Characterization of PDK2 Activity Against Protein Kinase B γ[†]

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ABSTRACT: Protein kinase B (PKB), also known as Akt, is a serine/threonine protein kinase controlled by insulin, various growth factors, and phosphatidylinositol 3-kinase. Full activation of the PKB enzyme requires phosphorylation of a threonine in the activation loop and a serine in the C-terminal tail. PDK1 has clearly been shown to phosphorylate the threonine, but the mechanism leading to phosphorylation of the serine, the PDK2 site, is unclear. A yeast two-hybrid screen using full-length human PKBγ identified protein kinase C (PKC) ζ, an atypical PKC, as an interactor with PKBγ, an association requiring the pleckstrin homology domain of PKBγ. Endogenous PKBγ was shown to associate with endogenous PKCζ both in cos-1 cells and in 3T3-L1 adipocytes, demonstrating a physiological interaction. Immunoprecipitates of PKCζ, whether endogenous PKCζ from insulin-stimulated 3T3-L1 adipocytes or overexpressed PKCζ from cos-1 cells, phosphorylated S472 (the C-terminal serine phosphorylation site) of PKBγ, in vitro. In vivo, overexpression of PKCζ stimulated the phosphorylation of approximately 50% of the PKBγ molecules, suggesting a physiologically meaningful effect. However, pure PKCζ protein was incapable of phosphorylating S472 of PKBγ. Antisense knockout studies and use of a PDK1 inhibitor showed that neither PKB autophosphorylation nor phosphorylation by PDK1 accounted for the S472 phosphorylation in PKCζ immunoprecipitates. Staurosporine inhibited the PKCζ activity but not the PDK2 activity in PKCζ immunoprecipitates. Together these results indicate that an independent PDK2 activity exists that physically associates with PKCζ and that PKCζ, by binding PKBγ, functions to deliver the PDK2 to a required location. PKCζ thus functions as an adaptor, associating with a staurosporine-insensitive PDK2 enzyme that catalyzes the phosphorylation of S472 of PKBγ. Because both PKCζ and PKB have been proposed to be required for mediating a number of crucial insulin responses, formation of an active signaling complex containing PKCζ, PKB, and PDK2 is an attractive mechanism for ensuring that all the critical sites on targets such as glycogen synthase kinase-3 are phosphorylated.

Protein kinase B (PKB),[1] also known as Akt, is a 60 kDa serine/threonine protein kinase which is found in three isoforms: PKBα, PKBβ, and PKBγ. PKB contains a pleckstrin homology domain (PH) at its N-terminus, a catalytic domain, and a short C-terminal tail, and as a member of the AGC family of kinases is closely related to protein kinase C (PKC) and protein kinase A (PKA). PKB may play a crucial role in signaling to many metabolic pathways as well as being potently anti-apoptotic (*1*). PKB can be activated by insulin and various mitogens through the activation of phosphatidylinositol 3-kinase (PI3K) (*1*). The products of PI3K, phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and phosphatidylinositol-3,4-bisphosphate (PIP_2), bind to the PH domain of PKB, recruiting the protein to the plasma membrane. Membrane localization leads to the phosphorylation of two residues within PKB that are critical for regulation of the activity of all three PKB isoforms. One phosphorylation site is found in the activation loop of the kinase domain (Thr-308 in PKBα, Thr-309 in PKBβ, and Thr-305 in PKBγ), and the other is in the C-terminal hydrophobic domain (Ser-473 in PKBα, Ser-474 in PKBβ, and Ser-472 in PKBγ). Phosphorylation of the two sites is required to fully activate PKB. The kinase responsible for phosphorylation of the threonine in the activation loop has been known for several years, this being the 3-phosphoinositide-dependent protein kinase-1 (PDK1) (*2*). The identity of the kinase that phosphorylates the second regulatory site in the hydrophobic C-terminal domain of PKB, PDK2, is still unknown. One suggestion is that PDK2 is attributable to PDK1. This has been proposed to occur through a small C-terminal fragment of protein kinase C-related kinase-2 (PRK2) which converts PDK1 into PDK2 (*3*, *4*). A second proposal is that a distinct PDK2 does not exist but that the serine phosphorylation occurs by virtue of PKB autophosphorylation (*5*). PDK2 kinases have also been suggested, these being integrin-linked kinase (*6*, *42*) or MAP kinase-activated protein kinase-2 (MAPKAP-K2) (*7*). However, other studies have cast strong doubt on all of these mechanisms (*8*−*10*, *18*).

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[1] Abbreviations: MAPKAP-K2, MAP kinase-activated protein kinase-2; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, phosphatidylinositol 3-kinase; PIF, PDK1-interacting fragment; PKB, protein kinase B; PKC, protein kinase C; PRK, protein kinase C-related kinase.

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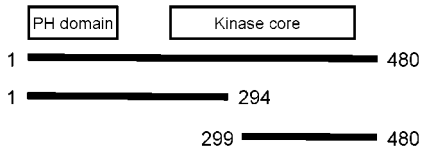

FIGURE 1: Schematic representation of the PKBγ constructs used in this study. Constructs were generated to cover the full-length (PKBγ 1−480), the N-terminal domain (PKBγ 1−294), and the C-terminal domain (PKBγ 299−480) of the PKBγ protein.

We have identified a model system that shows robust PDK2 activity. This system revealed that PKCζ functions as an adaptor, associating with a staurosporine-insensitive PDK2 enzyme which catalyzes phosphorylation of S472 of PKBγ without requiring either PDK1 activity or PKB autophosphorylation.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid System. The LexA yeast two-hybrid system was purchased from Clontech. The human matchmaker brain cDNA library was also from Clontech. Full-length human PKBγ was cloned from a human brain library using the 5′ oligonucleotide $^{5'}$ATG AGC GAT GTT ACC ATT GTG AAA GAA$^{3'}$ and the 3′ oligonucleotide $^{5'}$TTA TTC TCG TCC ACT TGC AGA GTA GGA$^{3'}$ via Pfu polymerase. The constructs were A-tailed and ligated into pGEM Teasy (Promega) and then sequenced. The inserts were then PCR'd via Pfu from this vector via the 5′ oligonucleotide $^{5'}$GCC GCC ATG GCG AAT TCG GCA ATT CGA$^{3'}$ and the 3′ oligonucleotide $^{5'}$GCA GGA GGC CGC CTC GAG ACT AGT GAT$^{3'}$. The PCR products, digested with *Eco*RI and *Xho*I, were then ligated into *Eco*RI−*Xho*I-digested pLexA to generate in-frame fusions with the LexA binding domain, confirmed by sequencing and expression of the full-length protein in yeast. The EGY48[p8opLacZ] yeast strain was first transformed with pLexA-PKBγ, tested for expression of the hybrid protein via Western blotting using the LexA antibody (Clontech). Subsequently, the EGY48[p8opLacZ, pLexA-PKBγ] were transformed with the pB42AD-brain cDNA library (100 μg) and plated out onto SD/dex/kan/-his/-trp/-ura. After 3 days of growth, the transformants were collected and 5×10^6 plated out onto SD/gal/raf/kan/-his/-trp/-ura/-leu selection medium plates supplemented with X-gal (80 μg/mL). Leu+LacZ+ colonies were collected over a period of 5 days. Library plasmids were rescued via transformation of KC8 bacteria grown on M9TrpDOAmp plates. Putative interacting library plasmids were reintroduced into EGY48[p8opLacZ, pLexA-PKBγ] as the positive control and with pLexA-laminin, pLexA-p53 as negative controls with the selection on SD/gal/raf/kan/-his/-trp/-ura/-leu/Xgal plates.

PKB Deletion Mutant Bait Plasmids. PKBγ 1−294 was produced by PCR with Pfu polymerase of the pLexA-PKBγ with the 5′ oligonucleotide $^{5'}$GCC GCC ATG GCG AAT TCG GCA ATT CGA$^{3'}$ and the 3′ oligonucleotide $^{5'}$GGC TGC ATC TGT GAT CTC GAG TTT GCA AAG TCC A$^{3'}$. PKBγ 299−480 was produced using the 5′ oligonucleotide $^{5'}$GGA CTT TGC AAA GAA GAA TTC ACA GAT GCA GCC A$^{3'}$ and the 3′ oligonucleotide $^{5'}$GCA GGA GGC CGC CTC GAG ACT AGT GAT$^{3'}$. Constructs were digested with *Eco*RI and *Xho*I and subsequently ligated into *Eco*RI−*Xho*I-digested pLexA. These constructs are shown in Figure 1.

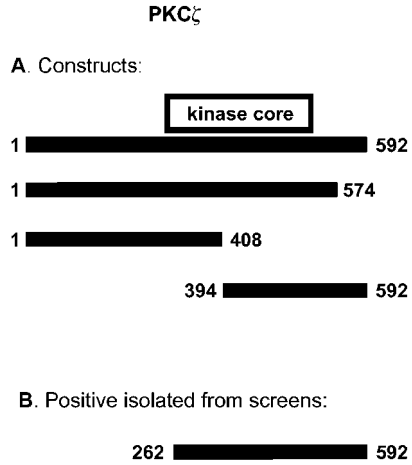

FIGURE 2: Schematic representation of the PKCζ constructs used in this study. (A) Constructs used. (B) Region of the PKCζ protein isolated in the yeast two-hybrid screen of the brain cDNA library.

PKC Deletion Mutant Prey Plasmids. Full-length PKCζ was isolated as a positive of a yeast two-hybrid screen previously conducted which had an additional 37 amino acids compared to published sequences. This as well as other PKCζ yeast two-hybrid clones served as the basis for PCR, using Pfu polymerase, to generate various PKCζ deletion mutants. These constructs are shown in Figure 2. All oligonucleotides are shown as forward and then reverse primers, respectively. PKCζ constructs: 1−408 was produced from full-length PKCζ using $^{5'}$GGT TTT CAT GAA TTC GAA GCG GAT$^{3'}$ $^{5'}$CGT TGT GTC ACC AGG CTC GAG GCC TTC CTT GAA$^{3'}$; 1−574 was produced from full-length PKCζ using $^{5'}$GGT TTT CAT GAA TTC GAA GCG GAT$^{3'}$ $^{5'}$CTC TGA CTG GTC GAT CTC GAG TAT GGC ATC CTC AT$^{3'}$; and 394−592 was obtained from a yeast two-hybrid screen. All constructs were digested with *Eco*RI and *Xho*I and ligated into *Eco*RI−*Xho*I-digested pB42AD.

HA- and Cmyc-Tagged pcDNA Constructs. pLexA and pB42AD constructs served as templates for Pfu polymerase-dependent PCR to generate the cmyc- and HA-tagged constructs listed above. pcDNA3.1+ (Invitrogen) was the mammalian expression vector used. Prior to ligation, the vector was digested with *Kpn*I and *Xba*I. Myc-tagged fusion constructs were generated via the 5′ oligonucleotide $^{5'}$AAC AAT TGT CGT AGG TAC CGG TAT GGA GGA GCA GAA GCT GAT CTC AGA GGA CCT GGT TAT TCG CAA CGG CGA CTG GCT G$^{3'}$ and the 3′ oligonucleotide $^{5'}$ACC TAA GAG TCA CTT CTA GAT TTG TAT ACA CT$^{3'}$ as suggested by Clontech. This PCR was very inefficient, and only one insert could be successfully amplified. This was digested with *Kpn*I and *Xba*I and ligated into the predigested pcDNA3.1. Once sequenced, this vector was cut with *Eco*RI and *Xho*I to generate a pcDNAmyc vector. pLexA vectors containing the remaining constructs were cut with *Eco*RI and *Xho*I to give rise to the insert, which was rescued from gels via a Qiagen gel extraction kit. The subsequent DNA was then ligated into pcDNAmyc. HA-tagged fusion proteins were generated by PCR using Pfu with the 5′ oligonucleotide $^{5'}$GTT AAC GAT ACC AGC GGT ACC CTG AGT ATG GAT GC$^{3'}$ and the 3′ oligonulceotide $^{5'}$ACC TAA GAG TCA CTC TAG AAT TTG TAT ACA CT$^{3'}$.

Transient Cell Transfection in Cos-1 Cells. Exponentially growing cos-1 cells (2×10^6) were transiently transfected with plasmid combinations as listed in the figures using the Effectene transfection reagent according to the manufacturer's protocol (Qiagen). After 48 h, cell lysates were prepared using lysis buffer (0.14 M NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 5 mM EDTA, 0.5% v/v Triton X-100, 0.1 mM PMSF, 7 μg/mL pepstatinA, 5 μg/mL leupeptin, 25 μg/mL aprotinin, pH 7.4). Lysis was allowed to proceed for 30 min on ice after which the cell debris was removed by centrifugation at 12000*g* for 5 min. Extracts were used immediately for immunoprecipitation.

Antisense Transfection. Phosphorothioate-modified oligodeoxynucleotides (ODNs) known to deplete PKBα, -β, and -γ were used in combination to deplete endogenous PKB from 3T3-L1 adipocytes. Transfection protocol and details of the ODNs used are described in Sale et al. (*11*). The ODN concentrations and incubation times utilized were routinely 5−10 μM and 5 days. Cells were incubated with or without 100 nM insulin at 37 °C for 5 min as indicated and extracted as described above for cos-1 cells.

Immunoprecipitation. Extracts were diluted in lysis buffer to give a 50 μL final volume (500 μg) and then further diluted in co-immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 5 μg/mL aprotinin, 0.5 mM PMSF, 0.1% v/v Tween 20, pH 7.4; 400 μL) with polyclonal HA (clone HA-7, 4 μg, Sigma) or cmyc (clone 9E10, 4 μg, Clontech) antibodies and protein G beads (20 μL, Sigma). After 6 h continuous gentle agitation at 4 °C, the beads were collected by pulse spin and then washed 3 times in co-immunoprecipitation buffer after which they were resuspended in PBS.

In Vitro Kinase Assays. Human recombinant PKBγ, expressed as an N-terminal His-tagged fusion protein in *E. coli* and purified using Ni−NTA agarose, was obtained from Upstate Biotechnology. Human recombinant PKCζ, expressed in insect cells using the baculovirus vector and purified to near-homogeneity (>95% pure as determined by SDS−PAGE), was also obtained from Upstate Biotechnology. In vitro kinase assays, using human recombinant PKBγ and PKCζ proteins, were carried out at 30 °C for 30 min in a buffer containing 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 20 μM ATP, and 5 μCi of [γ-^{32}P]ATP per assay (*12*). Where indicated, phosphatidylserine (Sigma) was added to the kinase mixture at a 200 μg/mL concentration.

In vitro kinase assays were also carried out with immunopurified PKCζ. Lysates from cos-1 cells or 3T3-L1 adipocytes were prepared using the lysis buffer as described above with the addition of 50 mM NaF, 40 mM β-glycerolphosphate, 10 mM Hepes, pH 7.3, 5 mM EDTA, and 1 μM microcystin. Cells were serum-starved for at least 24 h, and where appropriate, cells were stimulated with 100 nM insulin (3T3-L1 adipocytes) or 15% serum for 15 min (cos-1) prior to extraction. Immunoprecipitation of PKCζ was carried out as described above except that the immunocomplex, after being washed with co-immunoprecipitation buffer 3 times, was washed a further 2 times in kinase buffer lacking ATP. The kinase assay was carried out as described above with exogenous human recombinant PKBγ (50 ng) as substrate. Assay conditions were linear. Where necessary, staurosporine (5 μM) or 1 mM sphingosine (*13*) was added to the kinase assay.

All kinase reactions were stopped by the addition of 5× Laemmli sample buffer and subsequent boiling for 3 min. Samples were then subjected to 7.5% SDS−PAGE and transferred to nitrocellulose. Where necessary, filters were exposed to Amersham HybondN film at −80 °C prior to immunoblotting.

Immunoblotting. Immunoprecipitates were resolved by SDS−PAGE (*14*, *15*) and transferred to a nitrocellulose filter. The membranes were probed with various primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies as appropriate. Primary antibodies [cmyc (Clontech), monoclonal HA (Sigma), phospho PKB S472 (Calbiochem and New England Biolabs; the antibody was raised against PKBα phosphorylated on S473 but also recognizes PKBγ phosphorylated on S472), phospho-(Thr) PDK1 substrate antibody (New England Biolabs), PKCζ (Sigma)] were used according to the manufacturer's instructions. Blots were developed with the ECL system according to the manufacturer's instructions (Amersham).

Other Methods. Gel shift assays were performed as described previously (*14*). 3T3-L1 adipocytes were cultured and stimulated with insulin as described previously (*14*, *15*). Immunoprecipitations with the PKBγ antibody (Santa Cruz) were carried out according to the manufacturer's protocol.

RESULTS

Detection of Proteins Binding to PKBγ. To identify proteins able to interact with PKBγ, we employed the LexA yeast two-hybrid system to screen a human brain cDNA library. Full-length human PKBγ was fused to the LexA DNA binding domain of the Clontech pLexA vector and subsequently used in an interactor hunt with a brain cDNA library. Screening of 5×10^6 transformants yielded 111 Leu+LacZ+ colonies. Sequencing of the clones yielded various AGC kinases and several components of the proteasome complex. In this study, we focused solely on one of the AGC kinase interactors that was found, PKCζ.

PKBγ and PKCζ Interact in Mammalian Cos-1 Cells. To test whether PKBγ and PKCζ interact in vivo in mammalian cells, cmyc-tagged PKBγ and HA-tagged PKCζ were cotransfected into cos-1 cells. Transfected cells were lysed, and the lysates were incubated with cmyc antibodies to precipitate cmyc-tagged PKBγ. The immunoprecipitates were then separated by SDS−PAGE and immunoblotted with anti-HA antibodies to test for coprecipitation of HA-tagged PKCζ. As shown in Figure 3A, precipitation of PKBγ resulted in the co-immunoprecipitation of PKCζ (lane 2), confirming our above-described yeast two-hybrid binding results. That this association was not a result of nonspecific binding is evident from the observation that PKCζ, when transfected alone, was not precipitated by the cmyc antibodies (Figure 3A, lane 1).

To determine which regions of PKBγ were responsible for the interaction with PKCζ, various cmyc-tagged deletion mutants of PKBγ (see Figure 1) were cotransfected into cos-1 cells with HA-tagged PKCζ. Transfected cells were lysed and the cmyc-tagged PKBγ constructs immunoprecipitated and probed for PKCζ with the HA antibody. The N-terminal domain of PKBγ [PKBγ-(1−294)] was found to associate with PKCζ (Figure 3A, lane 3), whereas PKBγ-(299−480) did not (Figure 3A, lane 4). PKBγ was expressed at a higher

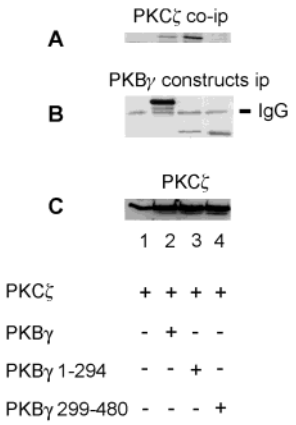

FIGURE 3: PKBγ interacts with PKCζ in cos-1 cells. Cos-1 cells were transiently transfected with the indicated PKBγ constructs (expressed as cmyc fusions) and PKCs (expressed as HA fusions). Cell extracts were immunoprecipitated with cmyc antibody, and after extensive washing, proteins were separated by SDS−PAGE, transferred to a nitrocellulose membrane, and probed for (A) HA to detect coprecipitated proteins or (B) cmyc to check for immunoprecipitation of the PKBγ constructs. Aliquots of the lysate were also analyzed for levels of expression of the HA-tagged PKCζ using HA antibody (C). The results are representative of seven independent experiments.

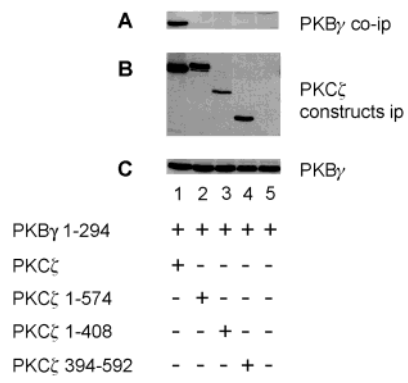

FIGURE 4: PKBγ requires the full-length PKCζ protein for interaction. Cos-1 cells were transiently transfected with cmyc-tagged PKBγ 1−294 either alone or in combination with various HA-tagged PKC constructs. Cell extracts were immunoprecipitated with HA antibody, and after extensive washing, proteins were separated by SDS−PAGE, transferred to a nitrocellulose membrane, and probed for (A) cmyc to detect coprecipitated proteins and (B) HA to determine the levels of immunoprecipitation of the HA constructs. The PKCζ 1−574 consistently ran at a slightly higher molecular weight than PKCζ. The cell extracts were probed for cmyc to check the expression of the PKBγ 1−294 construct (C). The results are representative of seven independent experiments.

level than PKBγ-(1−294) or PKBγ-(299−480) (Figure 3B). Therefore, the interaction between PKBγ-(1−294) and PKCζ was stronger than that for the wild-type PKBγ.

Experiments were then conducted to investigate the domain(s) within PKCζ required for binding to PKBγ. Cos-1 cells were transfected with the cmyc-tagged PKBγ-(1−294) construct along with various HA-tagged deletion mutants of PKCζ (see Figure 2 for details of the constructs). Transfected cells were lysed, immunoprecipitated with the HA antibody, and probed with the cmyc antibody (Figure 4A). A strong band was only observed with full-length PKCζ (Figure 4A, lane 1). The other constructs tested failed to bind, despite the similar levels of protein expression in all transfected cells (Figure 4B,C).

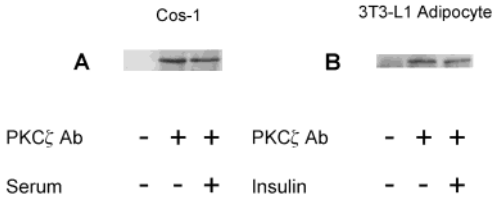

FIGURE 5: Endogenous PKBγ associates with endogenous PKCζ. (A) Untransfected cos-1 cells. (B) 3T3-L1 adipocytes. Cells were serum-starved for 24 h and then incubated for 15 min in the presence or absence of serum (cos-1 cells) or for 5 min in the presence or absence of 100 nM insulin (3T3-L1 adipocytes). Endogenous PKCζ was immunoprecipitated from cell lysates (0.5 mg of protein), and the immunoprecipitates were extensively washed. Proteins in the immunocomplex were Western-blotted with a PKBγ antibody. Control immunoprecipitations containing an equal amount of cell lysate and protein A beads but omitting the PKCζ antibody were also carried out. The results are representative of two independent experiments.

Endogenous PKBγ Associates with Endogenous PKCζ. We next considered whether the interaction between PKBγ and PKC occurred with the endogenous proteins in vivo. Cos-1 cells (un-transfected) were lysed and the lysates incubated with a PKCζ antibody to precipitate endogenous PKCζ. The immunoprecipitates were then probed with a PKBγ antibody. As shown in Figure 5A, precipitation of endogenous PKCζ resulted in the co-immunoprecipitation of endogenous PKBγ. Control experiments omitting the PKCζ antibody confirmed the absence of PKBγ (Figure 5A). To test if the endogenous proteins interacted in a different cell line, 3T3-L1 adipocytes were employed. 3T3-L1 adipocytes are a major model cell line for studying insulin signaling. Immunoprecipitation of endogenous PKCζ again co-immunoprecipitated endogenous PKBγ (Figure 5B). Stimulation of cos-1 cells with serum or 3T3-L1 adipocytes with insulin did not modulate the degree of association between PKBγ and PKCζ (Figure 5).

Immunopurified Endogenous PKCζ Complex Phosphorylates PKBγ on S472 in Vitro. A previous report (*16*) has shown that immunocomplexes containing PKCζ are capable of phosphorylating the PDK2 sites of PKCδ and PKCα. Therefore, we wished to investigate this possibility with respect to PKBγ. Pure PKBγ (human recombinant) was used as substrate. 3T3-L1 adipocytes were stimulated with and without insulin prior to extraction and immunopurification of endogenous PKCζ. The immunopurified PKCζ complex from insulin-stimulated cells heavily phosphorylated S472 of the PKBγ (Figure 6D, lane 7). Only slight T305 phosphorylation was observed (Figure 6E, lane 7). PKCζ complex immunopurified from 3T3-L1 adipocytes incubated without insulin did not phosphorylate S472 of PKBγ (Figure 6D, lane 3).

The possibility that the immunopurified PKCζ complex triggered autophosphorylation of the PKBγ on S472 was next considered. Autophosphorylation of the S472 residue as described by Toker and Newton (*5*) requires T305 phosphorylation, but little T305 phosphorylation was observed in our experiments (Figure 6E, lane 7). Thus, the immunopurified PKCζ complex did not appear to render the substrate PKB capable of autophosphorylating itself. The relatively low level of T305 phosphorylation observed, compared with the high level of phosphorylation of the exogenous PKBγ on S472, was likely to be due to co-immunoprecipitation of

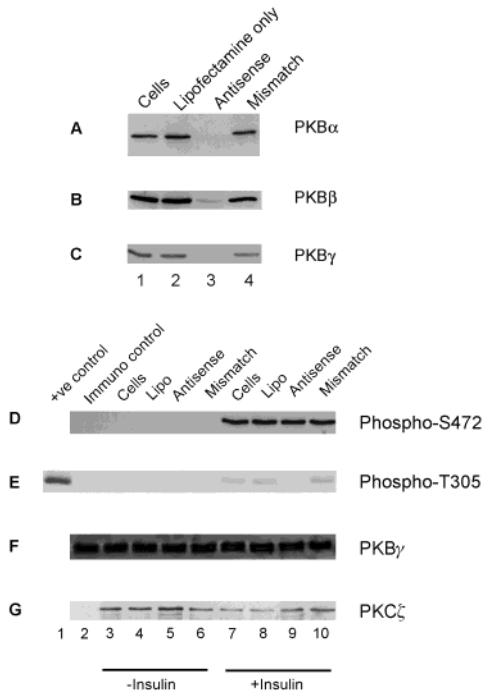

FIGURE 6: Phosphorylation of PKBγ on S472 by immunoprecipitates containing endogenous PKCζ does not involve PKB autophosphorylation. 3T3-L1 adipocytes were transfected with or without ODNs in the absence of serum and then incubated for 5 min in the presence or absence of 100 nM insulin. The ODNs were a mixture of 3 ODNs that together targeted all 3 PKB isoforms (antisense) or a mixture of 3 corresponding mismatch controls (mismatch). (A) Immunoblot with PKBα antibody. (B) Immunoblot with PKBβ antibody. (C) Immunoblot with PKBγ antibody. Endogenous PKCζ was immunoprecipitated from lysates of the cells (0.5 mg of protein), and the immunoprecipitates were extensively washed. The PKCζ immunoprecipitates were incubated with ATP and pure PKBγ protein as substrate, and samples were then immunoblotted with a PKB phospho S472 antibody (D), a phospho-(Thr) PDK1 substrate antibody to detect T305 phosphorylation of PKB (E), a PKBγ antibody to determine an equal amount of substrate (F), and a PKCζ antibody to determine levels of endogenous PKCζ immunoprecipitated (G). In (E), the positive control was immunopurified PKB activated by insulin as above. The immunoblots are representative of two individual experiments.

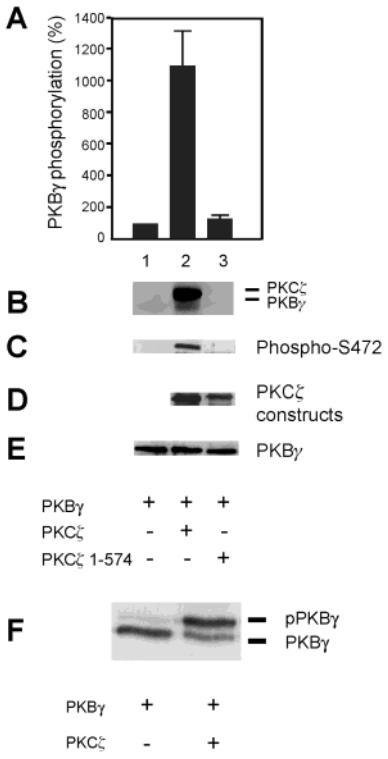

FIGURE 7: Immunoprecipitates containing PKCζ prepared from cos-1 cells overexpressing PKCζ phosphorylate PKBγ in vitro. PKBγ (cmyc-tagged) and PKCs (HA-tagged) were isolated by immunoprecipitation from 24 h serum-starved cos-1 cells transiently transfected with the respective constructs. PKBγ was incubated with [γ-^{32}P]ATP and the various PKCs as indicated. (A) Quantification of PKBγ phosphorylation. The level of PKBγ phosphorylation was determined by densitometric scanning of autoradiographs. Results are expressed as a percentage of phosphorylation of PKBγ alone and are the mean $\pm$ SEM of nine independent experiments. (B) Autoradiograph showing PKBγ and PKC phosphorylation. (C) Immunoblot with PKB phospho S472 antibody. (D) Immunoblot with HA antibody showing the levels of the HA-tagged PKC constructs. (E) Immunoblot with cmyc antibody showing the levels of cmyc-tagged PKBγ. Autoradiographs and blots from a representative experiment are shown. (F) Immunoblot showing the stoichiometry of PKBγ phosphorylation by PKCζ. Samples were resolved on a low bis-acrylamide SDS gel to separate the different phosphorylated forms and Western-blotted with the cmyc antibody. The amount of PKBγ phosphorylation by PKC was determined by densitometric scanning of the immunoblot.

endogenous active PKB along with the PKCζ. Therefore, it was a possibility that endogenous active PKB was catalyzing a trans phosphorylation of the PKBγ substrate. To exclude this, endogenous PKB was removed prior to extraction and immunoprecipitation via antisense knockout using a mixture of three phosphorothioate ODNs that in combination are known to target and deplete all three PKB isoforms (*11*). This procedure was successful in depleting endogenous PKBα and PKBγ to undetectable levels (Figure 6A and 6C, respectively) and 90% of endogenous PKBβ (Figure 6B). The procedure also removed the residual T305 phosphorylation. The PKB depletion was specific as shown by the carrier only (Figure 6A−C, lane 2) and mismatch phosphorothioate ODN (Figure 6A−C, lane 4) controls. Depletion of endogenous PKB had no effect on the ability of immunopurified endogenous PKCζ complex to phosphorylate S472 of PKBγ (Figure 6D, lane 9). Immunopurified endogenous PKCζ complex prepared from cells treated only with carrier (Figure 6D, lane 8) or from cells treated with a panel of three corresponding mismatch phosphorothioate ODNs (Figure 6D, lane 10) elicited a comparable level of S472 phosphorylation to endogenous PKCζ complex immunopurified from untreated cells (Figure 6D, lane 7).

PKCζ Complex Immunopurified from Cos-1 Cells Overexpressing PKCζ Phosphorylates S472 of PKBγ in Vitro. To further verify the results obtained with endogenous PKCζ above, overexpressed HA-tagged PKCζ was used instead with immunoprecipitation by the HA antibody. A low level of overexpression was used, only 2−3 orders of magnitude greater than endogenous expression (data not shown).The immunopurified HA-PKCζ was kinase-active in vitro (see also below) as has been reported previously (*16*), and for example phosphorylated MBP (data not shown), and underwent autophosphorylation (Figure 7B, lane 2). Immunoprecipitates containing HA-PKCζ significantly phosphorylated PKBγ (Figure 7A,B). The phosphorylation of PKBγ was abolished by truncating PKCζ, such that the C-terminal tail was removed (Figure 7A,B). Truncation of PKCζ in this fashion prevents it from associating with PKBγ (see above) and completely abrogated PKC activity (Figure 7B). Immuno-

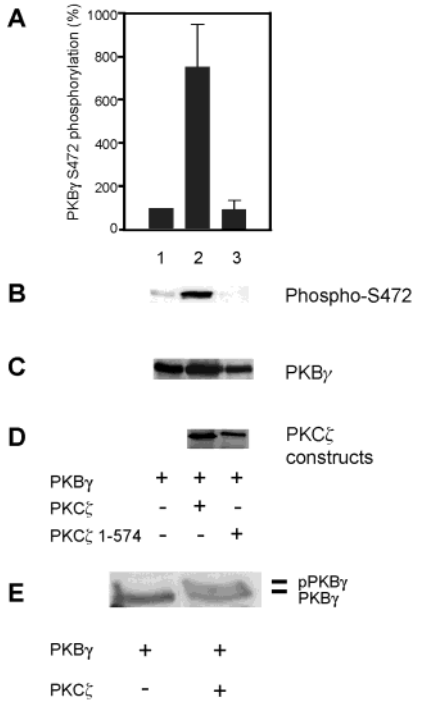

FIGURE 8: Overexpression of PKCζ in cos-1 cells promotes phosphorylation of PKBγ in vivo. Cos-1 cells were transiently transfected with cmyc-tagged PKBγ either alone or in combination with various HA-tagged PKC constructs. Cells were serum-starved for 24 h. PKBγ was immunoprecipitated with the cmyc antibody and immunoblotted with various antibodies. (A) Quantitation of PKBγ S472 phosphorylation. Samples were immunoblotted with a phospho S472 antibody. The level of PKBγ S472 phosphorylation was determined by densitometric scanning of the immunoblots. The results are expressed as the percentage of the S472 phosphorylation of PKBγ transfected without any PKC constructs and were corrected for any differences in the amount of PKBγ. Values are means ± SEM of three independent experiments. (B) Immunoblot with PKB phospho S472 antibody. (C) Immunoblot with the cmyc antibody showing the levels of cmyc-tagged PKBγ. (D) Immunoblot of cell extracts with the HA antibody showing the levels of HA-tagged PKC constructs. The immunoblots are from a representative experiment. (E) Immunoblot showing the stoichiometry of PKBγ phosphorylation by PKCζ. Samples were resolved on a low bisacrylamide SDS gel to separate the different phosphorylated forms and Western-blotted with the cmyc antibody. The amount of PKBγ phosphorylation by PKC was determined by densitometric scanning of the immunoblot.

blotting with a PKB phospho S472 antibody showed that phosphorylation of PKBγ by immunopurified HA-PKCζ was mirrored by large increases in S472 phosphorylation of PKBγ (Figure 7C, lane 2). Control immunoblots confirmed that the amounts of either PKBγ substrate or PKC construct were similar (Figure 7D,E). Truncated immunopurified HA-PKCζ did not elicit any S472 phosphorylation of PKBγ (Figure 7C, lane 3). Gel shift experiments were used to determine the stoichiometry of PKBγ phosphorylation by immunopurified PKCζ. Immunopurified PKCζ phosphorylated approximately 70% of the total PKBγ protein (Figure 7F).

Overexpression of PKCζ in Cos-1 Cells Stimulates Phosphorylation of S472 of PKBγ in Vivo. A number of other studies using a variety of cell types have concluded that overexpressed PKCζ is significantly active without ligand stimulation and have indicated that the overexpressed PKCζ is as much as 50−80% as active without ligand stimulation compared with ligand stimulation (*8*). Therefore, one would expect overexpression of PKCζ to elicit S472 phosphorylation of PKBγ in intact cells. Consequently, we next performed in vivo experiments in cos-1 cells transiently cotransfected with HA-PKCζ and cmyc-PKBγ. Extracts of serum-starved cos-1 cells were subjected to SDS−PAGE after immunoprecipitation with the cmyc antibody and transferred to nitrocellulose filters which were probed with a PKB phospho S472 antibody. Cotransfection of cmyc-PKBγ with HA-PKCζ enhanced S472 phosphorylation by ~8-fold (Figure 8A; Figure 8B, lane 2). Approximately 50% of the total cmyc-PKBγ protein was phosphorylated as determined by gel shift experiments (Figure 8E). Cotransfection with the truncated PKCζ 1−574 did not stimulate phosphorylation of PKBγ on S472 significantly above control values (Figure 8A; Figure 8B, lane 3).

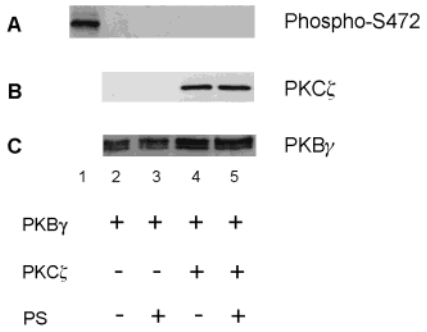

FIGURE 9: Pure PKCζ does not phosphorylate PKBγ on S472. Pure PKBγ protein was incubated with ATP, pure PKCζ, and phosphatidylserine as indicated. (A) Immunoblot with PKB phospho S472 antibody. The positive control was immunopurified endogenous PKB activated by insulin as above. (B) Immunoblot with PKCζ antibody. The filter was further probed with a PKBγ antibody to determine equal addition of the substrate to the kinase reaction. (C) The immunoblots are representative of two individual experiments.

Pure PKCζ Does Not Phosphorylate Pure PKBγ. It was important to ascertain whether PKCζ itself was responsible for phosphorylation of the PDK2 site of PKBγ. Pure PKCζ and PKBγ proteins (human recombinant, >95% pure) were used in in vitro kinase assays. Phosphatidylserine was required to cause full activation of the PKCζ protein. Control experiments using an exogenous substrate (MBP) showed that the PKCζ was highly active, with phosphatidylserine causing a 15-fold increase in the activity (data not shown). Immunoblotting with a PKB phospho S472 antibody showed that pure PKCζ was incapable of phosphorylating PKBγ on the S472 residue (Figure 9). Control experiments confirmed that the PKBγ was not already phosphorylated on S472 (Figure 9). Additionally, the source of the PKBγ was the same as that used to demonstrate S472 phosphorylation by the immunopurified PKCζ complex (see above). Moreover, PKCζ did not elicit any ^{32}P incorporation into PKBγ in experiments using [γ-^{32}P]ATP (data not shown). These results were supported by using staurosporine as an inhibitor (see below).

Inhibition of PDK1 Does Not Affect PDK2 Activity. PKCζ contains a PIF domain, and as such, it is plausible that any PDK1 that has immunoprecipitated with PKCζ may be acting as a PDK2 kinase. To test this, we employed the PDK1 inhibitor (PRK2-CT) which we recently identified (*17*). PRK2-CT is a C-terminal fragment of PRK2 that is naturally produced by caspase-3 cleavage during apoptosis. We previously found that PRK2-CT acts as a potent negative

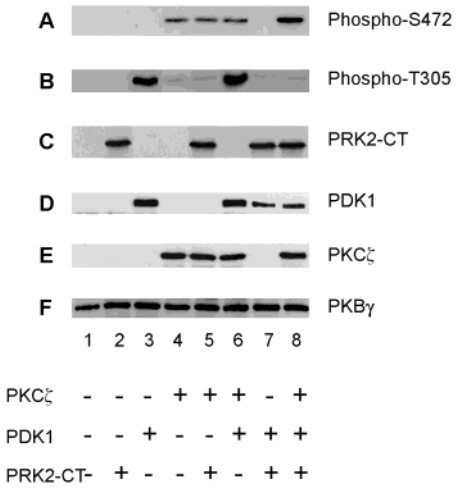

FIGURE 10: Inhibition of PDK1 does not affect PDK2 activity in immunoprecipitates of endogenous PKCζ. Cos-1 cells were serum-starved overnight and then incubated for 15 min in the presence of serum. Endogenous PKCζ together with cmyc-tagged PDK1 and HA-tagged PRK2-CT, from cells overexpressing these constructs, was isolated by immunoprecipitation from cell lysates (0.5 mg of protein). The immunopurified proteins were extensively washed and then incubated with ATP and pure PKBγ protein as substrate as indicated. (A) Immunoblot with PKB phospho S472 antibody. (B) Immunoblot with phospho-(Thr) PDK1 substrate antibody showing phosphorylation of PKB on T305. (C) Immunoblot with HA antibody showing the levels of the HA-tagged PRK2-CT construct. (D) Immunoblot with cmyc antibody showing the levels of cmyc-tagged PDK1. (E) Immunoblot with PKCζ antibody. (F) Immunoblot with PKBγ antibody. The immunoblots are representative of two individual experiments.

regulator of PDK1 autophosphorylation and PDK1 kinase activity against PDK1 substrates including PKCζ (*17*). In the present experiments, cos-1 cells were stimulated with serum, and endogenous PKCζ together with cmyc-tagged PDK1 and HA-tagged PRK2-CT, from cells overexpressing these constructs, was isolated by immunoprecipitation. After extensive washing of the immunoprecipitates, the kinase assay was conducted in the presence of sphingosine, a known PDK1 activator (*13*), and exogenous pure PKBγ. Control immunoblots confirmed that the amounts of PKBγ substrate, PKCζ, HA-PRK2-CT, and cmyc-PDK1 were similar in the various incubations (Figure 10C−F). As described above, immunoprecipitates containing endogenous PKCζ phosphorylated PKBγ on S472 (Figure 10A, lane 4). Immunoprecipitated cmyc-tagged PDK1 catalyzed marked phosphorylation of the T305 residue of PKBγ (Figure 10B, lane 3), demonstrating that the PDK1 was highly active under the assay conditions used. Immunopurified cmyc-PDK1 did not catalyze phosphorylation of PKBγ on S472 (Figure 10A, lane 3). When cmyc-PDK1 and endogenous PKCζ were present together, no difference in the level of S472 phosphorylation was observed compared to use of endogenous PKCζ alone (Figure 10A, lane 6 vs lane 4). Immunopurified HA-PRK2-CT did not elicit phosphorylation of either S472 (Figure 10A, lane 2) or T305 (Figure 10B, lane 2) of PKBγ. HA-PRK2-CT completely inhibited PDK1 activity as evidenced by the absence of T305 phosphorylation (Figure 10B, lane 7). HA-PRK2-CT did not inhibit the ability of immunopurified endogenous PKCζ to phosphorylate S472 of PKBγ (Figure 10A, lane 5 vs lane 4).

PDK2 Activity Present in Immunopurified Endogenous PKCζ Complex Is Staurosporine-Insensitive. We next tested

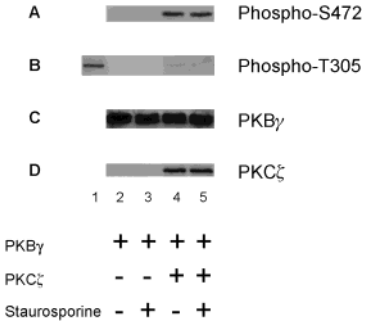

FIGURE 11: PDK2 activity in immunoprecipitates of endogenous PKCζ is staurosporine-insensitive. 3T3-L1 adipocytes were serum-starved for 2 days and then incubated for 5 min in the presence of 100 nM insulin. Endogenous PKCζ was immunoprecipitated from cell lysates (0.5 mg of protein), and the immunoprecipitates were extensively washed. The PKCζ immunoprecipitates were incubated with ATP, pure PKBγ protein as substrate, and staurosporine (5 μM) where indicated. (A) Immunoblot with PKB phospho S472 antibody. (B) Immunoblot with phospho-(Thr) PDK1 substrate antibody showing phosphorylation of PKB on T305. The positive control was immunopurified PKB activated by insulin as above. The filter was further probed with a PKBγ antibody to show equal amounts of substrate (C) and a PKCζ antibody to determine levels of endogenous PKCζ immunoprecipitation (D). The immunoblots are representative of two individual experiments.

whether PKCζ activity was required for activation of the PDK2 present in the PKCζ immunocomplex. Staurosporine was utilized because it is known to inhibit PKCζ. Endogenous PKCζ was immunoprecipitated from 3T3-L1 adipocytes which had been stimulated with insulin prior to extraction. Pure exogenous PKBγ was added to the kinase reaction. Phosphorylation of PKBγ on S472 by the immunopurified endogenous PKCζ complex was not inhibited by staurosporine (Figure 11A, lanes 4 and 5). Control experiments confirmed that PKCζ activity was completely inhibited under the conditions used (not shown). A recent report has also indicated that PDK2 activity is staurosporine-resistant (*18*).

DISCUSSION

PKBγ is one of the least studied PKB isoforms, and in this study, we have identified PKCζ as a protein which interacts with PKBγ. To our knowledge, this is the first example of a kinase that can regulate and/or interact with this isoform of PKB.

We found that PKBγ interacted with PKCζ via the N-terminal region of PKBγ both in yeast (not shown) and in mammalian cells. This encompassed the full pleckstrin homology (PH) domain of PKBγ and as such implies that PKBγ utilizes its PH domain to mediate protein−protein interactions with PKC, which is in agreement with previous findings with the truncated rat PKBγ isoform (*19*). Though PH domains bind to phospholipids such as PIP_3 and PIP_2 (*20*), there is ample evidence to suggest that they are also involved in regulating protein interactions in both a positive (*21*−*26*) and a negative fashion (*27, 28*). The PH domain of PKBα has been implicated in mediating the association with inosine 5′-monophosphate (*20*), PKCζ (*22*), the TCL1 oncogene (*25*), and possibly with itself (*21*). Considering that we have found that PKBγ also interacts with PKCζ, it is possible, therefore, that the PH domains of the PKB family members interact with the same proteins. Of fundamental

importance was to then determine the functional consequence of the association between PKBγ and PKCζ.

Once PKB is localized to the membrane in response to extracellular stimuli, it is phosphorylated on two residues that are critical for regulation of the activity: a threonine in the kinase domain and a serine within the C-terminal tail. Phosphorylation of the two sites is required to fully activate PKB. The kinase responsible for the phosphorylation of the threonine has been known for several years, this being the PDK1 kinase (*2*). The identification of the kinase that phosphorylates the serine, PDK2, has represented a major challenge for a number of years. One suggestion has been that PDK2 is attributable to PDK1. Thus, PDK1 has been suggested to interact with a PIF domain on another protein that converts the PDK1 activity into a PDK2 activity (*3, 4*). In a second proposal, the actual existence of a distinct PDK2 has been challenged, and it has been argued that serine phosphorylation occurs by virtue of PKB autophosphorylation (*5*). Specific PDK2 kinases have also been proposed, these being integrin-linked kinase (*6, 42*) and MAPKAP kinase-2 (*7*). However, other studies have cast strong doubt on all of these mechanisms (*8*−*10, 18, 29, 30*). For example, MAPKAP-K2 is activated in vivo by stress and other stimuli that fail to activate PKB while insulin fails to activate MAPKAP-K2 (*7, 8*). Additionally, the use of integrin-linked kinase mutants suggests that integrin-linked kinase does not possess an intrinsic S473 kinase activity (*10*). Moreover, integrin-linked kinase is not a typical protein kinase, and as such is expected to have low kinase activity. Indeed, in a very recent study, Hemmings and co-workers were unable to detect any significant kinase activity of overexpressed or endogenous integrin-linked kinase by autophosphorylation or on myelin basic protein (*18*).

Herein we describe a model system that shows high PDK2 activity in vitro. We used the model system to characterize the PDK2 activity and to determine whether it was attributable to PDK1, to autophosphorylation by PKB, or to a distinct kinase. The basis of the model system was that immunopurified PKCζ complex phosphorylated S472 of PKBγ in vitro whether the PKCζ was from endogenous (3T3-L1 adipocytes and cos-1 cells) or overexpressed (cos-1) sources. The phosphorylation of PKBγ on S472 achieved was of a high level as evidenced by gel mobility shift assays which showed that at least 50% of the PKBγ was phosphorylated. This was of sufficiently high efficiency to be expected if a physiologically relevant PDK2 activity was present in the immune complex. Additionally, endogenous PKBγ was shown to associate with endogenous PKCζ both in cos-1 cells and in 3T3-L1 adipocytes, demonstrating that the interaction between PKBγ and PKCζ occurs physiologically. Using the 3T3-L1 adipocyte system, the PDK2 activity in the immunopurified PKCζ complex was shown to be stimulated 18-fold by prior treatment of the cells with insulin (quantitation of results in Figure 6). However, pure PKCζ was found not to possess PDK2 activity. Moreover, inhibition of PKCζ activity in the model system with staurosporine did not inhibit the PDK2 activity. The latter result also shows that PKCζ activity was not required for activation of the PDK2 present in the PKCζ immunocomplex, consistent with results in embryonic stem cells (*29*). Our results also support another study which found that PDK2 activity was not inhibited by staurosporine in vivo in HEK293 cells (*18*). This is again consistent with the physiological PDK2 being present in our model system.

Using the PDK1 inhibitor PRK2-CT, we showed that the PDK2 activity was not attributable to PDK1. This result supports studies with PDK1$^{-/-}$ embryonic stem cells which retain high PDK2 activity (*29, 32*). The suggestion that phosphorylation of the hydrophobic motif of PKBα occurs by autophosphorylation requires PKBα to be catalytically competent and phosphorylated on the threonine site in the activation loop of the kinase domain (*5*). Similar experiments, however, have reached an opposing conclusion (*8*). In our experiments, the pure PKBγ used as substrate was not phosphorylated on T305. To exclude the possibility that endogenous active PKB in the PKCζ immunoprecipitates was catalyzing S472 phosphorylation in the substrate via a trans reaction, endogenous PKB was removed via antisense knockout prior to extraction and immunopurification of the PKCζ. This did not alter the ability of the immunopurified PKCζ complex to phosphorylate S472. Thus, autophosphorylation was ruled out as an explanation, and consequently PDK2 activity must be due to a definite kinase. Taken together, these results indicate that PKCζ functions as an adapter that delivers PDK2 activity to a required location. PKCζ is ideally suited to this function because it is not phosphorylated at its equivalent PDK2 site and instead possesses an aspartate residue.

In support of our results, Ziegler et al. showed likewise that a protein complex containing PKCζ is capable of phosphorylating S657 of PKCα and S662 of PKCδ, the corresponding positions to S472 of PKBγ (*16*), both in vitro and in vivo. Analogous to our study, purified PKCζ was unable to catalyze this reaction. Taken together, this suggests that a PKCζ-associated kinase is responsible for phosphorylation of the PDK2 site of various AGC kinases. Additionally, PKCζ is known to bind other AGC kinases in addition to PKB and the PKC isoforms, notably p70 S6K (*41*), so that the mechanism of delivery of PDK2 may well be a general mechanism. Moreover, our results have been obtained with both mouse and human PKCζ in two different cell lines indicating that the mechanism proposed is of wide-ranging importance.

Several reports have previously considered whether PKCζ had any identifiable effect on PKBα activity (*22, 32*). Doornbos et al. showed that in response to PDGF, PKCζ down-regulated PKBα activity (*22*), an effect they described as PI3K-independent. However, in an identical experiment, another study has contradicted these findings (*32*). It is indeed exceedingly unlikely that PKCζ could have a negative role on PKB phosphorylation when cells are stimulated by growth factors; otherwise PKB could conceivably exert no downstream effect. The data presented in Doornbos et al. are commensurate with overexpressed PKCζ sequestering out PKB and hence sterically blocking PKB activity.

It has been proposed that ceramide (*33*−*35*) induces inhibition of PKB through PKCζ (*32*). This inhibition was associated with diminished S472 phosphorylation of PKB. In conjunction with our results, this raises the possibility that the mechanism of action of ceramide is to displace PDK2 from the PKB/PKCζ/PDK2 complex. Many reports have described a positive role for PKCζ in signaling (*3, 36*−*38*). However, recently PKCζ has been suggested to be a bifunctional modulator (*39*) and may mediate very disparate

effects depending upon the stimulus. Indeed, our data indicate that one of the chief roles of PKCζ is as an adaptor protein. Different stimuli may lead to different proteins associating with PKCζ giving rise to diverse effects.

PKB and PKCζ have both been proposed to be involved in mediating a number of critical responses to insulin such as the inactivation of glycogen synthase kinase-3 and stimulation of the uptake of glucose. Our observations show that PKB and PKCζ come together to form a complex containing PDK2. Formation of such an active signaling complex may be crucial in ensuring that all the required sites in substrates such as glycogen synthase kinase-3 (*40*) become phosphorylated.

In summary, we have identified a model system that shows high PDK2 activity in vitro. The PDK2 activity was not due to PDK1 or PKB autophosphorylation and thus the results show the existence of a distinct PDK2 activity. The PDK2 activity was staurosporine-insensitive and physically associated with PKCζ. PKCζ thus appeared to function as an adaptor protein and by binding PKBγ to deliver PDK2 to one of it substrates. Whether other PKC isoforms can substitute in the role ascribed to PKCζ remains to be determined. The model system provides a basis for the further characterization of the PDK2 activity and provides a novel basis for purifying PDK2.

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