Cpk Is a Novel Class of Drosophila PtdIns 3-Kinase Containing a C2 Domain*

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We report the identification of a novel class of phosphatidylinositol (PtdIns) 3-kinases whose members contain C-terminal C2 domains. We have isolated Drosophila and murine genes (termed cpk and cpk-m respectively) by polymerase chain reaction amplification of cDNA libraries with degenerate primers corresponding to conserved regions of PtdIns kinases. The amino acid sequences of Cpk and Cpk-m are most similar to that of p110, a family of PtdIns 3-kinases that mediates the responses of cells to mitogenic stimuli. The Cpk and Cpk-m sequences are similar to a large, central region of p110, but differ from p110 at their N and C termini. The N termini of the Cpk proteins do not contain any recognizable protein motif, while the C termini contain "C2 domains," a feature unique among PtdIns kinases. Cpk has an intrinsic PtdIns kinase activity and can phosphorylate PtdIns and PtdIns-4-P, but not PtdIns(4,5)P_2, at the D3 position of the inositol ring. Cpk is the first PtdIns 3-kinase identified with this particular substrate specificity. We have identified two potential Cpk-binding proteins, p90 and p190, and have determined that both Cpk and p190 may be tyrosine phosphorylated. This finding suggests that Cpk function may be regulated by tyrosine kinases.

PtdIns3 kinases regulate diverse cellular processes, which include cell signaling, cell cycle progression, and intracellular protein sorting (reviewed in Herman et al., 1992; Kapeller and Cantley, 1994; Stephens et al., 1993; Kunz et al., 1993). PtdIns kinases phosphorylate phosphoinositides at distinct positions on the inositol ring: PtdIns 3-kinases phosphorylate the D3 hydroxyl group on the inositol ring, while PtdIns 4-kinases phosphorylate the D4 hydroxyl group (reviewed in Carpenter and Cantley (1990)). All PtdIns kinases contain a core region of sequence similarity, which is believed to be the catalytic domain. This domain, termed the "PtdIns kinase domain," shares limited sequence similarity with the catalytic domain of protein kinases (Hiles et al., 1992) and mutation of conserved residues results in loss of PtdIns kinase activity (Dhand et al., 1994; Schu et al., 1993). Surprisingly, the mechanism used by PtdIns kinases to modulate regulatory pathways remains unknown.

A number of receptor tyrosine kinases, src-like tyrosine kinases and viral oncoproteins bind and activate a cellular PtdIns 3-kinase (reviewed in Carpenter and Cantley (1990)). Studies of mutants that abrogate the binding of this PtdIns 3-kinase to these molecules suggest that PtdIns 3-kinases can mediate mitogenic and cell motility responses of cells to growth factors and oncoproteins. Purification of the polypeptide subunits of this PtdIns 3-kinase revealed that the enzyme exists as a heterodimeric complex composed of a 110- and an 85-kDa subunit (Carpenter et al., 1990; Fry et al., 1992; Morgan et al., 1990; Shibasaki et al., 1991). The 110-kDa subunit contains a C-terminal PtdIns kinase domain, as well as a small domain at its N terminus that is sufficient for binding to the 85-kDa subunit (Holt et al., 1994; Hiles et al., 1992; Klippel et al., 1994). The 85-kDa subunit serves as an adapter and binds activated growth factor receptors and other tyrosine phospholipid molecules through two Src homology 2 (SH2) domains (Hu et al., 1992; Klippel et al., 1992; McGlade et al., 1992; Reedijk et al., 1992; Yoakim et al., 1992; Yonezawa et al., 1992). The association of the enzyme with activated growth factor receptors may localize it to the plasma membrane, where its phospholipid substrates reside.

Recently, a number of genes have been identified that are similar to a large, central portion of p110, but which differ from p110 at their N and C termini (Stoyanov et al., 1995; Zhou et al., 1995). The product of one such gene, p110_y, can be activated in vitro by either the α or βγ subunits of heterotrimeric G proteins (Stoyanov et al., 1995). This observation suggests that different classes of receptors may utilize distinct mechanisms to activate p110-related enzymes (Stoyanov et al., 1995).

The substrate specificity of p110/p85 in vitro may differ from its substrate specificity in vivo. In vitro, p110/p85 can phosphorylate phosphatidylinositol (PtdIns) 4-phosphate [PtdIns(4)P] to produce phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] on the D3 hydroxyl group of the inositol ring, producing phosphatidylinositol 3-phosphate [PtdIns(3)P] and phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2] and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] (reviewed in Kapeller and Cantley (1994) and Stephens et al. (1993)). p110/p85 may phosphorylate a subset of its in vivo substrates in intact cells. Activation of p110/p85 in cells results in an increase in the cellular levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, but not PtdIns-3-P (Auger et al., 1989; Stephens et al., 1991; Traynor-Kaplan et al., 1988; Traynor-Kaplan et al., 1989). Therefore, it is possible that in vivo p110/p85 phosphorylates only a subset of the substrates that it is capable of phosphorylating in vitro.
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The stimulation of cells with growth factors or tumor antigens results in a rapid increase in the cellular concentrations of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ from a low basal level, which suggests that these molecules may represent novel second messengers. Several protein kinases have recently been described which may represent downstream effectors of the lipid products of PtdIns 3-kinases. The Akt protein kinase can be activated in vivo by treating cells with the mitogen platelet-derived growth factor, and the binding of PtdIns 3-kinase to the platelet-derived growth factor receptor is essential for Akt activation (Franke et al., 1995). Akt can be directly activated in vitro with PtdIns(3,4)-P₂, although the effects of PtdIns(3,4,5)-P₃ on Akt activity are not known. In addition to Akt, particular isoforms of protein kinase C may be activated by the lipid products of PtdIns 3-kinases. Protein kinase C isoforms δ, ε, η, and ξ can be activated in vitro with PtdIns(3,4,5)-P₃, but not with PtdIns(3,4,5)-P₃, not containing the 5'-P (Nakanishi et al., 1993). This construct drives expression of an 85-kDa Cpk hexahistidine fusion protein, named Pet.1. This protein was found to reside completely in inclusion bodies. Therefore, inclusion bodies were purified, solubilized in 1 × Laemmlisample buffer, and electrophoresed on a 8% preparative gel (Sambrook et al., 1989). Pet.1 protein was eluted from a gel slice, as described previously (Jessus and Beach, 1992) and then used to immunize rabbits (Berkeley Antibody Co.). The polyclonal rabbit sera, designated α-Cpk, was purified on an affinity column. The affinity-purified α-Cpk sera was prepared by coupling 2 mg of Pet.1 protein to an Affi-Gel 10 solid support, according to the manufacturer's instructions (Bio-Rad). This antibody column was used to immunoaffinity purify α-Cpk serum, as described previously (Harlow and Lane, 1988). The affinity purified serum was then incubated with whole cell BL21DE3 (lysS) lysates that had been immobilized to a PVDF membrane (Millipore). In this way, antibodies to that antigen were coupled to the membrane. Pet.1 protein was also used to immunize mice which were subsequently used to produce the monoclonal cell line α-Cpk.m1 (Program of Excellence, Monodonal Antibody Facility).

Preparation of Drosophila Lysates and Immunological Assays—Lysates were prepared by Dounce homogenizing 0–12 h Drosophila embryos in lysis buffer (lysis buffer: 20 mM HEPES pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM sodium fluoride, 0.25 mM sodium pyrophosphate (pH 7.5), 10 mM tetrasodium pyrophosphate, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10% glycerol, 10 μg/ml aprotinin, and 20 μg/ml leupeptin). The lysates were then frozen in aliquots at –70°C. Immediately prior to use, an aliquot was thawed, diluted with lysis buffer containing 1% Triton X-100, and the insoluble proteins were pelleted in a microcentrifuge. Cpk protein was detected by immunoblotting in the following manner. Proteins from lysates were resolved by SDS-PAGE on a 6% gel and then transferred to a PVDF membrane (Millipore) using high molecular weight transfer buffer (Harlow and Lane, 1988). The blots were incubated with the appropriate serum diluted in TBS-T (TBS-T: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) with 5% dry milk and 1% ovalbumin and then processed using an enhanced chemiluminescence kit (Amersham). A silver staining kit was used to visualize proteins in immunoprecipitates (Accurate Chemical and Scientific Corp.).

PtdIns Kinase Assays—Cpk protein was precipitated from either COS-7 cell or Drosophila lysates as described previously (Harlow and Lane, 1988). The precipitations were washed four times in lysis buffer containing 1.0% Triton X-100 and then twice in PtdIns kinase assay buffer (PtdIns kinase assay buffer: 30 mM HEPES pH 7.5, 30 mM magnesium chloride). PtdIns kinase assays in which PtdIns was used as the substrate were performed as described previously (Kaplan et al., 1987; Whitman et al., 1988). The PtdIns kinase assays were modified in the following manner for the determination of PtdIns-4-P, PtdIns(4,5)P₂, and PtdIns(4,5)P₃ substrate specificities. The Ptd Ins, PtdIns-4-P, and PtdIns(4,5)P₂ substrates were mixed with an equal amount of phosphatidylinositol and then sonicated to form vesicles. Preparation of vesicles with phosphatidylinositol assures that the physical properties of the PtdIns, PtdIns-4-P, and PtdIns(4,5)P₂ vesicles are approximately equivalent. The products of these kinase assays were resolved by TLC using Silica Gel 60 plates (Whatman) in a buffer consisting of chloroform:acetone:methanol:acetic acid:water (80:30:26:24:14). Cpk kinase assays were further modified by the addition of 0.05% CHAPS to the vesicle substrate and the PtdIns kinase assay buffer. The addition of CHAPS was determined to stimulate Cpk PtdIns kinase activity in vivo.

Determination of the Position on the Inositol Ring Phosphorylated by Cpk—PtdIns-3-P and PtdIns-4-P were resolved using TLC with a bovine serum albumin buffer system that has previously been described in detail (Walsh et al., 1993). [γ-32P]PtdIns-3-P and [γ-32P]PtdIns-4-P standards were generated in the following manner. [γ-32P]PtdIns-3-P was produced by phosphorylating PtdIns with γ-[32P]ATP using a constitutively active p110 mutant protein (p110*) whose construction and expression has been previously described (Hu et al., 1995). [γ-32P]PtdIns-4-P was produced by phosphorylating PtdIns with γ-[32P]ATP with lysis (20 μg) prepared from 0 to 12-h Drosophila embryos. PtdIns-3 kinases are generally the most abundant PtdIns kinases found in lysates. In order to verify that the major product of this reaction is indeed PtdIns-4-P, the reaction products were demonstrated to comigrate with an unlabeled PtdIns-4-P standard (Sigma), but could be resolved from the [γ-32P]PtdIns-3-P standard. The unlabeled PtdIns-4-P standard was...
visualized by iodine staining.

Expression of Proteins in COS-7 Cells—A plasmid was constructed that expressed Cpk-HA fusion proteins in COS-7 cells. Not I and Sma I sites were introduced into the cpk cDNA at the position of the stop codon using the primer dPIK 34 (dPIK 34: 5'-CCCGGGTCAGCGGC-CGCCGTTCCTGGACACCGCGCCCAG-3'), which corresponds to nucleotides 5755–5795 of the cDNA. A triple tandem copy of the HA1 epitope on a Not IDNA fragment was ligated into the Not I site (Tyers et al., 1992). An Spe I site was introduced at the position of the initiating methionine using the primer dPIK 29 (dPIK29: 5'-TTAGACGAGAC-TAGTATGTCAAATCAAGCG-3'), which corresponds to nucleotides 132–162 of the cpk cDNA. The resulting 5683-base pair Spe I/Sma I fragment was ligated into the Xba I/Sma I sites of the mammalian expression vector pCG. pCG is a derivative of pEVRF (Matthias et al., 1989) with a modified polylinker that contains the human cytomegalovirus enhancer/promoter region and the translation initiation region of the herpes simplex virus thymidine kinase gene. A kinase-deficient Cpk protein was constructed by changing lysine 1347 to arginine with the primer dPIK 27 (dPIK27: 5'-GTGGGACCTGATGCCGAATCTTTAC-GGGCTATCTTTAGGTGCGGA-3'). A constitutively active p110 mutant protein (p110*) was expressed as a control, and its construction and expression has been previously described (Hu et al., 1995).

RESULTS

Isolation of the Drosophila and Murine cpk cDNAs—We identified both the Drosophila and murine cpk genes by polymerase chain reaction amplification of cDNA libraries using degenerate primers corresponding to conserved amino acids in PtdIns kinase catalytic domains. DNA fragments of the expected size were obtained and analysis of the DNA sequences revealed open reading frames with a high degree of sequence identity to the catalytic domains of PtdIns kinases. These DNA fragments were used as probes to screen Drosophila and murine libraries and multiple large cDNA clones were obtained. These clones did not contain the 5' end of the Drosophila and murine cDNAs, and therefore the 5' ends were extended using anchored polymerase chain reaction (5' rapid amplification of cDNA ends). The Drosophila cpk cDNA is likely to be full-length for the following reasons. The size of the cDNA (6.9 kilobase pairs) is consistent with the size of the mRNA as estimated by Northern blot analysis (data not shown). Conceptual translation of the cDNA revealed a large open reading frame encoding a protein with a predicted molecular mass of 210 kDa. The first methionine in this open reading frame is encoded by the first ATG in the cDNA and it is preceded by an in-frame stop codon. The Drosophila and murine Cpk proteins are 34% identical and 48% similar (Fig. 1).

Analysis of the sequence of the Drosophila and murine Cpk proteins revealed that they are significantly more similar to the p110 family of PtdIns 3-kinases than to other families of PtdIns kinases. The Cpk genes are 31% identical and 43% similar to a...
large central region of p110 (Hilles et al., 1992). This region includes both the PtdIns kinase domain (Fig. 2A, black box) and an adjacent region in which p110 and Cpk can be distinguished from other PtdIns kinases (Fig. 2A, striped box). The Cpk proteins are also more similar to p110 family of PtdIns kinases in the most conserved portion of the catalytic domain (Fig. 2C). In this region, the Cpk proteins share approximately 45–50% identity with p110α, p110β, or p110γ. In contrast, the Cpk proteins share 35, 29, and 26% identity in this region with the Vps34, Pik1, and Tor2 PtdIns kinases, respectively.

The Cpk proteins differ from p110 at both their N and C termini, and therefore probably do not represent p110 homologues. The N termini of the Cpk proteins do not contain any recognizable domain, including the domain at the N terminus of the p110 that is responsible for binding to the p85 adapter molecule (Holt et al., 1994; Klippel et al., 1994). The N termini of the Cpk proteins are rich in the amino acids serine, glutamine, and proline, and the significance of this is unknown. The C termini of Cpk and Cpk-m contain a C2 domain (Fig. 2A). C2 domains are found in a diverse group of proteins and may mediate binding to phospholipids or other proteins. The C2 domains in Cpk and Cpk-m are 52% similar to each other, and approximately 38% similar to C2 domains present in protein kinase C, synaptotagmin, or rabphilin (Fig. 2B). The possible significance of the C2 domains in Cpk and Cpk-m is discussed below.

Detection of the Drosophila Cpk Protein in Lysates—Polyclonal sera that recognize the Drosophila Cpk were generated to characterize the biochemical properties of the Cpk. A portion of the Drosophila Cpk was produced as a fusion protein in E.

**Fig. 2. Domain structure of the Cpk proteins.** A, schematic diagram comparing the domain structures of Cpk with p110 (Hu et al., 1993; Stoyanov et al., 1995; Volina et al., 1994), Tor2 (Kunz et al., 1993), Pik1 (Flanagan et al., 1993) and Vps34 (Herman and Emr, 1990) PtdIns kinases. The PtdIns kinase domains are depicted in black, a region in which Cpk and p110 are related is depicted with stripes, and the C2 domain and p85 binding domains are indicated. B, comparison of the amino acid sequence of the C2 domains in Drosophila and murine Cpk with the C2 domains in rabphilin (Shirataki et al., 1993), synaptotagmin II (Geppert et al., 1991), and protein kinase C (Schaeffer et al., 1989). Rabphilin, synaptotagmin II, and protein kinase C are indicated by Rab, Syt II, and Pkc, respectively. Both conserved and identical residues are shaded. The following groups of amino acid were considered to be conserved: Ala, Val, Leu, Ile, Met; Asp, Glu; Lys, Arg; Asn, Gln; Phe, Tyr; Ser, Thr. C, comparison of the amino acid sequences of a part of the catalytic domains of Cpk and Cpk-m with the catalytic domains of p110a (Volina et al., 1994), p110β (Hu et al., 1993), p110γ (Stoyanov et al., 1995), Vps34 (Herman and Emr, 1990), Pik1 (Flanagan et al., 1993), and Tor2 (Kunz et al., 1993). Only identical amino acids are shaded. The amino acid numbers are indicated to the right of the sequence. Sequence similarity of the Cpk proteins with other proteins was obtained by searching data bases with the program FASTA (Pearson and Lipman, 1988).
coli and used to immunize rabbits. The resulting polyclonal serum (named α-Cpk) was affinity purified and used to probe an immunoblot of lysates prepared from 0 to 12-h Drosophila embryos. The α-Cpk immune serum recognizes a polypeptide migrating at approximately 210 kDa, the predicted molecular mass of the Cpk protein (Fig. 3A). p210 is not recognized by α-Cpk preimmune serum and is likely to be the Cpk gene product.

To verify that p210 is the Cpk gene product, we have generated an independent serum that recognizes Cpk protein. Rabbits were immunized with a peptide (P6) corresponding to a region of Cpk not present in the Cpk fusion protein described above. The resulting serum, which was designated α-P6, was used to precipitate protein from Drosophila lysates. The precipitates were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with the α-Cpk serum. Both α-Cpk or α-P6 immune sera precipitate a 210-kDa protein that represents the Cpk gene product. The α-Cpk serum also recognizes a 95-kDa protein on an immunoblot. We do not believe that this protein is an antigenically related PtdIns kinase, since it is not precipitated by the α-Cpk serum (lane 2). Preimmune and immune sera are indicated by P and I, respectively.

Drosophila Cpk Has PtdIns 3-Kinase Activity—Since Cpk is related to PtdIns kinases, it was of interest to determine whether it could phosphorylate PtdIns, and whether this phosphorylation occurred on the D3 or D4 position of the inositol ring. We have determined that Cpk protein precipitated from lysates prepared from Drosophila embryos can phosphorylate PtdIns (Fig. 4A). Cpk protein was precipitated from lysates using the α-Cpk and α-P6 sera. Half of each precipitate was assayed for PtdIns kinase activity and the other half was used to detect Cpk protein by probing an immunoblot with α-Cpk serum. B, wild-type (lane 1) and kinase deficient (lane 2) Cpk proteins which had been tagged with an HA epitope were expressed in COS-7 cells. The Cpk proteins were precipitated from lysates using an α-HA serum. Half of each precipitate was assayed for PtdIns kinase activity and the other half was used to detect Cpk protein by probing an immunoblot with α-Cpk serum. P and I indicate preimmune and immune sera, respectively. P represents either α-Cpk or α-P6 sera which had been preincubated with the P6 peptide (lane 6). The precipitates were split in half; half of each precipitate was assayed for PtdIns kinase activity, and the other half was used to detect Cpk protein by probing an immunoblot with α-Cpk serum. B, wild-type (lane 1) and kinase deficient (lane 2) Cpk proteins which had been tagged with an HA epitope were expressed in COS-7 cells. The Cpk proteins were precipitated from lysates using an α-HA serum. Half of each precipitate was assayed for PtdIns kinase activity and the other half was used to detect Cpk protein by probing an immunoblot with α-Cpk serum. P and I indicate preimmune and immune sera, respectively. I represents either α-Cpk or α-P6 sera which had been preincubated with the P6 peptide.
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7.5 nm, which is a value consistent with previously studies (Woscholski et al., 1994). The IC_{50} for wortmannin in inhibition of Cpk was 11 nm (data not shown). Also, p110 requires the addition of either Mg^{2+} or Mn^{2+} to in vitro kinase assays, although the enzyme is more active in the presence of Mg^{2+} (Volinia et al., 1995). In contrast, Cpk strictly requires the presence of Mg^{2+} in in vitro kinase assays, and the enzyme is inactive in the presence of Mn^{2+} (data not shown).

We have examined whether Cpk phosphorylates PtdIns at the same position of the inositol ring as p110, the D3 position (Fig. 5). PtdIns-3-P and PtdIns-4-P can be resolved by TLC using a borate buffer (Walsh et al., 1991). Cpk protein was precipitated from either Drosophila (Fig. 5A) or COS-7 cell (Fig. 5B) lysates and used to phosphorylate PtdIns. The \gamma^{32}P-labeled products of these reactions were separated by TLC. The Cpk products migrated at the position of \gamma^{32}P-PtdIns-3-P standard, but not a \gamma^{32}P-PtdIns-4-P standard and the mixtures were separated. The Cpk reaction products comigrated with \gamma^{32}P-PtdIns-3-P standard (lane 5). The Cpk reaction products are designated by Cpk-p.

**Fig. 5. Cpk protein phosphorylates PtdIns on the D3 hydroxyl group.** Cpk protein was precipitated from either Drosophila embryo (A) or COS-7 cell (B) lysates using \alpha-Cpk or \alpha-HA serum, respectively. The precipitated Cpk protein was used to phosphorylate PtdIns and the reaction products were separated by TLC, as described previously. The Cpk reaction products (lane 3) migrated at approximately the same position as a \gamma^{32}P-PtdIns-3-P standard (lane 2), but not a \gamma^{32}P-PtdIns-4-P standard (lane 1). Cpk reaction products were also mixed with either \gamma^{32}P-PtdIns-3-P (lane 5) or \gamma^{32}P-PtdIns-4-P (lane 4) standards and the mixtures were separated. The Cpk reaction products comigrated with \gamma^{32}P-PtdIns-3-P (lane 5). The Cpk reaction products are designated by Cpk-p.

**Fig. 6. Cpk protein can phosphorylate PtdIns and PtdIns-4-P.** Cpk protein was precipitated from Drosophila embryo lysates and the precipitate was divided into three aliquots. One aliquot was used to phosphorylate either PtdIns (lane 2), PtdIns-4-P (lane 4), or PtdIns(4,5)P_{2} (lane 6) using in vitro kinase assays. The reaction products were separated by TLC. A constitutively active p110 protein (p110*) served as a control and it was able to phosphorylate PtdIns (lane 1), PtdIns-4-P (lane 3), and PtdIns(4,5)P_{2} (lane 5) substrates. PIP_{2}, PIP_{3}, and PIP_{4} refer to phosphatidylinositol bisphosphate, phosphatidylinositol trisphosphate, respectively.

We investigated the possibility that either Cpk or any coprecipitating proteins are tyrosine phosphorylated. The p110/p85 PtdIns 3-kinase interacts with activated tyrosine kinase growth factor receptors, and both p85 and p110 are subsequently phosphorylated on tyrosine (reviewed by Stephens et al. (1993)). Cpk was precipitated from Drosophila lysates, the precipitated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with an \alpha-phosphotyrosine antisemur (Fig. 7C). The Cpk precipitations contained two \alpha-phosphotyrosine reactive polypeptides migrating at 190 and 210 kDa. This finding suggests that both Cpk and p190 may be tyrosine phosphorylated and raises the possibility that Cpk function may be regulated by tyrosine kinases.
We report the identification of a novel class of PtdIns 3-kinases whose members contain C-terminal C2 domains. We have isolated Drosophila and murine genes (termed Cpk and cpk-m, respectively) whose products share 34% sequence identity and 48% sequence similarity. The finding of a related genes in vertebrates and invertebrates suggests that Cpk may define a new class of conserved PtdIns 3-kinase and that Cpk regulatory pathways may also be conserved. In a recent independent study, MacDougall et al. (1995) also report the identification of this gene.

Cpk and cpk-m are more closely related to the p110 family of PtdIns 3-kinases than to other families of PtdIns kinases. Amino acids 863-1587 of Cpk are approximately 31% identical and 43% similar to a large, central region of mammalian p110 (Hil et al., 1992). Cpk and cpk-m differ from p110 at their N and C termini and therefore probably do not represent p110 homologues. The N termini of the Cpk proteins do not contain any recognizable domain, and do not resemble a domain at the N terminus of p110 that mediates its interaction with the p85 subunit (Holt et al., 1994; Klippel et al., 1994). Indeed, we have determined that Cpk does not form a complex with p85 when both proteins are coexpressed in COS-7 cells (data not shown).

The Drosophila and murine Cpk proteins contain a C2 domain at their C termini. C2 domains are found in a diverse group of proteins, including some isoforms of protein kinase C, synaptotagmin, and rabphilin (reviewed in Nishizuka (1992), Perin et al. (1990), and Shirataki et al. (1993)). C2 domains were initially described as Ca\(^{2+}\)-dependent phospholipid binding motifs that can mediate the translocation of proteins to membranes (reviewed in Nishizuka (1992)). Indeed, MacDougall et al. (1995) demonstrate that recombinant C2 domain derived from their PtdIns kinase clone (termed 68D) can weakly bind to phospholipids. In addition to binding phospholipids, C2 domains can also mediate interactions between proteins. A C2 domain from synaptotagmin, a synaptic vesicle protein, can bind AP-2 complexes (AP-2: adapter protein 2) with a high affinity (Zhang et al., 1994). AP-2 is a protein complex which is believed to play a role in the assembly of clathrin-coated pits and may target proteins to coated pits for recycling. Also, a C2 domain from protein kinase C can mediate its interaction with RACKs (RACKs: receptors for activated C kinases, reviewed in Lisovitch and Cantley (1994)). RACKs have been implicated in the regulation of the kinase activity and intracellular localization of protein kinase C (reviewed in Mochly-Rosen (1995)). Therefore, it is possible that the C2 domain in Cpk may function as a regulatory domain by binding lipids or proteins which modulate its intracellular localization or enzymatic activity.

We have characterized the enzymatic activity of the Cpk. PtdIns kinases phosphorylate PtdIns at either the D3 or D4 hydroxyl group of the inositol ring. Cpk can phosphorylate PtdIns at the D3 position of the inositol ring, in a manner similar to p110 PtdIns 3-kinases. We have also determined the substrate specificity of the Cpk. p110 can phosphorylate PtdIns, PtdIns-4-P, and PtdIns(4,5)P\(_2\) in vitro. In contrast to p110, Cpk can phosphorylate PtdIns and PtdIns-4-P, but not PtdIns(4,5)P\(_2\) in vitro. Cpk is the first PtdIns 3-kinase identified with this particular substrate specificity. The kinase assays were performed using Cpk derived from two sources, native protein purified from Drosophila lysates and recombinant protein purified from COS-7 cells. Also, we have determined that the PtdIns 3-kinase activity associated with Cpk is intrinsic by demonstrating that point mutations within the catalytic domain of Cpk result in an inactive enzyme. MacDougall et al. (1995) also report a similar in vitro substrate specificity associated with recombinant 68D purified from insect cells. We do not know to what extent the substrate specificity determined using in vitro kinase assays reflects the substrate specificity in cells.

The treatment of cells with mitogenic stimuli results in the rapid accumulation of the products of PtdIns 3-kinases from a low basal level. This observation suggests that these lipids may represent novel second messengers, although candidate effectors have only recently been described. Three protein kinases whose activity can be regulated by the lipid products of a PtdIns 3-kinase have been described. The Akt protein kinase is specifically activated by PtdIns-3-P (Franke et al., 1995), while particular isoforms of protein kinase C are activated by PtdIns(3,4)P\(_2\) or PtdIns(3,4,5)P\(_3\) (Nakanishi et al., 1993; Toker et al., 1994). It is possible that Cpk acts in a pathway in which
its lipid products regulate a currently unidentified effector.

Several PtdIns 3-kinases are known to be associated with regulatory proteins. For example, the p110 PtdIns 3-kinase associates with an 85-kDa subunit which is essential for the proper localization and activation of p110. p85 binds to tyrosine-phosphorylated growth factor receptors, and this interaction may localize p110 to the plasma membrane, where its lipid substrates reside (Hu et al., 1992; Klippel et al., 1992; McGlade et al., 1992; Reedijk et al., 1992; Yoakim et al., 1992; Yonezawa et al., 1992). In addition to functioning as an adapter, p85 is required for the enzymatic activity of p110; p110 must be associated with p85 to be fully active (Holt et al., 1994; Klippel et al., 1994). The Vps34 PtdIns 3-kinase associates with the Vps15 protein kinase and this complex regulates intracellular protein sorting (Stack et al., 1993). Vps34 is required for both the membrane localization and the PtdIns kinase activity of Vps34 (Stack et al., 1993). Therefore, it is possible that Cpk associates with a protein that can modulate its intracellular localization or enzymatic activity. We have identified two potential Cpk interacting proteins, p90 and p190. Interestingly, both Cpk and p190 are tyrosine phosphorylated. The only other PtdIns kinase known to be tyrosine phosphorylated is p190. Interestingly, both Cpk and p190 are tyrosine phospho-

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