure 4, lower panel). A single peak of radioactive glycerol phosphate was obtained, which co-eluted with the deacylated PtdIns3*P* produced by the action of recombinant p110α upon PtdIns. No additional products were observed, confirming that PI3-K-C2α specifically phosphorylates PtdIns and PtdIns4*P* at the D-3 position of their inositol ring.

Table 1. Determination of K_m and V_{max} values for recombinant PI3K-C2α

Lipid	PtdSer	K_m (μM)	V_{max} (pmol/min per mg of protein)
K_m for lipid			
PtdIns	−	122	990
PtdIns	+	64	200
PtdIns4*P*	+	25	240
K_m for ATP			
PtdIns	−	15	6800
PtdIns	+	32	805
PtdIns4*P*	+	54	880

Sensitivity to inhibitors of PI3-K activity

The compounds wortmannin and LY294002 have been used extensively to assess the involvement of PI3-K in many different physiological processes. To date, all cloned human PI3-Ks have been found to be sensitive to wortmannin action at nanomolar concentrations. The effect of these inhibitors on the lipid kinase activity of PI3-K-C2α was examined, using PtdIns as a substrate, in the presence of increasing concentrations of each inhibitor. In agreement with previous studies, p110α activity was found to be sensitive to wortmannin at low nanomolar concentrations (IC_{50} = 5 nM), with maximal attenuation of activity obtained at 100 nM (Figure 5, top panel). In contrast, the observed IC_{50} using PI3-K-C2α was 420 nM, with maximal inhibition only being obtained using wortmannin at 10 mM. For LY294002, the IC_{50} using p110α was 0.8 μM and maximal inhibition was obtained at 30 μM (Figure 5, lower panel). Again, PI3-K-C2α was less sensitive than p110α to the action of the inhibitor (IC_{50} = 19 μM). The lipid kinase activity of PI3-K-C2α was maximally attenuated using 1 mM LY294002.

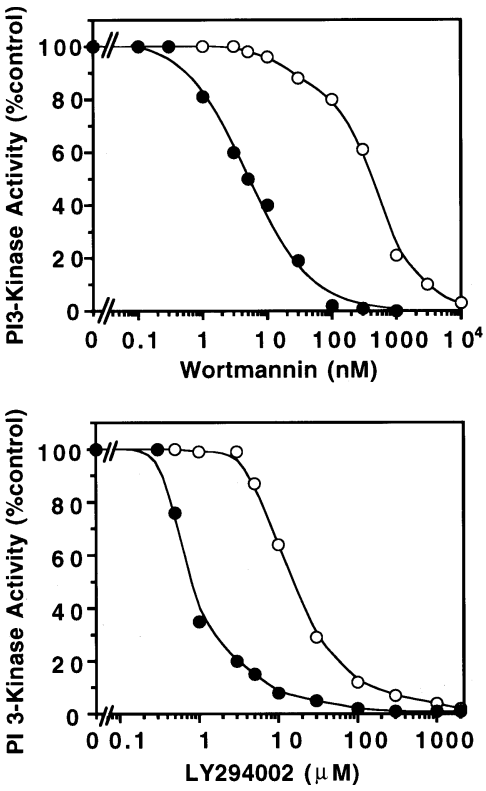

Figure 5 Sensitivity of PI3-K-C2α to wortmannin and LY294002

The lipid kinase (PI3-K; PI 3-Kinase) activity of recombinant p110α (●) and PI3-K-C2α (○) was investigated in the presence of varying concentrations of wortmannin (upper panel) or LY294002 (lower panel), using PtdIns as substrate. The reaction products were extracted, analysed by TLC and visualized by autoradiography. The formation of PtdIns3*P* was determined by scintillation counting. Each activity is expressed relative to enzyme activity in the absence of drug.

DISCUSSION

In the present study we describe the cloning and biochemical characterization of PI3-K-C2α, a novel human PI3-K of 190 kDa. Based on the presence of a C-terminal C2 domain, PI3-K-C2α, like *Drosophila* PI3-K_68D/cpk [21,27] and the two murine PI3-Ks m-cpk [27] and p170 [28], can be defined as a class II PI3-K [20] (Figure 2).

The amino acid sequence of PI3-K-C2α is 90.2% and 90.8% identical with that of two recently cloned murine PI3-Ks, m-cpk [27] and p170 [28] respectively. This level of sequence identity suggests that PI3-K-C2α is the human homologue of a single mouse PI3-K. Despite the overall sequence identity between human PI3-K-C2α, murine p170 and m-cpk, alignment of their N-termini shows marked heterogeneity. The p170 sequence lacks the first 175 amino acid residues of both PI3-K-C2α and m-cpk (Figure 2). In contrast, m-cpk lacks 28 amino acid residues that are present in both PI3-K-C2α and p170. Analysis of the two murine cDNAs suggests that the differences between the two proteins may be the result of differentially splicing a single gene.

The putative catalytic domain (HRI) and the PIK domain (HRII) of PI3-K-C2α are related to similar regions found in all class I, II and III PI3-Ks. However, PI3-K-C2α appears to lack any clear consensus sequences that would delineate other shared functional domains. For example, PI3-K-C2α lacks the N-terminal motif present in p110α, p110β and p110δ, which mediates binding to p85 adaptors [49a]. Furthermore, PI3-K-C2α does not contain the type II polyproline motif previously identified in PI3-K_68D/cpk, although it does contain a large number of proline residues, which might allow an interaction with SH3-domain-containing proteins through a non-consensus binding motif [50].

The class II PI3-Ks are characterized by their C-terminal C2 domain. C2 domains were originally identified in protein kinase C, where they were shown to mediate Ca^{2+}-dependent phospholipid binding [29]. Subsequently, C2 domains, often occurring in tandem, have also been defined in a variety of other proteins, including synaptotagmin, rabphillin 2A and cytosolic phospholipase A_2 [51]. In these proteins the C2 domain may confer similar biochemical properties. Functional heterogeneity has, however, been observed among isolated C2 domains despite their high degree of sequence similarity. In addition to phospholipid binding, Ca^{2+} stimulates an association between synaptotagmin and syntaxin through the first C2A domain [52]. In contrast, the second C2B domain is inactive in these assays, suggesting that its function is not regulated by intracellular Ca^{2+} levels. Indeed, the second C2 domain of synaptotagmin was shown to display a Ca^{2+}-independent binding to clathrin AP2 and polyinositol phosphates [53,54]. Studies have since demonstrated, however, that the second C2 domain of synaptotagmin can also respond in a Ca^{2+}-triggered manner by mediating a dimerization event [55,56]. The PI3-K_68D C2 domain, like the second C2 domain of synaptotagmin, only binds acidic phospholipids in a cation-independent manner [21]. It will be of interest to determine if the biochemical properties of the PI3-K-C2α domain are more similar to the first or the second synaptotagmin C2 domain.

The biochemical analysis described here has shown that PI3-K-C2α can phosphorylate PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 at the D3 position of the inositol ring *in vitro* (Figure 4). When the lipids were presented alone, PI3-K-C2α phosphorylated PtdIns and PtdIns4*P* but not PtdIns(4,5)P_2. This is consistent with the reported lipid specificity of PI3-K_68D and cpk [21,27]. In contrast, when the substrates were presented together with PtdSer, although the degree of PtdIns and PtdIns4*P* phosphorylation was lower, PI3-K-C2α was now able to phosphorylate PtdIns(4,5)P_2 (Figure 4). Analysis of the K_m and V_{max} data demonstrates that PtdIns and PtdIns4*P* can serve as equivalent substrates for the PI3-K-C2α enzyme (Table 1). Unfortunately, due to the low level of PtdIns(4,5)P_2 phosphorylation, similar quantification could not be performed. It remains a possibility, however, that the efficiency of PtdIns(4,5)P_2 phosphorylation could be further enhanced by the inclusion of additional lipids in the micelle, or by variation of the phospholipid carrier/substrate ratio. The activation of protein kinase C family members by PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 has also been shown to be found to be similarly dependent upon presentation of PIs with PtdSer [46]. The K_m for lipids and ATP, obtained using recombinant PI3-K-C2α, is, in general, similar to that previously reported for p110α [57].

The substrate specificity of the murine homologue has only been reported by Virbasius et al., who concluded that PtdIns was the major lipid substrate of p170 *in vitro* [28]. These results are somewhat surprising given the degree of sequence identity between the catalytic domains of PI3-K-C2α and p170 (Figure 2). One explanation for this difference could be that Virbasius et al. [28] used a crude brain lipid extract substrate in contrast with the purified lipid preparations we have used in the analysis of substrate specificity. It is clear from the results of this study that the degree of substrate phosphorylation and even substrate specificity can vary depending upon micelle composition. This may explain why ligand stimulation of p110α activity is thought to generate PtdIns(3,4,5)P_3 preferentially *in vivo* [11], yet recom-

binant p110α phosphorylates PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 *in vitro* (Figure 4). Although it could be argued that use of the crude membrane extracts may be more physiological, it does not allow for an accurate quantitative comparison of the lipids present in the two preparations. Under these circumstances, a comparative assessment of the efficiency with which an enzyme could utilize a particular lipid substrate cannot be made with confidence. Furthermore, a crude lipid preparation may contain factors that influence the lipid kinase activity of the recombinant protein. Ultimately, metabolic labelling experiments, together with a means of activating PI3-K-C2α *in vivo*, will resolve the issue of which phospholipid is the major physiological substrate for this enzyme.

The involvement of PI3-Ks in various processes of cell physiology has relied more and more on the use of the inhibitors wortmannin and LY294002. Although initially described as an inhibitor of myosin light-chain kinase at micromolar concentrations [58], wortmannin was later shown to potently inhibit platelet-derived growth factor receptor-associated PI3-K activity at 100-fold lower concentrations (2–5 nM). Used in the low nanomolar range, wortmannin blocks the generation of 3-phosphorylated lipids both *in vitro* and in whole cells such as murine fibroblasts [44]. On the basis of such findings, wortmannin began to be used extensively as a specific inhibitor of PI3-K activity, thereby implicating the formation of 3-phosphorylated lipids in a number of physiological responses. When the effect of wortmannin on the lipid kinase activity of recombinant PI3-K-C2α was examined, we found that the concentrations of the compound required to inhibit PI3-K-C2α activity were more similar to those previously used to inhibit myosin light-chain kinase (IC_{50} = 170–200 nM) [58,59]. Similarly, data presented using the p170 enzyme suggested that it is also less sensitive to wortmannin treatment than p110α [28]. LY294002 is a structurally unrelated inhibitor of PI3-K activity and PI3-K-C2α is also less sensitive than p110α to this inhibitor. The difference in sensitivity between PI3-K-C2α and p110α is, however, less than that observed with wortmannin. The relevance of this differential sensitivity is unclear. Studies using p110α have shown that wortmannin inactivates kinase activity by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction and consequently present in all PI3-Ks [60]. Molecular modelling and structural studies are required to elucidate the nature of this wortmannin resistance. Our results with PI3-K-C2α contrast not only with those derived from studies of class I and III human PI3-Ks but also with those obtained using *Drosophila* PI3-K_68D, which displays a sensitivity to wortmannin similar to that of p110α [21]. The concentrations of both wortmannin and LY294002 required to abolish PI3-K-C2α activity are therefore likely to have precluded its inhibition in many experiments where PI3-K activity has been studied. Our findings may thus require many investigators to re-evaluate the possible involvement of PI3-Ks in their physiological studies. Questions relating to the precise role that PI3-K-C2α plays in cells must therefore await the identification of more suitable inhibitors of its lipid kinase activity.

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