# Dual Role of Phosphatidylinositol-3,4,5-trisphosphate in the Activation of Protein Kinase B

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Protein kinase B (PKB) is a proto-oncogene that is activated in signaling pathways initiated by phosphoinositide 3-kinase. Chromatographic separation of brain cytosol revealed a kinase activity that phosphorylated and activated PKB only in the presence of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)$P_3$]. Phosphorylation occurred exclusively on threonine-308, a residue implicated in activation of PKB in vivo. PtdIns(3,4,5)$P_3$ was determined to have a dual role: Its binding to the pleckstrin homology domain of PKB was required to allow phosphorylation by the upstream kinase and it directly activated the upstream kinase.

PKB (also known as c-Akt or Rac-PK) was originally identified as the transforming oncogene in a retrovirus from a spontaneous thymoma in an AKR mouse (*1*). The oncogene product contains all of the coding sequence of PKB, with an $NH_2$-terminal fusion 60 bases upstream of the initiator methionine to the viral *gag* gene (*2*). This fusion creates a myristoylation signal, which causes a relocation of PKB from the cytosol to the plasma membrane (*3*). This altered localization is likely to explain the deregulated oncogenic activity of the viral fusion protein because targeting PKB to the plasma membrane confers constitutive activity (*4*). PKB contains a pleckstrin homology (PH) domain at its $NH_2$-terminus, a domain that has been implicated in binding inositol lipids (*5*). PKB appears to be activated as a consequence of increased phosphoinositide 3-kinase (PI3K) activity in cells stimulated with mitogens, because chemical inhibitors of PI3K or dominant-negative subunits of PI3K both block the activation of PKB (*6–8*), and transfection of cells with a constitutively active form of PI3K causes activation of PKB (*9*). Potential targets of PKB include glycogen synthase kinase–3 (*10*) and p70 ribosomal protein S6 kinase (*6*). PKB is also crucial in mediating cell survival (*11*). The exact mechanism of activation of PKB is not completely understood. Activation is usually accompanied by phosphorylation (*12*), and incubation of activated PKB with serine-threonine phosphatases abolishes activity (*4*). However, the lipid products of PI3K may also bind (*13*) and activate (*7, 14, 15*) PKB. Furthermore, an intact PH domain is necessary for activation of PKB by growth factors and constitutively active PI3K (*7, 9*), but this has been disputed (*4*). We therefore examined the relative contributions of lipid binding and phosphorylation of PKB on its activation in a defined in vitro system.

Lysates from COS1 cells transiently transfected with hemagglutinin (HA)-tagged PKB (*16*) were incubated with synthetic dipalmitoyl PtdIns(3,4,5)$P_3$ [(dipalmitoyl)-sn-phosphatidyl-D-myo-inositol-(3,4,5)-trisphosphate]. Addition of either PtdIns(3,4,5)$P_3$ or an adenosine triphosphate (ATP)–regenerating system to the cell lysate had little effect on the activity of PKB that was subsequently immunoprecipitated and assayed (Fig. 1). Similarly, if PKB was first immunoprecipitated through the HA tag and PtdIns(3,4,5)$P_3$ was added either alone or in combination with ATP to the immunoprecipitate, there was no activation of PKB. However, if both PtdIns(3,4,5)$P_3$ and the ATP-regenerating system were added to the cell lysate, PKB that was subsequently affinity-purified had increased kinase activity, both toward itself and an exogenous substrate, histone h2B (Fig. 1A). For optimal activation of PKB, lipid, ATP, and detergent were all required. Greater activation was achieved in vitro after addition of PtdIns(3,4,5)$P_3$ and ATP than after stimulation of cells with epidermal growth factor, but in both cases the

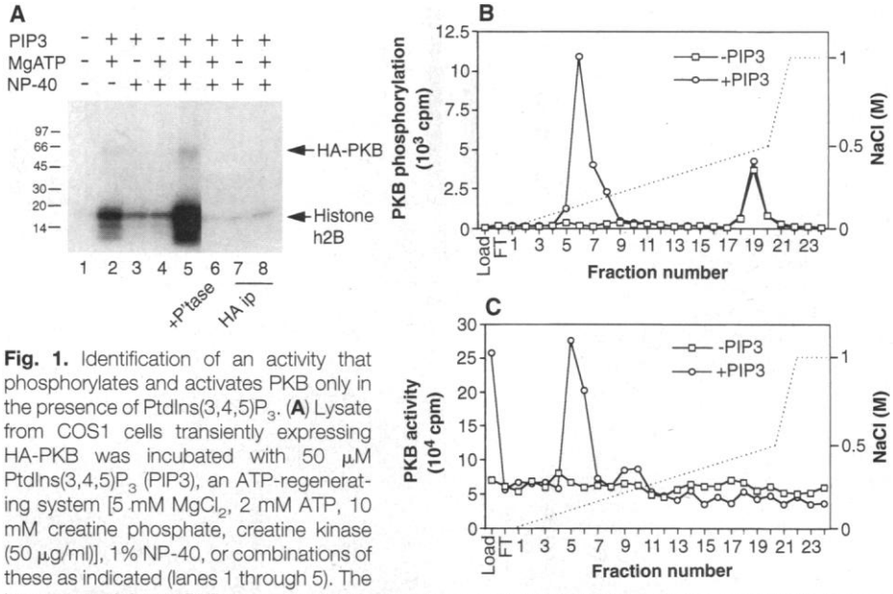


**Fig. 1.** Identification of an activity that phosphorylates and activates PKB only in the presence of PtdIns(3,4,5)$P_3$. (**A**) Lysate from COS1 cells transiently expressing HA-PKB was incubated with 50 μM PtdIns(3,4,5)$P_3$ (PIP3), an ATP-regenerating system [5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, creatine kinase (50 μg/ml)], 1% NP-40, or combinations of these as indicated (lanes 1 through 5). The immunoprecipitated PKB was then assayed for kinase activity with histone h2B as a substrate. In lanes 7 and 8 the PKB was first immunoprecipitated, and the washed immunoprecipitate (ip) was incubated with PtdIns(3,4,5)$P_3$ and an ATP-regenerating system and then assayed with histone h2B as a substrate. The sample in lane 6 is from an identical experiment to that shown in lane 5, except that after immunoprecipitation, PKB was incubated with 200 U of lambda phosphatase (New England Biolabs) for 30 min. The phosphorylated PKB and histone h2B bands are indicated by arrows. Molecular size markers are in kilodaltons. (**B** and **C**) Brain cytosol (20 mg) was fractionated by chromatography on a Mono Q column (Pharmacia). Each fraction was assayed for the ability to cause phosphorylation (B) or activation (C) of purified recombinant Glu-Glu–tagged (*26*) PKB in the presence (circles) or absence (squares) of PtdIns(3,4,5)$P_3$.

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activity was abolished by incubation of the affinity-purified PKB with a serine-threonine–specific protein phosphatase [Fig. 1A and (*17*)]. Three different sources of the dipalmitoyl form of the biological isomer of PtdIns(3,4,5)$P_3$ all had a similar effect on PKB. However, a stearoyl-arachidonoyl–substituted version of PtdIns(3,4,5)$P_3$ [(1-stearoyl,2-arachidonoyl)-sn-phosphatidyl-D-myo-inositol-(3,4,5)-trisphosphate] was the most potent in the activation of PKB (*17*), and because this is likely to be the major species of PtdIns(3,4,5)$P_3$ within the cell (*18*), this lipid was used for further characterization of the events involved in the activation of PKB under these conditions (*19*).

The above results indicated that both PtdIns(3,4,5)$P_3$ binding to either PKB or an upstream component, together with phosphorylation of PKB, are required for full activation of PKB. To dissect these possibilities, we chromatographed lysates from a number of different rat tissues on a MonoQ column and tested fractions for their ability to phosphorylate and activate purified PKB in a PtdIns(3,4,5)$P_3$- and ATP-dependent manner, which would reflect the presence of an activator or upstream kinase (*20*). Activity from rat brain cytosol was resolved into two peaks of activity that phosphorylated PKB. The peak eluting at 475 mM NaCl phosphorylated PKB both in the absence and presence of PtdIns(3,4,5)$P_3$ and was present in all rat tissues tested. However, the peak eluting at 150 mM NaCl phosphorylated PKB exclusively in the presence of PtdIns(3,4,5)$P_3$. This latter activity overlapped with, but did not exactly coincide with, an activity that activated PKB, also in a PtdIns(3,4,5)$P_3$-dependent manner (Fig. 1C) (*21*). The amount of the PtdIns(3,4,5)$P_3$-sensitive PKB kinase activity varied in cytosol from eight different tissues tested, with brain and thymus being the richest sources (*17*).

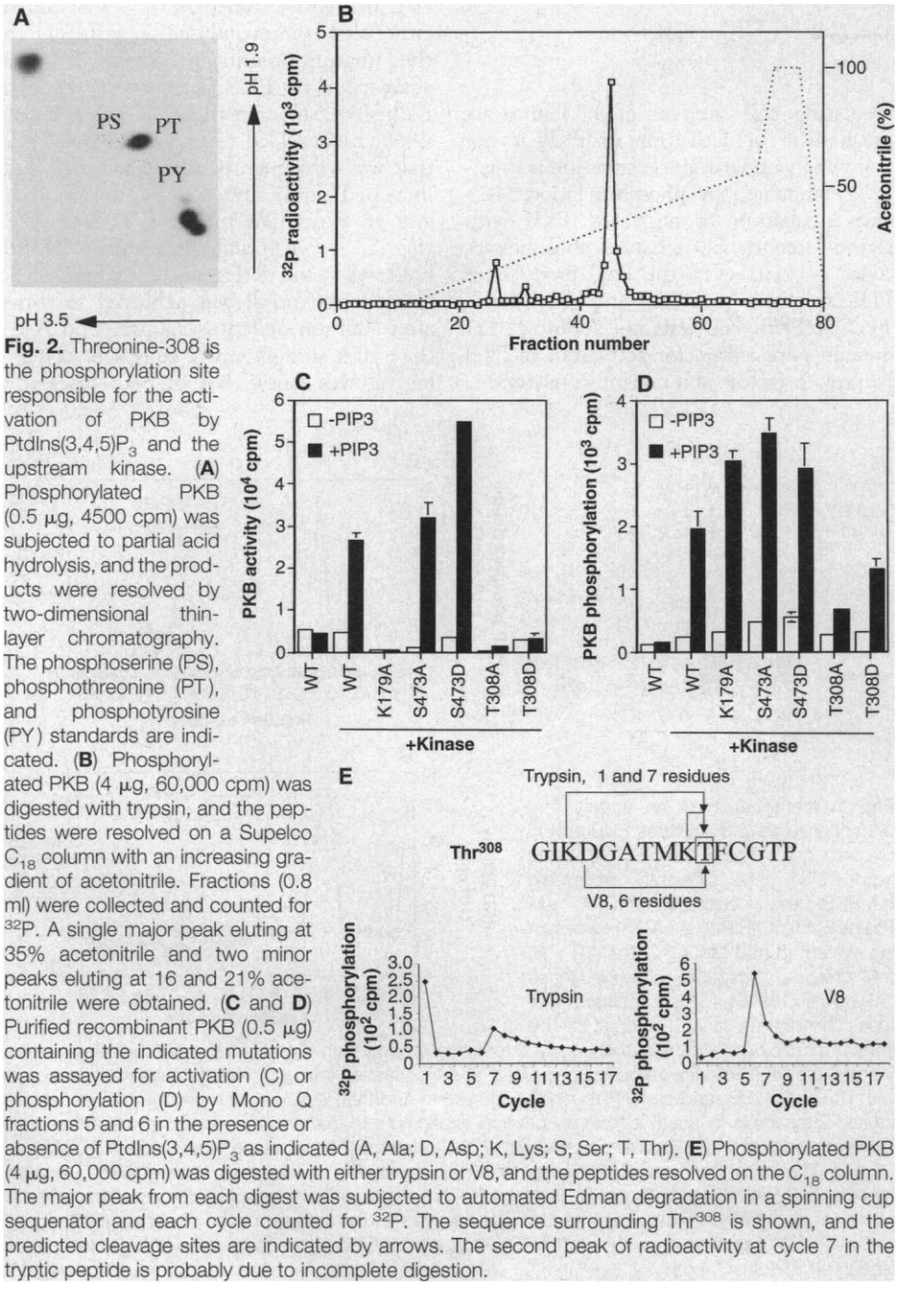


**Fig. 2.** Threonine-308 is the phosphorylation site responsible for the activation of PKB by PtdIns(3,4,5)$P_3$ and the upstream kinase. (**A**) Phosphorylated PKB (0.5 μg, 4500 cpm) was subjected to partial acid hydrolysis, and the products were resolved by two-dimensional thin-layer chromatography. The phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) standards are indicated. (**B**) Phosphorylated PKB (4 μg, 60,000 cpm) was digested with trypsin, and the peptides were resolved on a Supelco $C_{18}$ column with an increasing gradient of acetonitrile. Fractions (0.8 ml) were collected and counted for $^{32}$P. A single major peak eluting at 35% acetonitrile and two minor peaks eluting at 16 and 21% acetonitrile were obtained. (**C** and **D**) Purified recombinant PKB (0.5 μg) containing the indicated mutations was assayed for activation (C) or phosphorylation (D) by Mono Q fractions 5 and 6 in the presence or absence of PtdIns(3,4,5)$P_3$ as indicated (A, Ala; D, Asp; K, Lys; S, Ser; T, Thr). (**E**) Phosphorylated PKB (4 μg, 60,000 cpm) was digested with either trypsin or V8, and the peptides resolved on the $C_{18}$ column. The major peak from each digest was subjected to automated Edman degradation in a spinning cup sequenator and each cycle counted for $^{32}$P. The sequence surrounding Thr$^{308}$ is shown, and the predicted cleavage sites are indicated by arrows. The second peak of radioactivity at cycle 7 in the tryptic peptide is probably due to incomplete digestion.

Phosphoamino acid analysis of the phosphorylated PKB revealed that threonine was the only amino acid phosphorylated under these conditions (Fig. 2A). Phosphopeptide mapping showed that the majority of the [$^{32}$P]phosphate was incorporated into a single phosphopeptide under these conditions (Fig. 2B), which also contained only phosphothreonine (*17*). Two residues of PKB are phosphorylated in cells stimulated with growth factors, Thr$^{308}$ and Ser$^{473}$ (*12*). We therefore mutated either of these residues to alanine or aspartate and examined the effect of these mutants in allowing phosphorylation and activation by the active fractions from Fig. 1. Mutant PKB in which Ser$^{473}$ was changed to Ala (S473A) was activated to the same extent as wild-type PKB, a mutant in which Ser$^{473}$ was changed to Asp (S473D) was activated to a greater extent, whereas PKB with mutations at Thr$^{308}$ was not activated by the upstream kinase (Fig. 2C). Mutations at Ser$^{473}$ also had little effect on the phosphorylation of PKB mixed with active fractions, whereas mutations at Thr$^{308}$ inhibited phosphorylation of PKB (Fig. 2D). The residual phosphorylation of the Thr$^{308}$ mutants resulted from the increased phosphorylation of the two minor phosphopeptides in wild-type PKB (*17*). Mutation of the ATP-binding site of PKB (Lys$^{179}$ to Ala) eliminated its ability to phosphorylate an exogenous substrate (Fig. 2C), but it was still phosphorylated when mixed with active fractions in a PtdIns(3,4,5)$P_3$-dependent manner to the same degree as wild-type PKB (Fig. 2D). Tryptic and V8 proteolytic digestion of the phosphorylated PKB, followed by Edman degradation of the separated phosphopeptides in a spinning cup sequenator (*22*), also gave results indicative of Thr$^{308}$ being the major site of phosphorylation (Fig. 2E).

The above results suggested that the role of PtdIns(3,4,5)$P_3$ could either be to promote activation of the kinase that phosphorylates PKB on Thr$^{308}$, or to bind to PKB itself and change its conformation to allow phosphorylation of Thr$^{308}$ by the up-

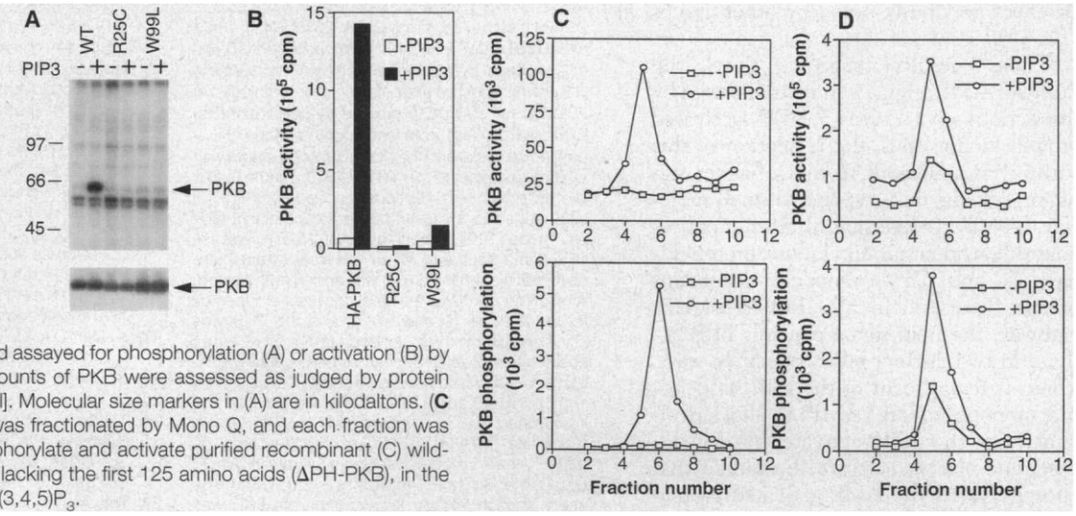


**Fig. 3.** Requirement of the PH domain of PKB for phosphorylation and activation by the upstream kinase in a PtdIns(3,4,5)$P_3$-dependent manner. (**A** and **B**) HA-tagged PKB containing the indicated mutations was transiently expressed in COS1 cells and affinity-purified by using the HA tag (WT, wild type; C, Cys; L, Leu; R, Arg, W, Trp). The immunoprecipitate was added to Mono Q fractions 5 and 6 and assayed for phosphorylation (A) or activation (B) by these fractions. Equivalent amounts of PKB were assessed as judged by protein immunoblotting [(A) lower panel]. Molecular size markers in (A) are in kilodaltons. (**C** and **D**) Brain cytosol (20 mg) was fractionated by Mono Q, and each fraction was assayed for the ability to phosphorylate and activate purified recombinant (C) wild-type PKB or (D) a PKB mutant lacking the first 125 amino acids (ΔPH-PKB), in the presence or absence of PtdIns(3,4,5)$P_3$.

stream kinase. Because phospholipids bind specifically to the PH domain of PKB (*13*, *15*), we assessed the effects of PH domain mutations and truncations on phosphorylation and activation in this system. Two mutations in the PKB PH domain, $Trp^{99}$ to Leu and $Arg^{25}$ to Cys, abolished the phosphorylation and activation of PKB by the upstream kinase in the presence of PtdIns(3,4,5)$P_3$ (Fig. 3, A and B). This finding suggested the possibility that the PH domain of PKB could be masking the ability of the upstream kinase to phosphorylate $Thr^{308}$, and that binding of PtdIns(3,4,5)$P_3$ might relieve this constraint. A PKB mutant lacking the first 125 amino acids (ΔPH-PKB) was phosphorylated and activated by the upstream kinase in the absence of PtdIns(3,4,5)$P_3$ (Fig. 3D). However, the ability of the upstream kinase to phosphorylate and activate ΔPH-PKB was still enhanced in the presence of PtdIns(3,4,5)$P_3$ (Fig. 3D). This suggests that PtdIns (3,4,5)$P_3$ has two functions in the activation of PKB—direct binding to the PH domain of PKB, which allows phosphorylation by the upstream kinase, and direct activation of the upstream kinase itself.

Further evidence for this dual mode of regulation was provided by examining the lipid specificity for phosphorylation and activation of full-length PKB and the ΔPH-PKB mutant by the upstream kinase. The biologically relevant stereoisomer of PtdIns(3,4,5)$P_3$ (termed DD-PIP3) had a median effective concentration ($EC_{50}$) of about 3 to 5 μM in stimulating phosphorylation and activation of full-length PKB, whereas its enantiomer [(2-arachidonoyl, 3-stearoyl) sn-phosphatidyl-L-myoinositol-(3,4,5)-trisphosphate, termed LL-PIP3] was essentially inactive (Fig. 4A). However, the $EC_{50}$ of DD-PIP3 in stimulating phosphorylation and activation of ΔPH-PKB was 0.3 to 0.5 μM, and its enantiomer also activated to a smaller extent (Fig. 4B). PtdIns(3,4)$P_2$ caused activation and phosphorylation (albeit only at high concentrations) of full-length PKB, but it was inactive at causing phosphorylation or activation of the ΔPH-PKB mutant (Fig. 4C). Activation and phosphorylation by PtdIns (3,4)$P_2$ was dependent on the presence of the upstream kinase (*17*). PtdIns(4,5)$P_2$ or a diacetyl form of PtdIns(3,4,5)$P_3$ were both unable to cause phosphorylation or activation of either full-length or truncated PKB (*17*).

Deletion of the PH domain of PKB has been reported to either enhance the activity of PKB (*4*, *23*) or to have no effect on its basal or stimulated activity (*4*), depending on the cell type tested. The discrepancy in these results may reflect the amount or distribution of the upstream kinase in various cell types. The upstream kinase was more sensitive to PtdIns(3,4,5)$P_3$ than PKB, and PtdIns(3,4)$P_2$ did not activate this kinase (Fig. 4); therefore the effect of PtdIns(3,4)$P_2$ is likely to be on PKB itself,

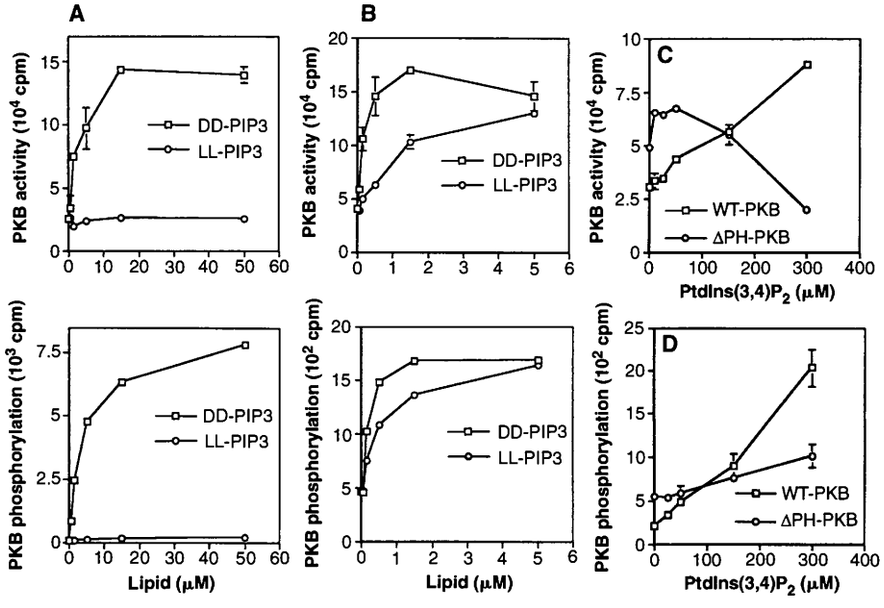


**Fig. 4.** Similar but distinct lipid specificities of the PKB PH domain and the upstream kinase. (**A**) Wild-type PKB (0.5 μg) or (**B**) the ΔPH-PKB mutant (0.5 μg) was incubated with 10 μl of Mono Q fractions 5 and 6 and various amounts of either the biological form of PtdIns(3,4,5)$P_3$ (DD-PIP3) or its enantiomer (LL-PIP3) as indicated. Activation (upper panels) and phosphorylation (lower panels) of PKB was assessed. (**C** and **D**) Wild-type PKB (0.5 μg) or the ΔPH-PKB mutant (0.5 μg) was incubated with various concentrations of dipalmitoyl PtdIns(3,4)$P_2$, and activation (C) and phosphorylation (D) of PKB was determined.

an effect previously noted by other groups (*14*, *15*).

These results suggest that the PtdIns(3,4,5)$P_3$ signal can be transduced by the actions of a PtdIns(3,4,5)$P_3$-activated protein kinase; thus, the regulation of this pathway is analogous to that of other signaling systems that respond to small molecule signals (adenosine 3′,5′-monophosphate–dependent protein kinase, protein kinase C, and $Ca^{2+}$/calmodulin-dependent protein kinases). In the PtdIns(3,4,5)$P_3$ pathway, the lipid signal controls PKB activity in two distinct but cooperative ways, which is reminiscent of the role of adenosine monophosphate (AMP) to allosterically activate the AMP-activated protein kinase, and also to activate the kinase that phosphorylates the AMP-activated protein (*24*). This may ensure tight regulation of PKB at the correct membrane localization, and it will be interesting to see whether this dual regulatory principle will apply to other targets in the PI3K signaling pathway.

*Note added in proof*: A PtdIns(3,4,5)$P_3$-dependent protein kinase has been purified (*25*) that may be related to the upstream kinase described here.

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16. PSG5(HA)PKB was obtained from B. Burgering (Utrecht University, Utrecht, The Netherlands). The cDNA encoding PKB was subcloned into pBluescript, and phosphorylation site and PH domain mutants were obtained with a strategy based on the polymerase chain reaction [with the use of *Pfu* DNA polymerase (Stratagene) and low cycle numbers]. The ΔPH-PKB mutant lacks the first 125 amino acids. All mutations were checked by sequencing. The appropriate cDNAs were then subcloned into a mammalian expression vector [pCMV3(EE)] and a baculovirus transfer vector [pAcoG1(EE)] encoding $NH_2$-terminal EEEEFMPME (Glu-Glu) tags (*25*) (E, Glu; F, Phe; M, Met; P, Pro).
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19. Details of the synthesis of dipalmitoyl- and stearoyl-arachidonoyl PtdIns(3,4,5)$P_3$ and their isomers will be published separately.
20. Rat tissues (5 g) were homogenized in 5 volumes of 20 mM tris (pH 7.5), 1 mM EDTA, 25 mM NaF, 1 mM dithiothreitol (DTT), 1 mM NaVn, leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), aprotinin (10 μg/ml), and 100 μM pefabloc and centrifuged at 20,000*g* for 30 min to prepare cytosolic extracts. Extracts (~20 mg) were loaded onto a Mono Q column (Pharmacia) and the bound proteins eluted with a 20-ml gradient to 500 mM NaCl in the same buffer. We collected 1-ml fractions, and portions (10 μl) were added to 0.1 μg of purified recombinant Glu-Glu–tagged PKB, 5 mM $MgCl_2$, 2 mM ATP, and 1% NP-40 in a volume of 40 μl. The stearoyl-arachidonoyl PtdIns(3,4,5)$P_3$ (5 μM) was added as indicated. After 20 min at 30°C, the reactions were stopped by the addition of 10 mM EDTA, and the PKB was affinity-purified with an antibody to the Glu-Glu tag coupled to protein G–Sepharose beads. After washing the beads with tris-buffered saline (TBS) containing 1% NP-40 and 1 mM EDTA, we assayed the immobilized PKB in a volume of 30 μl with 50 μM "Crosstide" (GRPRTSSFAEG, a peptide based on the $NH_2$-terminus of GSK-3) as a substrate (A, Ala; E, Glu; F, Phe; G, Gly; P, Pro; R, Arg; S, Ser; T, Thr). The ability of each fraction to phosphorylate PKB was determined in a similar manner, except the concentration of ATP was reduced to 10 μM, and 2.5 μCi of [$\gamma^{32}$P]ATP was added to each assay. After affinity purification of the PKB, the beads were washed with TBS containing 1% NP-40 and 1 mM EDTA and then analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.
21. There are at least two potential reasons why the two peaks of phosphorylation and activation reproducibly do not exactly coincide. The Glu-Glu–tagged PKB from Sf9 cells purifies as a heterogeneous mixture of phosphoproteins that is partially active. These species may exhibit differences in their ability to become phosphorylated versus their ability to become activated. An alternative possibility is that there is a second activity eluting earlier in the gradient that is required in addition to phosphorylation of PKB for activation, and it is when both this peak and the kinase peak overlap that the activation of PKB is seen. The two peak fractions were pooled for subsequent analysis of this activity.
22. Radiolabeled peptides were sequenced in a Beckman 890C spinning cup sequenator in the presence of Polybrene and horse myoglobin. Each cycle fraction was blown dry in a chemical fume hood, redissolved in 6 ml of scintillation fluid and counted for 20 min, and corrected for background.
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# Transmission of Hepatitis C by Intrahepatic Inoculation with Transcribed RNA


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More than 1% of the world's population is chronically infected with hepatitis C virus (HCV). HCV infection can result in acute hepatitis, chronic hepatitis, and cirrhosis, which is strongly associated with development of hepatocellular carcinoma. Genetic studies of HCV replication have been hampered by lack of a bona fide infectious molecular clone. Full-length functional clones of HCV complementary DNA were constructed. RNA transcripts from the clones were found to be infectious and to cause disease in chimpanzees after direct intrahepatic inoculation. This work defines the structure of a functional HCV genome RNA and proves that HCV alone is sufficient to cause disease.


In 1989, HCV, the viral agent believed to be responsible for most posttransfusion non-A, non-B hepatitis, was molecularly cloned (*1*). HCV is an enveloped positive-strand RNA virus classified in the family Flaviviridae (*2*). Characterization of HCV genome organization and expression has progressed rapidly since its discovery (*3*). The HCV genome RNA is ~9.6 kb and consists of a 5′ nontranslated region (NTR) that functions as an internal ribosome entry site, a long open reading frame (ORF) encoding a polyprotein of >3000 amino acids, and a 3′ NTR. The genome RNA was originally thought to terminate with polyadenylate [poly(A)] or polyuridylate [poly(U)] tracts, but recent studies have revealed the presence of an internal poly(U)/polypyrimidine [poly(U/UC)] tract followed by a highly conserved 98-base sequence (*4*, *5*). The HCV polyprotein is processed by host signal peptidase and two viral proteinases to yield at least 10 different structural and nonstructural (NS) proteins (Fig. 1). Properties of many of the HCV-encoded replication enzymes, such as the serine proteinase, RNA helicase, and polymerase have begun to emerge as part of intensive efforts to devel-


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