with PDK1 as the in vivo kinase responsible for mediating Thr^{229} phosphorylation in the catalytic domain of $p70^{s6k}$. The kinases responsible for mediating activation of $p70^{s6k}$ have been difficult to identify because of the multiple and hierarchical regulatory steps required to bring about its activation (*4*, *9*, *14*). Initially, it had been suggested that Thr^{229} phosphorylation was regulated by a kinase that was activated directly or indirectly by PI3K in a wortmannin-sensitive manner (*17*). However, our studies indicate that the Thr^{229} kinase is constitutively active, wortmannin-resistant (*9*), and dependent on prior phosphorylation of Thr^{389} to provide access to Thr^{229} (*13*). These latter requirements are fulfilled by PDK1 (Fig. 4E) (*4*, *9*). Activation of $p70^{s6k}$ appears to be first mediated by phosphorylation of the (Ser/Thr)-Pro sites in the autoinhibitory domain, which facilitates phosphorylation at Thr^{389} by disrupting the interaction of the COOH- and NH_2-termini of the kinase, thereby allowing phosphorylation of Thr^{229} (Fig. 4E) (*4*, *9*). A key step in this process is Thr^{389} phosphorylation, which appears to be positively regulated by a wortmannin-sensitive, PI3K-dependent input, possibly through PKB, and is suppressed by a rapamycin-activated Thr^{389} phosphatase (*9*), through inhibition of mTOR (Fig. 4E). Many members of the AGC family of Ser/Thr kinases share the same conserved catalytic domain of $p70^{s6k}$ and PKB (Fig. 1A) (*6*), suggesting that PDK1 may be a member of a family of kinases that mediate activation-loop phosphorylation of AGC protein kinases. Consistent with this possibility, we have identified a number of PDK1-like cDNAs (*18*).

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12. The 293 cells were seeded at 10^6 per 10-cm plate in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) 24 hours before transfection as previously described (*9*). Transfections were performed by titrating each construct to achieve consistent and uniform expression, typically no more than 1 μg of $p70^{s6k}$, PDK1, or PKB were transfected together with pRK5 or pCMV5 carrier DNA to 5 μg total (*9*). After calcium phosphate transfection, the cells were washed with DMEM and quiesced for 24 hours before extraction or treatments described in the text. Samples were extracted in buffer A [50 mM tris (pH 7.5), 50 mM NaCl, 10 mM NaF, 10 mM β-glycerol-phosphate, 10 mM $NaPP_i$ (P_i, inorganic phosphate), 0.5 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% TX-100, and 10 μg of leupeptin and aprotinin per milliliter]. We co-immunoprecipitated Myc-PDK1 and Myc-$p70^{s6k}$ or HA-ΔPHPKB by mixing extract from independently transfected cells in buffer A with the appropriate monoclonal antibody for each epitope tag. After immunoprecipitation with protein G–Sepharose, the samples were washed twice with buffer A, twice with buffer A containing 500 mM NaCl, and finally with buffer B [50 mM tris (pH 7.5), 10 mM NaCl, 1 mM DTT, 10% glycerol, 1 mM benzamidine, and 0.2 mM PMSF]. The washed immunoprecipitates were resuspended in buffer B containing 10 mM $MgCl_2$ for assays described. The $p70^{s6k}$, PKB, and $p44^{mapk}$ activities were assayed as described in (*1*, *19*).
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Protein Kinase B Kinases That Mediate Phosphatidylinositol 3,4,5-Trisphosphate–Dependent Activation of Protein Kinase B

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Protein kinase B (PKB) is activated in response to phosphoinositide 3-kinases and their lipid products phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] and PtdIns(3,4)P_2 in the signaling pathways used by a wide variety of growth factors, antigens, and inflammatory stimuli. PKB is a direct target of these lipids, but this regulation is complex. The lipids can bind to the pleckstrin homologous domain of PKB, causing its translocation to the membrane, and also enable upstream, Thr^{308}-directed kinases to phosphorylate and activate PKB. Four isoforms of these PKB kinases were purified from sheep brain. They bound PtdIns(3,4,5)P_3 and associated with lipid vesicles containing it. These kinases contain an NH_2-terminal catalytic domain and a COOH-terminal pleckstrin homologous domain, and their heterologous expression augments receptor activation of PKB, which suggests they are the primary signal transducers that enable PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 to activate PKB and hence to control signaling pathways regulating cell survival, glucose uptake, and glycogen metabolism.

Phosphoinositide 3-kinases (PI3Ks) are a diverse family of enzymes capable of 3-phosphorylating inositol phospholipids (*1*). One subfamily can be activated by receptors through various signal transduction mechanisms. These enzymes phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 in vitro and apparently preferentially phosphorylate the

latter in vivo, yielding PtdIns(3,4,5)P_3 (*2*, *3*). As a result many receptors can stimulate accumulation of PtdIns(3,4,5)P_3. PtdIns-(3,4)P_2 can often be detected under similar circumstances. The existence of PtdIns-(3,4,5)P_3 5-phosphatases in many cells, combined with the (sometimes) relatively slower accumulation of PtdIns(3,4)P_2 than of PtdIns(3,4,5)P_3, suggests that at least some of the PtdIns(3,4)P_2 is derived from PtdIns(3,4,5)P_3 (*4*); however, some is probably generated directly by hormone-sensitive lipid kinases.

PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are potential participants in intracellular signaling pathways but the primary target or targets of these lipids are not clear. However, several signal transducers are activated as a consequence of PI3K activity, including PKB (*5*, *6*), protein kinase Cs (PKCs) (*7*–*9*), and the small GTP-hydrolyzing protein rac (*10*). Specific PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 binding proteins have been identified and a subgroup of pleckstrin homologous (PH) domains may be specialized for binding these lipids (*11*). However, binding of 3-phosphorylated lipids to the PH domain in PKB cannot alone account for regulation of PKB (*12*). PKB can be phosphorylated on Thr^{308} and Ser^{473} in a PI3K-dependent fashion, and one or more upstream kinases phosphorylate and contribute to the activation of PKB (*13*). A protein kinase or kinases that phosphorylate Thr^{308} only when PKB is bound to PtdIns(3,4,5)P_2 or PtdIns(3,4)P_2 has been partially purified (*14*, *15*); the upstream kinases are themselves activated by PtdIns-(3,4,5)P_3, implying a complex two-level regulation by PtdIns(3,4,5)P_3 (*14*). We describe experiments aimed at characterizing PtdIns(3,4,5)P_3- and PtdIns(3,4)P_2-sensitive PKB kinases and the mechanism by which they are regulated.

[^{32}P]PtdIns(3,4,5)P_3 binding protein or proteins copurify with PKB kinase activity and four distinct forms of PKB kinase can be resolved (Fig. 1). All four activities phosphorylate and activate phosphorylation of myelin basic protein (MBP) by PKB in the presence of the biological stereoisomers of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 {for example, (1-stearoyl 2-arachidonyl)-*sn*-phosphatidyl-D-*myo*-inositol 3,4,5-trisphosphate [D-D-S/A-PtdIns(3,4,5)P_3]} (*16*) (Fig. 2A). The purified kinases are (i) inactive against PKB in the presence of the enantiomers of these lipids or PtdIns(4,5)P_2; (ii) active at lower concentrations of stearoyl arachidonyl than dipalmitoyl versions of these lipids; and (iii) equally effectively activated by D-D-S/A-PtdIns(3,4,5)P_3 and its diastereoisomer differing only in the arrangement of the chiral center in the glycerol backbone [D-L-S/A-PtdIns(3,4,5)P_3], which suggests that, although interaction depends on the nature of the fatty acids, it is not dictated by their precise stereochemistry with respect to the water-soluble headgroup (Fig. 2B).

With the same assay conditions used for the PKB phosphorylation studies, we investigated the association of both the PKB kinases (Fig. 2D) and PKB itself (Fig. 2C) with the lipid vesicles in these assays. The PKB kinases associated with the lipid vesicles containing very low molar percentages of the PtdIns(3,4,5)P_3 stereoisomers [0.003% for D-D-S/A-PtdIns(3,4,5)P_3] and D-D-P/P-PtdIns(3,4)P_2 but not PtdIns(4,5)P_2 or L-L-P/P-PtdIns(3,4)P_2. This is consistent with the observations that the PKB kinase or kinases can bind [^{32}P]PtdIns(3,4,5)P_3 and that phosphorylation of a water-soluble, 30-mer peptide, based on the sequence of PKB around Thr^{308}, was inhibited by PtdIns-(3,4,5)P_3 or PtdIns(3,4)P_2 (*17*).

Under the same assay conditions PKB also associated with the lipid vesicles (Fig. 2C). However, substantially higher concentrations of 3-phosphorylated lipids were required to detect translocation of PKB; the lipid specificity of this event was distinct from that for translocation of the PKB ki-

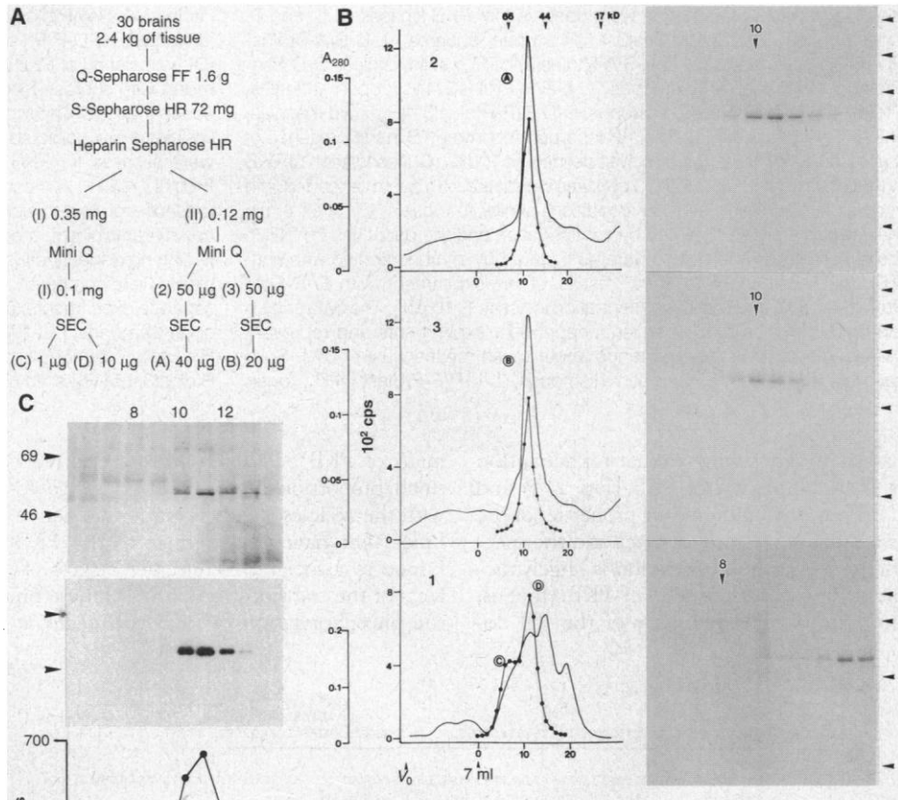

Fig. 1. Purification of PKB kinases from sheep brain. (**A**) Flow diagram for purification of PKB kinases A to D from sheep brain cytosol (*27*) and records for the quantity of protein carried through each step. The overall recovery of activity from the initial cytosol fractions was 16%. Further details can be found at the Science Website (www.sciencemag.org). (**B**) Analysis of the active fractions eluting from the final SEC. The native sizes were estimated to be 58, 58, 68, and 54 kD and their SDS-denatured sizes were estimated to be 57, 57, 70, and 55 kD (the positions to which 220-, 97-, 69-, 46-, and 31-kD standards had migrated during SDS-PAGE are indicated). (**C**) Copurification of PKB kinase activity with a [^{32}P]PtdIns(3,4,5)P_3 binding protein. A partially purified preparation of PKB kinase was applied to a SEC (*28*) and fractions were analyzed for PtdIns(3,4,5)P_3-dependent PKB kinase activity [bottom; ^{32}P counts per second (cps) in PKB] (*25*), [^{32}P]PtdIns(3,4,5)P_3 binding {middle, a renatured immunoblot was incubated with [^{32}P]PtdIns-(3,4,5)P_3} (*29*), or total proteins [by silver staining of an SDS-polyacrylamide gel (top)].

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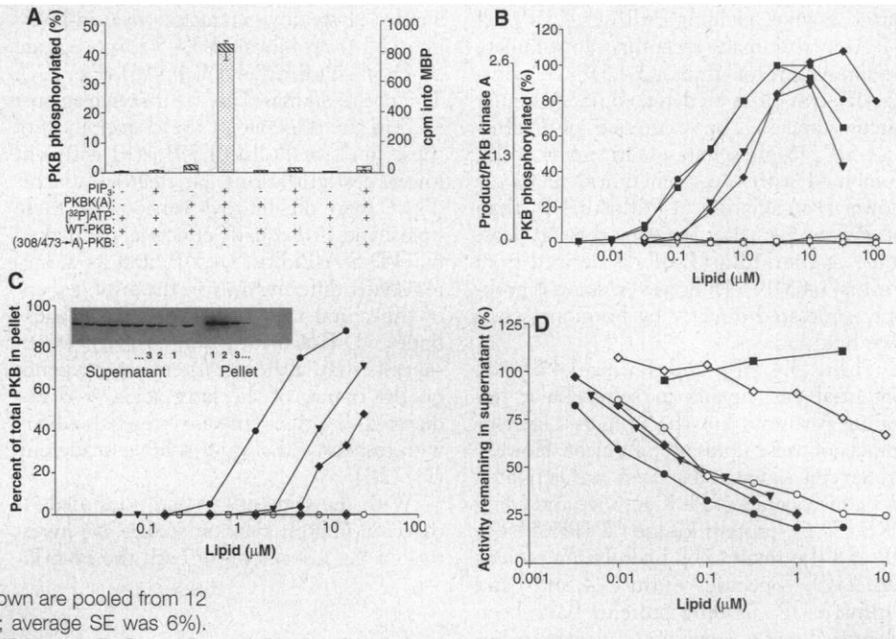

Fig. 2. Characterization of PKB kinases A to D. (**A**) PtdIns(3,4,5)P_3-dependent activation of PKB. Assays were run in two stages. The first stage was run with mixed lipid vesicles either with or without D-D-S/A-PtdIns(3,4,5)P_3 (final concentration, 5 μM) and in the presence or absence of PKB kinase A (6 nM), wild-type (EE)-PKB (2.5 μM), or Thr^{308} to Ala–Ser^{473} to Ala-(EE)-PKB and [γ-^{32}P]ATP (adenosine triphosphate) (50 μM). Percentage of PKB phosphorylated is indicated by stippled bars. The assays were stopped and PKB proteins were immunoprecipitated with anti-(EE) beads, washed, and then incubated with [γ-^{32}P]ATP (10 μM) and MBP (7 μM) to determine the activity of the immobilized PKB (hatched bars). Results are from a single experiment and are represented as means ± SE (n = 3 to 5); four other experiments gave similar results. PKB kinases B, C, and D gave very similar results. (**B**) Phospholipid specificity of activation of PKB phosphorylation. Assay mixtures contained a constant concentration of mixed lipid vesicles (*27*) with the indicated concentrations of inositol phospholipids, (EE)-PKB (2.5 μM), PKB kinase A (5 nM), and [γ-^{32}P]ATP (1 μM; total volume, 12 μl). The data shown are pooled from 12 separate experiments and are means (n = 3 to 6; average SE was 6%). Identical patterns of activation were observed for PKB kinases B, C, and D. Open circles, L-L-S/A-PtdIns(3,4,5)P_3; open squares, L-D-S/A-PtdIns-(3,4,5)P_3; solid squares, D-L-S/A-PtdIns(3,4,5)P_3; solid circles, D-D-S/A-PtdIns(3,4,5)P_3; open diamonds, L-L-P/P-PtdIns(3,4)P_2; open triangles, PtdIns(4,5)P_2 (brain); solid diamonds, D-D-P/P-PtdIns(3,4)P_2; solid triangles, D-D-P/P-PtdIns(3,4,5)P_3. The ^{32}P incorporated into PKB in the presence of 4 μM D-D-S/A-PtdIns(3,4,5)P_3 was defined as 100%. (**C**) Association of PKB with lipid vesicles. (EE)-PKB (40 nM) was incubated with sucrose-loaded lipid vesicles (or their vehicle) under conditions similar to those in (B). After 4 min the vesicles were sedimented by centrifugation and the quantities of PKB in the supernatants and pellets were quantitated by immunoblotting with anti-(EE). Inset immunoblots show results of an experiment with D-D-S/A-PtdIns(3,4,5)P_3 (maximum concentration in assay 1, 16 μM). The data shown are pooled from a total of seven independent experiments and represent means (n = 2 to 3; average range about those means was 11.0%). Solid squares, D-PtdIns(4,5)P_2; open diamonds, L-L-P/P-PtdIns(3,4)P_2; open circles, L-L-S/A-PtdIns(3,4,5)P_3; solid diamonds, D-D-P/P-PtdIns(3,4)P_2; solid triangles, D-D-P/P-PtdIns(3,4,5)P_3; solid circles, D-D-S/A-PtdIns(3,4,5)P_3. (**D**) Association of PKB kinases with lipid vesicles. PKB kinase A (5 nM) was mixed with sucrose-loaded lipid vesicles (or their vehicle) containing various concentrations of inositol phospholipids as shown (*30*). After 4 min at 30°C the vesicles were collected by centrifugation and portions of the supernatants were assayed for PKB kinase activity in the presence of 5 μM D-D-S/A-PtdIns(3,4,5)P_3. A mean of 8.2% of the total activity was sedimented in the presence of lipid vesicles containing no added inositol phospholipids; the activity remaining in the supernatant after centrifugation of lipid vesicles containing no added inositol lipids defined the 100% value to which other treatments were compared. Data shown are means (n = 4 to 6, pooled from 16 separate experiments; average SE was 8%). Solid squares, D-PtdIns(4,5)P_2; open diamonds, L-L-P/P-PtdIns(3,4)P_2; open circles, L-L-S/A-PtdIns(3,4,5)-P_3; solid diamonds, D-D-P/P-PtdIns(3,4)P_2; solid triangles, D-D-P/P-PtdIns(3,4,5)-P_3; solid circles, D-D-S/A-PtdIns(3,4,5)P_3.

nases but very similar to that for activation of phosphorylation of PKB (Fig. 2, B and C). Thus, the lipid binding properties of the two kinases are quite distinct and the specificity of the phosphorylation is largely dictated by the recruitment of PKB (that is, the affinity and specificity of the PH domain of PKB). Probably only a relatively small proportion of the PKB is associated with the vesicles at concentrations of active lipids that have caused most of the PKB kinase to associate. Hence, the biggest effects of the translocation of PKB kinase on the phosphorylation of PKB will occur at very low concentrations of PtdIns(3,4,5)P_3, whereas at higher concentrations the translocation of PKB may become the major factor. These properties explain why membrane-targeting effectively activates PKB (*18*); presumably there is adequate basal turnover of 3-phosphorylated lipid in these cells to ensure there is some PKB kinase already in place.

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                                                    ADADALQHTAQPFQPR                ILGE
  1 MARTTSQLYDAVPIQSSVVLCSCPSPSMVRSQTESSTPPGIPGGSRQGPAMDGTAAEPRPGAGSLQHAQPPFQPRKKRPEDFKFGKILGE

                GSFSTVVLAR
 91 GSFSTVVLARELATSREYAIKILEKRHIIKENKVPYVTRERDVMSRLDHPFFVKLYFTFQDDEKLYFGLSYAKNGELLKYIRKIGSFDET

                                                    ANHFVGTAQYVSPELLTER
181 CTRFYTAEIVSALEYLHGKGIIHRDLKPENILLNEDMHIQITDFGTAKVLSPESKQARANSFVGTAQYVSPELLTEKSACKSSDLWALGC

271 IIYQLVAGLPPFRAGNEYLIFQKIIKLEYDFPEKFFPKARDLVEKLLVLDATKRLGCEEMEGYGPLKAHPFFESVTWENLHQQTPPKLTA

361 YLPAMSEDDEDCYGNYDNLLSQFGCMQVSSSSSSHSLSASDTGLPQRSGSNIEQYIHDLDSNSFELDLQFSEDEKRLLLEKQAGGNPWHQ

                                QLLLTEGPHLYYVDPVNK
451 FVENNLILKMGPVDKRKGLFARRRQLLLTEGPHLYYVDPVNKVLKGEIPWSQELRPEAKNFKTFFVHTPNRTYYLMDPSGNAHKWCRKIQ

541 EVWKQRYQSHPDAAVQ
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Fig. 3. Primary structure of a PKB kinase (*31*). A potential ORF defined by cDNAs isolated from our human U937 cell library is shown (EMBL accession number Y15056). The four peptide sequences derived from the 57-kD sheep brain PKB kinase A are shown in boldface above the sequence. The area of similarity to other protein kinase catalytic domains is boxed in a solid line; the area of similarity to other PH domains is boxed in a dashed line.

A preparation of PKB kinase A was blotted onto nitrocellulose and treated with trypsin in situ. The liberated peptides were subjected to analysis by NH_2-terminal sequencing and mass spectrometry (*19*). Four peptides were defined and used to search the databases and a family of human expressed sequence tag (EST) sequences were identified. Combined use of the peptide sequences (to fix the reading frame) and information from further sequencing of the EST clones and of cDNAs isolated from a human U937 cell cDNA library (*20*) defined a cDNA with a potential open reading frame (ORF) containing all four peptides (allowing for species differences) and

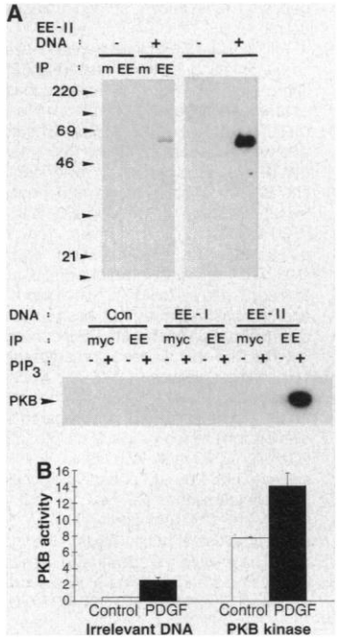

Fig. 4. Expression of PKB kinases in COS-7 and aortic endothelial cells. (**A**) Mammalian expression vectors containing NH_2-terminal EE-tagged versions of the PKB kinase (*23*) [(EE)-II refers to the complete ORF and (EE)-I refers to the kinase-inactive splice variant] were transiently expressed in COS-7 cells (*24*). Proteins were purified via their (EE)-tags (antibodies to myc were used as controls) and were detected by immunoblotting. Polyvinylidene difluoride filters, probed with anti-(EE) (detection by enhanced chemiluminescence; right) were then stained with Coomassie blue [left; similar results were obtained with (EE)-I]. Samples were assayed for PKB kinase activity in the presence of lipid vesicles either with or without D-D-S/A-PtdIns(3,4,5)P_3 and [γ-^{32}P]ATP (300 nM; 3 μM and 1 μM final concentrations, respectively). Autoradiogram shows ^{32}P in PKB. IP, immunoprecipitated with; PIP_3, PtdIns(3,4,5)P_3. (**B**) PAE cells were cotransfected with (EE)-PKB and either (myc)-PKB kinase (*23*) or an irrelevant DNA. After 12 hours the cells were transferred into serum-free medium and after a further 12 hours some were stimulated with PDGF for 45 s. The activity of PKB in anti-(EE) immunoprecipitates prepared from lysates of the transfected cells was quantitated with MBP as a substrate (*6*); data are presented from a single experiment (means ± SE; n = 4) representative of four.

encoding a protein with a predicted molecular size of 63 kD (Fig. 3).This predicted protein contains an NH_2-terminal protein kinase domain and a COOH-terminal PH domain (Fig. 3). We have also isolated cDNAs from a rat brain library that encode a protein with 94% identity at the amino acid level of this ORF (EMBL accession number Y15748) and have identified a closely related sequence in the database from *Drosophila* (EMBL accession number Y07908) (*21*).

Genomic sequence information in the databases regarding the PKB kinase gene allowed us to define a precise chromosomal localization (human chromosome 16p13.3) (*22*) and some, but not all, intron-exon boundaries. It is clear from this information and from sequencing a number of cDNAs that this locus gives rise to a complex pattern of alternatively and apparently incompletely spliced transcripts. One human transcript is precisely equivalent to the ORF defined in Fig. 3 except that it is missing the exon encoding the substrate recognition motif of the protein kinase domain (residues 238 to 263; peptide sequence data showed that our purified enzyme contained this motif) (Fig. 3). We used this cDNA to generate a catalytically inactive version of the PKB kinase.

We have constructed mammalian expression vectors encoding NH_2-terminally tagged PKB kinase and its catalytically inactive variant (*23*). EE-tagged versions of these proteins were expressed in and purified from COS-7 cells (*19*) (Fig. 4). The protein with the intact protein kinase domain phosphorylated PKB in a PtdIns(3,4,5)P_3-sensitive manner, indicating that this activity resided in the ORF defined in Fig. 3.

Coexpression of (EE)-PKB with (myc)-ERK-2 or (myc)-PKB kinase in porcine aortic endothelial (PAE) cells resulted in an increase in both the basal and platelet-derived growth factor (PDGF)–stimulated activity of PKB but had no effect on the activity of ERK-2 (*24*), despite the fact that ERK-2 is activated by PDGF in a wortmannin-sensitive fashion in these cells (*25*). In the presence of the transfected upstream kinase, the fold increase in PKB activity in response to PDGF fell; however, the difference between control and PDGF-stimulated PKB activities increased by about threefold (Fig. 4). This suggests that the PKB kinase we have purified and cloned can participate in the pathway by which PDGF activates PKB. The increase in the basal PKB activity in the presence of PKB kinase is most simply explained by our observation that the upstream kinase appears to be very sensitive to PtdIns(3,4,5)P_3. Presumably, under the conditions of our experiments the cells are still sufficiently basally activated that during long periods of exposure (the 24-hour transfection protocol) to very high levels of PKB kinase, significant amounts of the heterologous PKB can become phosphorylated and hence active.

Our results indicate that PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 activate PKB by causing a translocation to the membrane of PKB itself and an upstream Thr^{308}-directed protein kinase. These results confirm the function of PH domains in propagation of the PI3K signal in cells (*11*, *26*) and suggest that positional information is of particular importance in this pathway.

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16. The formal name of the biological stereoisomer PtdIns(3,4,5)P_3 is (for a specific fatty acid combination) (1-stearoyl, 2-arachidonyl)-*sn*-phosphatidyl-D-*myo*-inositol 3,4,5-trisphosphate [D-D-S/A-PtdIns(3,4,5)P_3]. We have abbreviated this, while retaining all the relevant discriminatory precision to define specific isomers, to D-D-S/A-PtdIns(3,4,5)P_3, with the second D referring to the chirality of the glycerol backbone—that is, D-L-S/A-PtdIns(3,4,5)P_3 is (2-arachidonyl, 3-stearoyl)-*sn*-phosphatidyl-D-*myo*-inositol 3,4,5-trisphosphate. Dipalmitoyl derivatives are abbreviated to D-D-P/P-PtdIns (3,4,5)P_3. The enantiomer of D-D-S/A-PtdIns(3,4,5) P_3 is L-L-S/A-PtdIns(3,4,5)P_3.
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20. Human and mouse EST sequences relating to the PKB kinase gene can be found in The Institute for Genome Research database (THC 193570). Further cDNAs encoding PKB kinases were identified by screening a human U937 cell oligo(dT)-primed library (in λZAPII; Stratagene) and a rat brain library [both oligo(dT) and random primed in UniZAPXR; Stratagene] with a ^{32}P-labeled 0.3-kb Eco RI–Hind III fragment derived from IMAGE clone 526583. Positive plaques were identified and purified and the cDNAs were excised as pBluescript-based plasmids. DNA sequencing was performed on an Applied Biosystems automatic sequencer at the Babraham Institute Microchemical Facility.
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23. Residues 51 to 556 of the human ORF defined in Fig. 3 were placed in frame with an NH_2-terminal EE-tag or a myc-tag in the pCMV3 transient expression vector (*19*) by standard polymerase chain reaction–based cloning strategies. Versions were also constructed with a cDNA missing the nucleotides coding for residues 238 to 263 (this was made with IMAGE clones 510982 and 526583, referred to in the text as the catalytically inactive splice variant). All constructs were verified by sequencing.
24. COS-7 and PAE cells were transfected by electroporation. After treatment with trypsin, the cells were washed once with complete medium and two times with PBS and then resuspended in electroporation medium (*19*). Circular DNA (30 μg) and 0.6 × 10^7 cells were shocked once at 250 V, 960 μF in a cuvette (0.4-cm gap; Bio-Rad) in the absence of antibiotics. COS-7 cells were replated in complete medium and harvested 36 hours later. A lysate was prepared [1% (w/v) NP-40, 0.15 M NaCl, 20 mM Hepes-NaOH (pH 7.4 at 5°C), leupeptin (10 μg ml^{-1}), pepstatin A (10 μg ml^{-1}), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (1.0 to 2.0 mg of protein per milliliter), centrifuged at 10,000*g* for 30 min at 0°C] and precleared [samples were mixed with 40 μl of packed anti-(myc)-protein Sepharose at

4°C], and the final supernatant was adjusted to 0.3 M NaCl before being mixed with 40 μl of packed anti-(EE) protein G–Sepharose beads (per milliliter of supernatant) for 3 hours at 4°C. The beads were washed five times with lysis buffer containing 0.3 M NaCl, and then four times with the same solution without NP-40 and containing 0.15 M NaCl and 0.02% (w/v) Tween 20. Proteins were eluted in the final wash buffer supplemented with eluting peptide (150 μg ml^{-1}) (*19*) by washing three times with 1 volume (compared with packed beads) of buffer (each time the beads were incubated with the eluting solution for 20 min on ice). PAE cells were transfected by a similar protocol [30 μg of pCMV3-(EE)PKB(Ser^{473} to Asp) vector (*14*) or 3.0 μg of pCMV3 (myc)-PKB kinase vector or irrelevant DNA] except they were serum-starved for 12 hours before stimulation with PDGF (2 ng ml^{-1}) for 45 s; assays were conducted as described in (*6*). In parallel experiments to examine the effects of transient expression of PKB kinase on another protein kinase cascade, cells were cotransfected with (myc)–ERK-2. Anti-(myc)–directed immunoprecipitates were assayed as described by S. Cook *et al.* [*EMBO J.* **12**, 3475 (1993)].

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27. The assay mixture used to purify PKB kinase activities contained 1 μl of column fraction [γ-^{32}P]ATP (5 μCi, 1 μM final concentration); assay buffer [0.1 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 30 mM Hepes (pH 7.4)]; 2.5 μM (EE)-PKB [from stock PBS containing 1 mM dithiothreitol (DTT), 1 mM EGTA, and then mixed 1:1 (v/v) with glycerol; the kinase was purified from SF9 cells infected with clonal, recombinant baculovirus through its (EE)-tag and the eluting peptide was removed by gel filtration]; and phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine with or without D-D-S/A-PtdIns(3,4,5)P_3 [final concentrations in the assay mixture of 100, 100, 20, and 15 μM, respectively; the vesicles were prepared by sonicating a dry film of the lipids into 25 mM Hepes (pH 7.4) and were stored at 4°C for up to 3 days]. The assays were run for 12 min at 30°C, stopped by the addition of 400 μl of ice-cold 1% Triton X-100, 0.3 M NaCl, 10 mM EDTA, 1 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM EGTA, 0.01% azide, 25 mM Hepes (pH 7.4), and then 30 μl of anti-(EE) beads [4 μl of packed beads per assay mixture; protein G–Sepharose covalently crosslinked to a saturating quantity of anti-(EE) monoclonal antibody]. The tubes were mixed at 4°C for 25 min and then washed once with the above stop buffer and the ^{32}P content of each tube was quantitated with a Geiger counter. Column fractions were diluted so that a maximum of 40 to 45% of the total [γ-^{32}P]ATP was consumed in any assay. In assays designed to more accurately quantitate PKB kinase activity, the total volume was 12 μl with all additions scaled up in proportion; otherwise all steps were the same except the assay mixtures were incubated with anti-(EE) beads for 1 hour [resulting in a mean of 87% of (EE)-PKB being recovered on the anti-(EE) beads], they were washed three times, and their ^{32}P content was defined by liquid scintillation counting. The extent of PKB phosphorylation was estimated by measuring the ^{32}P incorporated into PKB and knowing the specific radioactivity of [γ-^{32}P]ATP in the assay mixture and the concentration of PKB (determined by analysis of both Coomassie-stained gels in the presence of standard amounts of bovine serum albumin and the maximum ^{32}P that can be incorporated into a known amount of PKB from [γ-^{32}P]ATP of defined specific radioactivity by an excess of PKB kinase).
28. A high-performance liquid chromatography–size exclusion column (SEC) was prepared with a Biosilect column (V_T 11.6 ml; Bio-Rad). Samples (35 to 45 μl) were loaded, the flow was 40 μl min^{-1}, and 80-μl fractions were collected. The SEC buffer contained 0.15 M NaCl, 20 mM Hepes (pH 7.4), 0.5 mM EGTA, 0.1 mM EDTA, 1% betaine, 0.03% Tween 20, 0.01% azide, 2 mM β-glycerophosphate, 1 mM DTT, and pepstatin A, leupeptin, aprotinin, and antipain (all 2 μg ml^{-1}).
29. [^{32}P]PtdIns(3,4,5)P_3 binding was assayed by protein immunoblotting (samples heated to 50°C with SDS sample buffer). The filter was incubated in phosphate-buffered saline (PBS) containing 1% NP-40, 1 mM EGTA, and 0.01% azide for 12 hours at 4°C. The filter was incubated for 30 min (room temperature) in the above solution, which also contained 0.1% cholate, phosphatidylserine and phosphatidylcholine at 50 μg ml^{-1}, 1 mM $MgCl_2$, and 1 mM DTT. [^{32}P]PtdIns(3,4,5)P_3 {prepared from recombinant p101/p120-PI3K, PtdIns(4,5)P_2, and [γ-^{32}P]ATP (*19*)} was sonicated into solution (10 μCi in 10 ml) and applied to a filter for 20 min at room temperature and then washed away with fresh solution (five times over 5 min) and finally with PBS containing 1% NP-40. The filter was air dried and autoradiographed.
30. To assay association of PKB and PKB kinases with lipid vesicles and the effects of different lipids on the phosphorylation of PKB, we prepared the lipid vesicles by sonicating dry lipid films into 0.2 M sucrose, 20 mM KCl, 20 mM Hepes (pH 7.4 at 30°C), 0.01% azide (to give 200 μM phosphatidylcholine, 150 μM phosphatidylserine, 20 μM phosphatidylethanolamine, 10 μM sphingomyelin) plus the indicated concentrations of inositol lipids in the final assay mixture. These were mixed with the relevant kinases in an assay buffer containing bovine serum albumin (1 mg ml^{-1}), 0.12 M NaCl, 1 mM EGTA, 0.2 mM calcium, 1.5 mM $MgCl_2$, 1 mM DTT, 0.01% azide, 5 mM KCl, 20 mM Hepes (pH 7.4 at 25°C), and about 50 nM free calcium (all final concentrations in the assay mixture) with or without [γ-^{32}P]ATP (1 μM final concentration) and (EE)-PKB (2.5 μM final concentration). If the assays were to estimate association of the kinases with the lipid vesicles, then after 4 min at 30°C the assay mixtures were centrifuged (200,000*g* for 20 min). Portions of the supernatants were removed for assays or immunoblotting. The pellets were rinsed rapidly with assay buffer, recentrifuged, and dissolved in SDS sample buffer. Phosphorylation of PKB or PKBs was quantitated as described above.
31. Single-letter abbreviations for amino acid residues are as follows: A, Ala;, C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Role of Ceruloplasmin in Cellular Iron Uptake

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Individuals with hereditary ceruloplasmin (Cp) deficiency have profound iron accumulation in most tissues, which suggests that Cp is important for normal release of cellular iron. Here, in contrast to expectations, Cp was shown to increase iron uptake by HepG2 cells, increasing the apparent affinity for the substrate by three times. Consistent with its role in iron uptake, Cp synthesis was regulated by iron supply and was increased four- to fivefold after iron depletion. Unlike other iron controllers that are posttranscriptionally regulated, Cp synthesis was transcriptionally regulated. Thus, iron-deficient cells could increase Cp synthesis to maintain intracellular iron homeostasis, so that defects would lead to global accumulation of iron in tissues.

Iron is essential for many different biological processes, often functioning as a protein-bound redox element. However, iron in excess of cellular needs is extremely toxic, and its levels are precisely regulated (*1*). Defective regulation due to hereditary hemochromatosis or secondary iron overload leads to hepatic iron excess and injury, most likely due to iron-stimulated free radical reactions (*2*). Alterations of iron pools are implicated in neurodegenerative disease, aging, microbial infection, atherosclerosis, and cancer (*3*). The balance required to maintain appropriate intracellular iron concentration has led to the utilization of multiple mechanisms that regulate, primarily at the posttranscriptional level, the synthesis of iron transport and storage proteins (*4*).

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Ceruloplasmin (Cp) is a 132-kD monomeric copper oxidase implicated in iron metabolism because of its catalytic oxidation of Fe^{2+} to Fe^{3+} (ferroxidase activity) (*5*). Also, copper-deficient swine develop anemia that is overcome by Cp injection (*6–8*). Cp ferroxidase activity accelerates iron incorporation into apo-transferrin (*9, 10*), which may cause a negative concentration gradient of iron and cellular iron release. Patients with aceruloplasminemia have hemosiderosis characterized by low serum iron, high serum ferritin, massive iron deposition in multiple organs, and neurological deficit and diabetes (*11–13*), further implicating Cp in release of iron from tissue (*14–16*). In addition, multicopper oxidases homologous to Cp [fet3 in *Saccharomyces cerevisiae* (*14, 17, 18*) and fio1 in *S. pombe* (*19*)] facilitate high-affinity iron uptake by yeast. Thus, the yeast oxidases and Cp may promote iron fluxes but in opposite orientations with respect to the cell surface (*15*).