Activation of protein kinase B β and γ isoforms by insulin *in vivo* and by 3-phosphoinositide-dependent protein kinase-1 *in vitro*: comparison with protein kinase B α

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The regulatory and catalytic properties of the three mammalian isoforms of protein kinase B (PKB) have been compared. All three isoforms (PKBα, PKBβ and PKBγ) were phosphorylated at similar rates and activated to similar extents by 3-phosphoinositide-dependent protein kinase-1 (PDK1). Phosphorylation and activation of each enzyme required the presence of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, as well as PDK1. The activation of PKBβ and PKBγ by PDK1 was accompanied by the phosphorylation of the residues equivalent to Thr^{308} in PKBα, namely Thr^{309} (PKBβ) and Thr^{305} (PKBγ). PKBγ which had been activated by PDK1 possessed a substrate specificity identical with that of PKBα and PKBβ towards a range of peptides. The activation of PKBγ and its phosphorylation at Thr^{305} was triggered by insulin-like growth factor-1 in 293 cells. Stimulation of rat adipocytes or rat hepatocytes with insulin induced the activation of PKBα and PKBβ with similar kinetics. After stimulation of adipocytes, the activity of PKBβ was twice that of PKBα, but in hepatocytes PKBα activity was four-fold higher than PKBβ. Insulin induced the activation of PKBα in rat skeletal muscle *in vivo*, with little activation of PKBβ. Insulin did not induce PKBγ activity in adipocytes, hepatocytes or skeletal muscle, but PKBγ was the major isoform activated by insulin in rat L6 myotubes (a skeletal-muscle cell line).

INTRODUCTION

Protein kinase B (PKB) [1] is the cellular homologue of a viral oncoprotein v-Akt that causes leukaemia in mice [2], and PKB isoforms are overexpressed in a significant percentage of human cancers. For example, PKBβ is overexpressed in about 15% of ovarian cancers [3] and 12% of pancreatic cancers [4], whereas PKBα is overexpressed in about 3% of breast cancers [5]. The reason why PKB is oncogenic in high copy number has recently become apparent with the discovery that it provides a survival signal that protects cells from apoptosis induced by a number of agents, including UV radiation [6], withdrawal of the survival factor insulin-like growth factor-1 (IGF1) from neuronal cells [7], withdrawal of serum factors while c-*myc* is expressed artificially [8] and by detachment of cells from the extracellular matrix [9]. One of the cellular targets that PKB may phosphorylate to protect cells from apoptosis is BAD [10,11]. This protein, in its dephosphorylated form, interacts with the Bcl family member Bcl-x_L and induces apoptosis of some cells [12]. However, after phosphorylation at Ser^{136} by PKB it dissociates from Bcl-x_L, and apoptosis is prevented [10]. PKB may also induce the expression of anti-apoptotic proteins such as Bcl-2 and c-Myc [13]. High PKB expression and activity may therefore allow tumour cells to flourish in media containing low concentrations of extracellular survival signals. For this reason there is increasing interest in developing drugs that inhibit particular isoforms of PKB, such as PKBβ, because such drugs may cause tumour cells that overexpress this isoform to undergo apoptosis.

A potential problem in using specific inhibitors of PKB isoforms to treat cancer is that the PKBα isoform is known to be activated by insulin [14,15], and there is increasing evidence that this protein kinase plays a critical role in mediating many of the actions of this hormone. For example, PKB appears to mediate the insulin-induced activation of the cardiac isoform of 6-phosphofructo-2-kinase which underlies the stimulation of cardiac-muscle glycolysis by this hormone [16,17]. PKB also appears to mediate the insulin-induced inhibition of glycogen synthase kinase 3 ('GSK3'); this is thought to contribute to the dephosphorylation (activation) of glycogen synthase [15] and protein initiation factor eIF2B [18] and hence to the stimulation of glycogen synthesis and protein synthesis by insulin (reviewed in [19]). In transfection-based experiments, PKB also mimics other actions of insulin, such as the enhancement of glucose uptake in adipocytes [20] and L6 myotubes [21] that results from the translocation of glucose transporter GLUT4 from an intracellular compartment to the plasma membrane, and the increased rate of amino acid uptake into muscle cells [21]. For these reasons there is a concern that drugs which inhibit particular PKB isoforms might cause diabetes.

There are three mammalian isoforms of PKB, namely PKBα [1], PKBβ [3] and PKBγ [22], but nearly all studies of the regulation and specificity of this enzyme have so far focused on the PKBα isoform. The activation of PKBα by insulin or IGF1 results from its phosphorylation at two residues, Thr^{308} and Ser^{473}, which are phosphorylated by distinct enzymes [23]. The mechanism of activation involves first, the insulin/IGF1-induced activation of PtdIns 3-kinase, which produces the second messenger PtdIns(3,4,5)P_3 at the plasma membrane [24]. This is followed by the interaction of PKBα [via its pleckstrin homology (PH) domain] with PtdIns(3,4,5)P_3, leading to its recruitment from the cytosol to the membrane [25]. The interaction of PtdIns(3,4,5)P_3 with PKBα also alters its conformation in such a way that Thr^{308} can be phosphorylated by the membrane-associated 3-phosphoinositide-dependent protein kinase-1

Abbreviations used: PKB, protein kinase B; PDK1, 3-phosphoinositide-dependent protein kinase-1; GST, glutathione S-transferase; HA, haemagglutinin antigen; IGF1, insulin-like growth factor-1; A.T.C.C., American Tissue Culture Collection; MMLV, Maloney-murine-leukaemia virus; CH-, carboxyhexyl; PH, pleckstrin homology.

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(PDK1) [26–29]. PDK1 contains a PH domain capable of binding inositol phospholipids, such as PtdIns(3,4,5)P_3, and the interaction of PDK1 with 3-phosphoinositides may contribute to the activation of PKBα *in vivo* [27,29]. The insulin/IGF1-induced phosphorylation of Ser^{473}, like the phosphorylation of Thr^{308}, is prevented by inhibitors of PtdIns 3-kinase [23], suggesting that the phosphorylation of this residue may occur by an analogous mechanism. However, the Ser^{473} kinase has not yet been characterized.

Recently PDK1 has been found to phosphorylate p70 S6 kinase at the residue in the kinase catalytic domain equivalent to Thr^{308} of PKBα, and this resulted in activation of p70 S6 kinase [30,31]. Other protein kinases in the PKB/p70 S6 kinase subfamily have similar sequences in this region, suggesting PDK1 may have multiple functions *in vivo*.

PKBβ and PKBγ are the products of different genes that are 81 and 83 % identical in amino acid sequences with PKBα. Like PKBα, they possess an N-terminal PH domain and a residue equivalent to Thr^{308} (Thr^{309} in PKBβ; Thr^{305} in PKBγ). Ser^{473} (and the sequence surrounding it) is also conserved in PKBβ, and the activation of PKBβ in response to insulin/IGF1 is accompanied by the phosphorylation of Thr^{309} and Ser^{474} [32]. However, intriguingly, PKBγ lacks a residue equivalent to Ser^{473}, because it is truncated by 23 residues at the C-terminus. In the present study we have investigated the regulatory and catalytic properties of PKBβ and PKBγ, and used specific antisera to the isoforms of PKB to study the activation of PKBα, PKBβ and PKBγ in the major insulin-responsive tissues.

MATERIALS AND METHODS

Materials

Human PDK1 was expressed as a glutathione S-transferase (GST) fusion protein in 293 cells [27]. Rapamycin and PD 98059 were purchased from Calbiochem (La Jolla, CA, U.S.A.), collagenase (type 1) from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), XL 400 medium from JRH Biosciences (St. Albans, Herts., U.K.) and Moloney-murine-leukaemia-virus (MMLV) reverse transcriptase from Promega (Southampton, U.K.). All inositol phospholipids were from the sources described previously [26]. The 20-residue peptide inhibitor of cAMP-dependent protein kinase (PKI) and all other peptides were synthesized at the Department of MRC Protein Phosphorylation Unit, University of Dundee, by Mr. F. B. Caudwell. Sources of other materials are given in [23].

Buffers

Buffer A was 50 mM Tris/HCl (pH 7.5)/1 mM EGTA/1 mM EDTA/1 % (w/w) Triton-X 100/1 mM sodium orthovanadate/50 mM NaF/5 mM sodium pyrophosphate/0.27 M sucrose/1 μM microcystin-LR/0.1 % (v/v) β-mercaptoethanol/'complete' proteinase inhibitor cocktail (one tablet/50 ml).

Buffer B was 50 mM Tris/acetate (pH 7.5)/0.27 M sucrose/0.1 mM EDTA/0.1 mM EGTA/10 mM β-mercaptoethanol/400 μM PMSF/400 μM benzamidine/5 μg/ml leupeptin/1 % (w/w) Triton-X 100.

Buffer C was 50 mM Tris/acetate (pH 7.5)/0.1 mM EDTA/0.1 mM EGTA/10 mM β-mercaptoethanol/200 μM PMSF/200 μM benzamidine.

Cloning, expression and purification of PKB isoforms

A full-length human PKBα construct was isolated from a human skeletal-muscle cDNA library (Clontech, Palo Alto, CA, U.S.A.) using the oligonucleotides 5′-CGGGATCCATGAGCGACGTGGCTATTGTG-3′ and 5′-GCTCAAAAAGCTTCTCATGGTC-3′ to subject the N-terminal portion of the PKBα gene to PCR, and the oligonucleotides 5′-GACCATGAGAAGCTTTTTGAGC-3′ and 5′-CGGGTACCGTTAAGCGTCGAAAAGGTCAA-3′ to subject the C-terminal portion to PCR. The two PCR products were ligated to generate a full-length PKBα gene, which was subcloned into pBluescript SK and sequenced. A haemagglutin-antigen (HA) epitope tag was incorporated into the N-terminus of the PKBα gene by PCR and the resulting construct was subcloned into the pEBG-2T GST mammalian expression vector [33]. Kidney 293 cells were transfected with this construct and the expressed GST-PKBα was purified by glutathione–Sepharose affinity chromatography [26].

A full-length human PKBβ with a C-terminal His_6 tag was isolated from the clone HCEPD60 [American Tissue Culture Collection (A.T.C.C.) catalogue number 101317] by PCR using the oligonucleotide 5′-AGGATCCACCATGAATGAGGTGTCTGTCATC-3′ and 5′-TCTAGAATTCAATGGTGATGGTGATGGTGCTCGCGGATGCTGGCC-3′. The 3′ end of the sequence was then manipulated to produce a PKBβ sequence corresponding to that described in [3] followed immediately by a His_6 tag (i.e. …AGC/ATC/CGC/GAG/CAC/CAT/CAC/CAT/CAC/CAT/TGA/TTCTAGAAAGCCGAATTC coding for …SIREHHHHHH). This PKBβ-His_6 sequence was cloned as a *Bam*H1/*Eco*R1 fragment into the *BamH*1/*Eco*R1 sites of pFASTBAC (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) for generation of recombinant baculovirus. Human His-PKBβ was expressed as follows. Sf9 cells (A.T.C.C., CRL 1711) were cultured at 27 °C in XL 400 medium supplemented with 5 % foetal-bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Monolayers of Sf9 cells (4×10^8 cells) were infected with recombinant baculovirus encoding PKBβ at a multiplicity of infection equivalent to 5. At 80 h after infection, monolayers were washed with ice-cold 10.5 mM KH_2PO_4/1 mM Na_2HPO_4/0.14 M NaCl/40 mM KCl, pH 6.2, and lysed in Buffer A. The lysate was clarified by centrifugation (25000 ***g***, 4 °C, 30 min) and the supernatant (200 mg of protein) applied to a 3 ml column of Ni^{2+}/nitrilotriacetate–agarose equilibrated in Buffer C. After washing with Buffer C containing 20 mM imidazole and 0.5 M NaCl, PKBβ was eluted with Buffer C containing 150 mM imidazole and 0.5 M NaCl. Fractions (2 ml each) were collected, and the protein-containing fractions were pooled and concentrated to 1–2 ml by dialysis against Buffer C containing 50 % (v/v) glycerol.

A full-length clone encoding human PKBβ, corresponding to the shorter variant of PKBβ, published by Cheng et al. [3], was prepared as follows. The N-terminal portion of the gene from the initiating methionine until the unique *Sma*1 site (nt 928) was amplified from a human muscle cDNA library purchased from Clontech, using the following primers: 5′-CG GGATCC ATG AATGAGGTGTCTGTCATC-3′ and 5′-CACGCTCCCGGGACAGGTG-3′. The resulting PCR product was subcloned into the *Bam*H1/*Sma*1 site of pBluescript. The 3′ end of the PKBβ gene was completed by ligating the appropriate fragments of PKBβ obtained from the expressed sequence tags AA055543 and AA459943 (Image Consortium, St. Louis, MO, U.S.A.). A full-length clone of PKBβ was then constructed with an HA epitope at the N-terminus using standard cloning techniques, and the DNA sequence was verified. This construct was subcloned into the *Bam*H1/*Kpn*1 site of pEBG2T in order to express PKBβ as a N-terminal GST fusion protein in 293 cells (GST–PKBβ).

Human PKBγ was amplified by PCR on the basis of the published sequence [22] from total RNA isolated from L6 myotubes. cDNA was synthesized in a 25 μl reaction mixture,

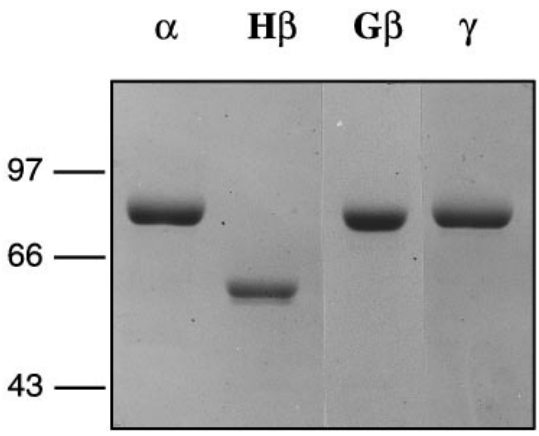

Figure 1 Purification of GST–PKBα, His–PKBβ, GST–PKBβ and GST–PKBγ

A 3 μg portion (each) of purified GST–PKBα, His–PKBβ, GST–PKBβ and GST–PKBγ was electrophoresed on a SDS/10%-polyacrylamide gel and stained with Coomassie Blue. Lane 1, GST–PKBα (α); lane 2, His–PKBβ (Hβ); lane 3, GST–PKBβ (Gβ); lane 4, GST–PKBγ (γ). The positions of the molecular-mass markers glycogen phosphorylase (97 kDa), BSA (66 kDa) and ovalbumin (43 kDa) are indicated on the left.

using 1 μg of total L6 RNA as a template and MMLV reverse transcriptase according to the manufacturer's protocol. A full-length PKBγ clone was isolated by PCR, and the PCR reaction was then set up using the following oligonucleotides: 5′-GGACTAGTGCCACCATGTACCCATACGATGTGCCA-GATTACGCCAGCGATGTTACCATCGTTAAAG-3′ and 5′-GGATCGATTATAGTGGACACTTTTCAGGTGGTGTT-ATTG-3′. The resulting PCR product possesses an *Spe*1 site followed by an HA tag prior to the PKBγ coding sequence. This was subcloned into the pCR2.1-Topo vector (InVitrogen, San Diego, CA, U.S.A.) and sequenced. PKBγ was then subcloned as an *Spe*1 fragment into the eukaryotic pEBG-2T vector [33] to generate a construct for expression of a GST–PKBγ fusion protein. PKBγ was also subcloned as an *Eco*R1 fragment into pCMV5 vector [34] to generate a construct for expression of HA–PKBγ. GST–PKBβ and GST–PKBγ were expressed in 293 cells and purified as described for GST–PKBα [26].

Activation and phosphorylation of PKB isoforms by PDK1 *in vitro*

This was carried out as described previously for GST–PKBα [26]. A reaction was set up containing 0.2 mg of either His–PKBβ or GST–PKBγ, in 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/0.1 % (v/v) β-mercaptoethanol/2.5 μM PKI/1 μM microcystin-LR/10 mM magnesium acetate/100 μM ATP/100 μM phosphatidylcholine/100 μM phosphatidylserine in the presence of 10 μM PtdIns(3,4,5)P_3 in a total volume of 1.8 ml. The reaction was initiated with 0.2 ml of PDK1 (160 units/ml). After incubation for 1 h at 30 °C, EDTA was added to a final concentration of 20 mM to stop the reaction. The activated enzymes were snap-frozen in liquid N_2 and stored in aliquots at −80 °C. One unit of PDK1 activity is defined as that amount required to increase the activity of GST–PKBα by 1 unit in 1 min in the presence of PtdIns(3,4,5)P_3 [26].

Mapping the PDK1 phosphorylation site on His–PKBβ and GST–PKBγ

PKBβ and PKBγ (both at a concentration of 2 μM) were phosphorylated by PDK1 (65 units/ml) in the presence of the D-

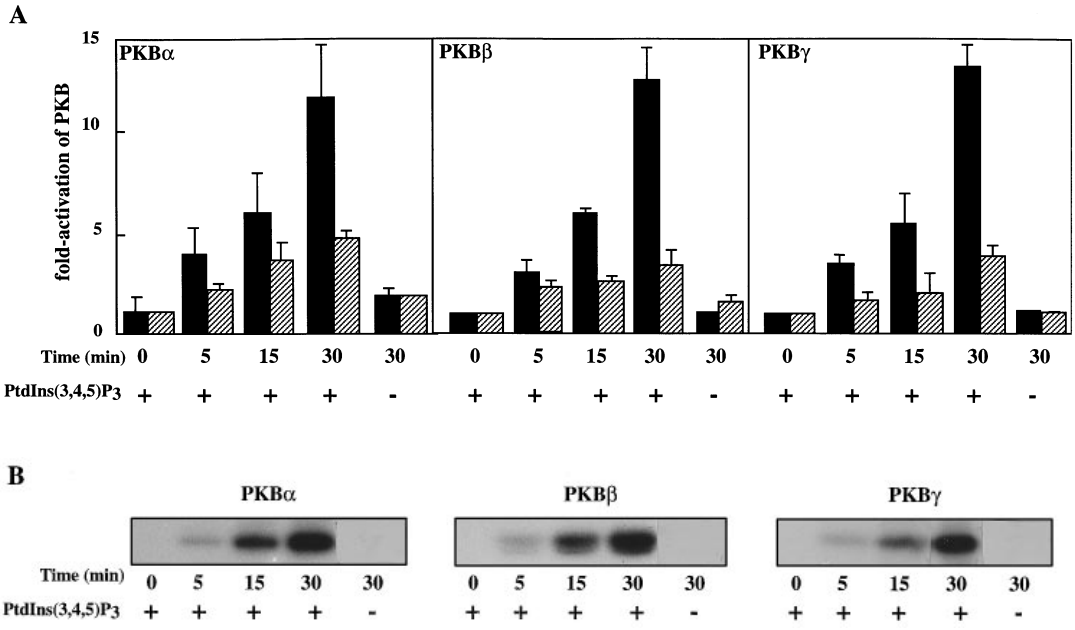

Figure 2 Activation and phosphorylation of the three PKB isoforms by PDK1

(**A**) GST–PKBα, His–PKBβ and GST–PKBγ were incubated with PDK1 in the presence (+) or absence (−) of *sn*-1-stearoyl-2-arachidonyl D-PtdIns(3,4,5)P_3 (solid bars) or *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P_3 (hatched bars) and MgATP. (**B**) Same as (**A**), except that Mg[γ-^{32}P]ATP (500 c.p.m./pmol) was used in the presence of *sn*-1-stearoyl-2-arachidonyl D-PtdIns(3,4,5)P_3. At the times indicated, the reactions were stopped by the addition of Triton X-100 to a final concentration of 1.25% (w/w) and PKB assayed (**A**), or stopped by the addition of 1% (w/w) SDS followed by SDS/PAGE and autoradiography (**B**). The data in (**A**) are presented as fold-activation of PKB relative to PKB activity in the absence of PDK1 (0.65 ± 0.07 unit/mg for PKBα; 0.15 ± 0.03 unit/mg for PKBβ; 1.67 ± 0.23 units/mg for PKBγ) and are given as means ± S.E.M. for three determinations.

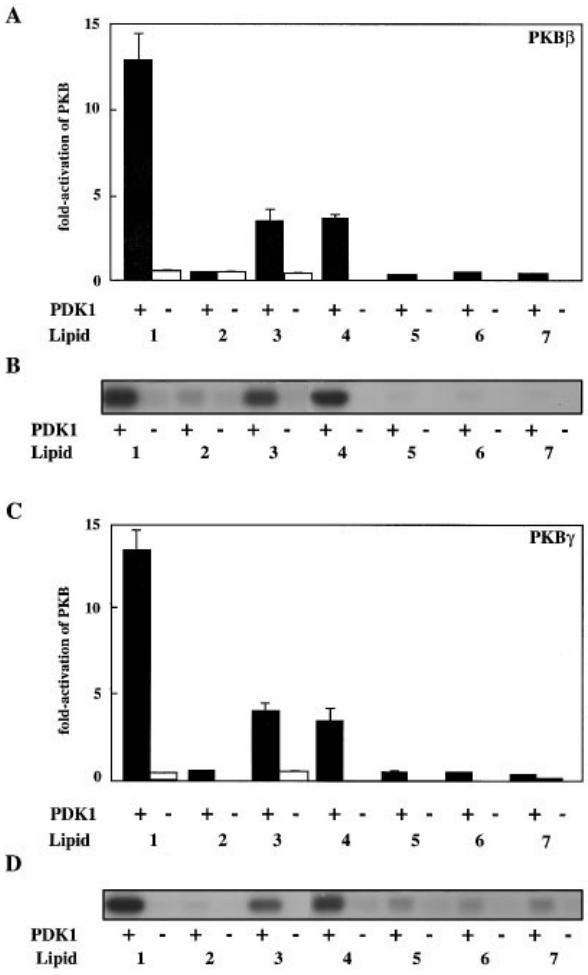

Figure 3 Activation and phosphorylation of PKBβ and PKBγ by PDK1 requires PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2

His–PKBβ (**A**,**B**) and GST–PKBγ (**C**,**D**) were incubated for 30 min at 30 °C in the presence (closed bars) or absence (open bars) of PDK1, Mg[γ-^{32}P]ATP (500 c.p.m./pmol) and phospholipid vesicles containing 100 μM PtdCho, 100 μM PtdSer and various PtdIns lipids (numbered 1–7, see below) all at a concentration of 10 μM. In (**A**) and (**C**) the reactions were terminated by adding 1.25% (w/w) Triton X-100 and PKB activity was assayed. The results are presented as the increase in PKBβ or PKBγ activity relative to a control incubation in which PDK1 and lipid were omitted (0.15 $\pm$ 0.03 unit/mg for PKBβ, 1.67 $\pm$ 0.23 units/mg for PKBγ) and are given as means $\pm$ S.E.M. for four (**A**) or three (**C**) separate experiments. All assays were performed in triplicate. In (**B**) and (**D**) the reactions were terminated by adding 1% SDS and the samples were then subjected to SDS/PAGE, stained with Coomassie Blue, and the phosphorylation of PKB assessed by autoradiography. Lane 1, *sn*-1-stearoyl-2-arachidonyl D-PtdIns(3,4,5)P_3; lane 2, *sn*-1-stearoyl-2-arachidonyl L-PtdIns(3,4,5)P_3; lane 3, *sn*-1,2-dipalmitoyl D-PtdIns(3,4,5)P_3; lane 4, *sn*-1,2-dipalmitoyl D-PtdIns(3,4)P_2; lane 5, PtdIns(4,5)P_2 (purified from Folch brain fraction); lane 6, *sn*-1,2-dipalmitoyl-D-PtdIns3*P*; lane 7, no PtdIns lipid added.

enantiomer of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P_3 and [γ-^{32}P]ATP (500 c.p.m./pmol) [26]. The reactions were stopped after 1 h by adding 1% (w/w) SDS and 1% (v/v) β-mercaptoethanol. After incubation at 95 °C for 5 min, the samples were alkylated with 4-vinylpyridine, electrophoresed, eluted from the gel, digested with trypsin [23] and chromatographed on a Vydac C_{18} column. Phosphoamino acid analysis was carried out as described in [35].

Transfection of HA–PKBγ into 293 cells and immunoprecipitation

293 cells were cultured on 10-cm-diameter dishes and transfected with the pCMV5 HA-PKBγ construct using a modified calcium phosphate method [23]. At 24 h after transfection, the cells were deprived of serum for 16 h, then incubated with or without 100 nM wortmannin for 10 min, followed by stimulation with or without 50 ng/ml IGF-1. The cells were lysed in 1 ml of ice-cold Buffer A, centrifuged at 20000 ***g*** for 5 min, and HA–PKBγ was immunoprecipitated from aliquots of lysate (containing 25 μg of protein) using 2 μg of anti-HA antibody coupled to 5 μl of Protein G–Sepharose. Immunoprecipitates were incubated and washed as described in [23].

Assay of PKBβ and PKBγ

Activated His–PKBβ and GST–PKBγ were assayed routinely using the peptide GRPRTSSFAEG (30 μM), termed 'Crosstide', as substrate [15]. Michaelis constants (K_m) and V_{max} values were determined from double-reciprocal plots of $1/v$ against $1/s$, where v is the initial rate of phosphorylation and s is the substrate concentration. The standard errors for all reported kinetic constants were within $< \pm 20\%$.

Preparation of isoform-specific PKB antibodies

PKBα-specific antibodies were raised in sheep against the N-terminal 176 residues of PKBα (which include the PH domain) expressed as a GST fusion protein in *Escherichia coli*. This construct was prepared by Dr. A. Casamayor (MRC Protein Phosphorylation Unit, University of Dundee). The antiserum was first passed through a GST–carboxyhexyl (CH)-Sepharose column to remove anti-GST antibodies, and the flowthrough was then affinity-purified by chromatography on CH-Sepharose to which GST–PKBα (1–176) had been attached covalently. This antibody is termed the PKBα PH-domain antibody. To prevent binding of the PKBα PH-domain antibody to GST–PKBγ (via any anti-GST antibodies still present), this antibody was incubated at ambient temperature for 1 h with GST (20 μg of GST/μg of antibody) before immunoprecipitation and immunoblotting.

Antibodies specific for PKBβ or PKBγ were raised in sheep against the peptide RYDSLGSLELDQRTH (corresponding to residues 455–469 of PKBβ) and RMNCSPTSQIDNI (corresponding to residues 116–128 of PKBγ), and affinity-purified on CH-Sepharose columns to which the appropriate peptide was coupled covalently. All three antibodies are available from UBI (Lake Placid, NY, U.S.A.).

Preparation of cell and tissue extracts

Rat L6 myotubes [36] and primary rat adipocytes [37] were cultured, stimulated and lysed as described previously. Primary rat hepatocytes were also cultured, incubated and lysed as described previously [38,39], and stimulations were carried out by the addition of various concentrations of insulin directly into the incubation following a 10 min preincubation in the presence or absence of 100 nM wortmannin. Rats which had been deprived of food for 16 h were anaesthetized and injected with insulin (1 unit/kg). At various times, hind-limbs were freeze-clamped, and the animals were immediately killed. Hind-limbs were then powdered [37] and homogenized in Buffer A. All cell and tissue extracts were centrifuged at 20000 ***g*** for 5 min, and the supernatants snap-frozen in liquid N_2 and stored in aliquots at -80 °C. Protein concentrations were determined by using the method of Bradford [40].

Immunoprecipitation of endogenous PKB isoforms from cell lysates

The amount of cell lysate used for each immunoprecipitation was: L6 myotubes (100 μg of protein), adipocytes (150 μg of protein), skeletal muscle (500 μg of protein), hepatocytes (2.3 mg of protein). The lysates were incubated at 4 °C for 1 h on a shaking platform with 5 μg of isoform-specific PKBα (PH-domain antibody), PKBβ or PKBγ antibodies coupled to 5 μl of Protein G–Sepharose. The immunoprecipitates were washed and assayed for PKB activity using Crosstide as substrate [15]. One unit of activity was that amount which catalysed the phosphorylation of 1 nmol of substrate in 1 min.

RESULTS

Cloning and expression of protein kinase isoforms

Human PKBα, PKBβ and rat PKBγ were cloned, expressed as GST fusion proteins in human embryonic kidney 293 cells, and purified on glutathione–Sepharose. Human PKBβ was also expressed in Sf9 cells with six histidine residues at its C-terminus (His–PKBβ) and purified by chromatography on nickel–agarose. All three isoforms were more than 80% pure as judged by SDS/PAGE (Figure 1). A total of 1.4 mg of His–PKBβ was obtained from one 15 cm-diameter dish of Sf9 cells, and 40 (10 cm diameter) dishes of 293 cells yielded 2 mg of GST–PKBα or GST–PKBγ and 1 mg of GST–PKBβ. The nucleotide sequence of human PKBα differed at one position from that previously reported [1] which changed Ser[478] to a Gly residue, and the sequence of rat PKBγ differed at two positions from the published sequence [22], changing Asn[10] to Gly, and Pro[396] to Ala. The equivalent residues in PKBα and PKBβ are also Gly and Ala respectively.

Phosphorylation and activation of PKB isoforms by PDK1

PDK1 phosphorylated and activated all three PKB isoforms at similar rates *in vitro*, and this was dependent on the presence of PtdIns(3,4,5)P_3. The extent of phosphorylation reached a plateau at about 0.5 mol of phosphate/mol of protein and each isoform was activated >10-fold (Figure 2). The maximal specific activities towards Crosstide were 21.4 ± 0.2 units/mg (PKBα), 4.1 ± 0.05 units/mg (His–PKBβ), 3.75 ± 0.001 units/mg (GST–PKBβ) and 64.6 ± 6.3 units/mg (PKBγ). Possible reasons for the low specific activity of PKBβ and the high specific activity of GST-PKBγ are considered further in the Discussion.

Lipid requirement for the phosphorylation and activation of PKBβ and PKBγ by PDK1

The ability of a panel of PtdIns derivatives to support the PDK1-catalysed phosphorylation and activation of His–PKBβ and GST–PKBγ when presented in a lipid-vesicle background containing phosphatidylcholine and phosphatidylserine was investigated. Similar to findings for PKBα [26,27], His–PKBβ and GST–PKBγ were only phosphorylated and activated by PDK1 in the presence of the D-enantiomers of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 (Figure 3). Both PtdIns(3,4,5)P_3 derivatives and one PtdIns(3,4)P_2 derivative were able to support the activation

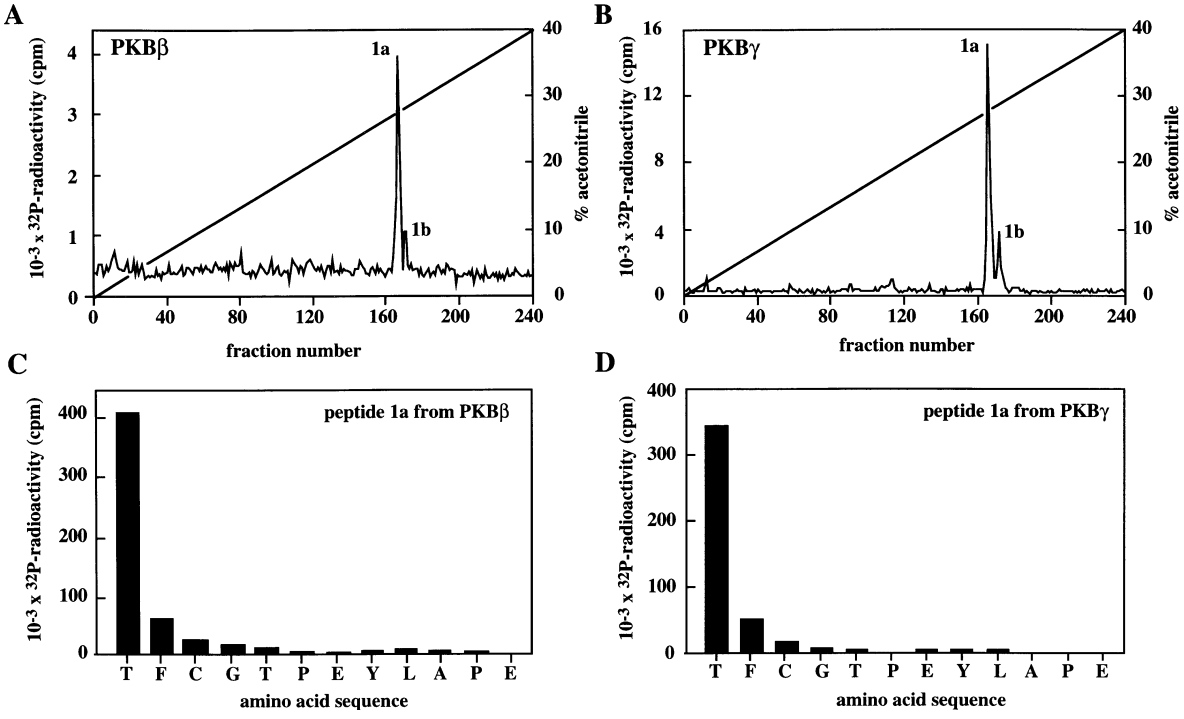

Figure 4 ***In vitro*** **labelling of PKBβ and PKBγ with PDK1**

His–PKBβ (**A**) and GST–PKBγ (**B**) phosphorylated by incubation with PDK1 (65 units/ml) and Mg[γ-^{32}P]ATP (500 c.p.m./pmol) for 1 h were alkylated, digested with trypsin then applied to a C_{18} column equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min and 0.4 ml fractions were collected. In (**C**) and (**D**), aliquots of peptide 1a from (**A**) and (**B**) respectively were coupled to a Sequelon arylamine membrane and analysed by solid-phase sequencing on an Applied Biosystems 470A sequencer [47] to measure ^{32}P radioactivity released after each cycle of Edman degradation. Further aliquots were analysed by conventional gas-phase sequencing analysis to identify the amino acid sequences.

Table 1 Substrate specificities of PKBβ and PKBγ

The phosphorylated residue is shown in **bold** type and the altered residues are underlined. The activities are presented relative to that measured at 30 μM Crosstide for both His–PKBβ (5.55 $\pm$ 1.03 units/mg) and GST–PKBγ (8.13 $\pm$ 0.18 units/mg).

	Relative PKB activity			
	PKBβ		PKBγ	
Peptide	5 μM	30 μM	5 μM	30 μM
1. GRPRTS**S**FAEG	49	100	41	100
2. RPRTS**S**F	31	78	40	95
3. KPRTS**S**F	0.2	0.7	3.4	5.4
4. RPKTS**S**F	0	0.4	1.8	2.5
5. RPRTS**S**A	0.1	1	5.1	7.4
6. RPRTS**T**F	6	39	22	37
7. RPRAA**T**F	9	29	12	36

of PKBβ and PKBα by PDK1. As found for PKBα, the naturally occurring derivative of PtdIns(3,4,5)P_3 [the *sn*-1-stearoyl-2-arachidonyl D-PtdIns(3,4,5)P_3 derivative; Figure 3, lipid 1] was more effective than the dipalmitoyl derivative (Figure 3, lipid 3; and see Figure 2A). The L-enantiomer of the *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)P_3 (Figure 3, lipid 2), PtdInds(4,5)P_2 (lipid 5) or PtdIns3*P* (lipid 6) were ineffective at inducing activation of either isoform. Moreover, as reported previously for PKBα [26], none of the PtdIns derivatives tested induced any activation or phosphorylation of His–PKBβ or GST–PKBγ in the absence of PDK1 (Figure 3).

Identification of the residues on PKBβ and PKBγ phosphorylated by PDK1 *in vitro*

^{32}P-labelled His–PKBβ and GST–PKBγ phosphorylated by PDK1 to a stoichiometry of 0.4–0.5 mol of phosphate/mol of protein (Figure 2) were digested with trypsin and chromatographed on a C_{18} column (Figures 4A and 4B). One major ^{32}P-labelled peptide eluted at 28 % acetonitrile was observed in each case. These peptides were co-eluted with the tryptic phosphopeptide from PKBα that contains Thr^{308} (residues 308–328 [23]), were found to contain phosphothreonine only (results not shown) and, when subjected to solid-phase sequencing, ^{32}P radioactivity was released after the first cycle of Edman degradation (Figures 4C and 4D). The amino acid sequence of these peptides was also established by conventional gas-phase sequencing (Figures 4C and 4D). These results establish that PKBβ and PKBγ are phosphorylated by PDK1 at Thr^{309} and Thr^{305} respectively, the equivalent residues to Thr^{308} of PKBα. The minor tryptic phosphopeptide from PKBγ (peptide 1b in Figure 4B) was also found to be phosphorylated at Thr^{305} and to arise from incomplete cleavage of the Lys–Thr bond between residues 304 and 305 (results not shown). Peptide 1b is ten residues longer at the N-terminus than peptide 1a, commencing at Glu^{294} (results not shown).

Substrate specificities of PKBβ and PKBγ

It has been shown previously that PKBα and PKBβ have similar specificities towards a panel of synthetic peptides [32]. In the present study the specificities of PDK1-activated His–PKBβ and GST–PKBγ were also found to be similar (Table 1). In addition, PKBα, PKBβ and PKBγ phosphorylated Crosstide with the same K_m value (4 μM), whereas the more specific peptide

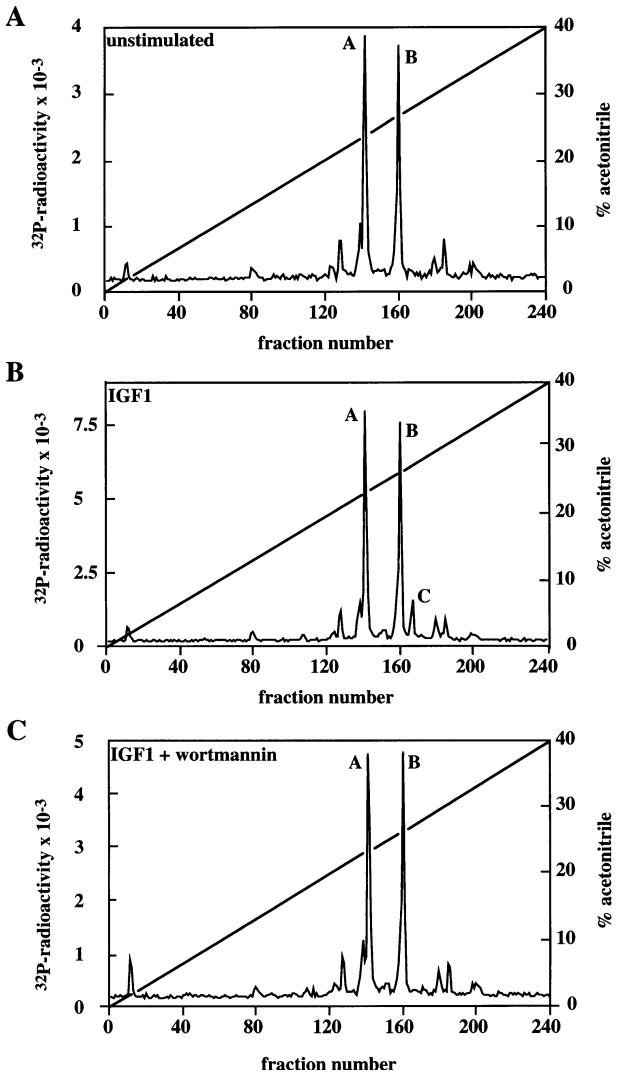

Figure 5 ^{32}P-labelling of HA–PKBγ in 293 cells

293 cells transfected with the HA–PKBγ pCMV5 construct were washed with phosphate-free DMEM, incubated for 4 h with [^{32}P]P_i (1 mCi/ml), treated for 10 min with or without 100 nM wortmannin, then stimulated for 10 min with or without 50 ng/ml IGF1. HA–PKBγ was immunoprecipitated from the lysates, alkylated with 4-vinylpyridine, subjected to SDS/PAGE and autoradiographed. Bands corresponding to HA–PKBγ were excised from the gel, digested with trypsin and chromatographed on a C_{18} column as described in the legend to Figure 4. Tryptic peptide maps of ^{32}P-labelled HA–PKBγ are shown for unstimulated cells (**A**), IGF1-stimulated cells (**B**) and cells treated with wortmannin prior to IGF1-stimulation (**C**). The three major ^{32}P-labelled peptides are marked.

substrate, RPRAATF [41], was phosphorylated by PKBα with a K_m of 25 μM and by PKBβ and PKBγ with a K_m of 11 μM (results not shown). However, there appeared to be some subtle differences in the substrate specificity between the PKB isoforms. The PKBγ isoform seems to tolerate better than PKBβ (Table 1) and PKBα (results not shown; see [41]) the substitution of either of the Arg residues N-terminal to the phosphorylated residue or the C-terminal Phe. PKBγ, purified from insulin-stimulated L6 myotubes by Mono Q chromatography and immunoprecipitation (see below), possessed a substrate specificity identical with that of GST–PKBγ activated *in vitro* with PDK1 (results not shown). These experiments establish that, like PKBα [41] and PKBβ [32],

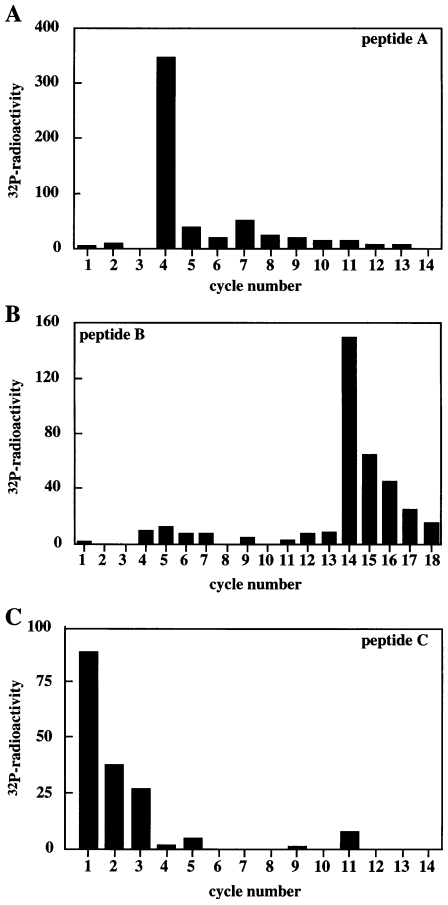

Figure 6 Identification of phosphorylation sites on HA–PKBγ

Peptides A, B and C from Figure 5 were analysed by solid-phase sequencing as described in the legend to Figure 4 to identify ^{32}P radioactivity released at each cycle of Edman degradation.

the minimum consensus sequence for phosphorylation by PKBγ is RXRXXS/TF.

IGF1 induces the activation of HA–PKBγ in 293 cells and its phosphorylation at Thr^{305}

It has been shown that PKBα [23] and PKBβ [32] are phosphorylated constitutively at two residues, Ser^{124}(α)/Ser^{125}(β) and Thr^{450}(α)/Thr^{451}(β) and that activation by insulin or IGF1 is accompanied by the phosphorylation of Thr^{308}(α)/Thr^{309}(β) and Ser^{473}(α)/Ser^{474}(β). However, the activation of PKBγ has never been studied previously in any cell, and hence the site(s) that is (are) phosphorylated are unknown. IGF1 stimulation of 293 cells overexpressing HA–PKBγ induced a 10-fold activation of this enzyme after 10 min, which was abolished if the cells were pre-incubated with 100 nM wortmannin before stimulation with IGF1 (results not shown). This experiment was then repeated, but after incubating 293 cells with [^{32}P]P_i. ^{32}P-labelled HA–PKBγ was immunoprecipitated from the lysates, digested with trypsin, and the resulting peptides were analysed by C_{18} chromatography (Figure 5). Two major ^{32}P-labelled peptides (termed A and B) were seen in unstimulated 293 cells (Figure 5A) which were eluted at 24 and 26 % acetonitrile respectively. Stimulation with IGF1 in the absence of wortmannin induced the ^{32}P-labelling of another peptide (termed C) that was eluted at 28 % acetonitrile (Figure 5B). The phosphorylation of peptide C was prevented by incubation of cells with wortmannin before incubation with insulin (Figure 5C).

Peptide A, a phosphoserine-containing peptide, was eluted at a similar position to the tryptic peptide from PKBα containing Ser^{124} and, when subjected to solid-phase sequencing, ^{32}P radioactivity was released after the fourth cycle of Edman degradation (Figure 6A). This is consistent with peptide A arising from tryptic cleavage between Arg^{116} and Met^{117} of PKBγ, with phosphorylation at Ser^{120} (the residue equivalent to Ser^{124} in PKBα). Peptide B was a phosphothreonine-containing peptide, and ^{32}P radioactivity was released after 14 cycles of Edman

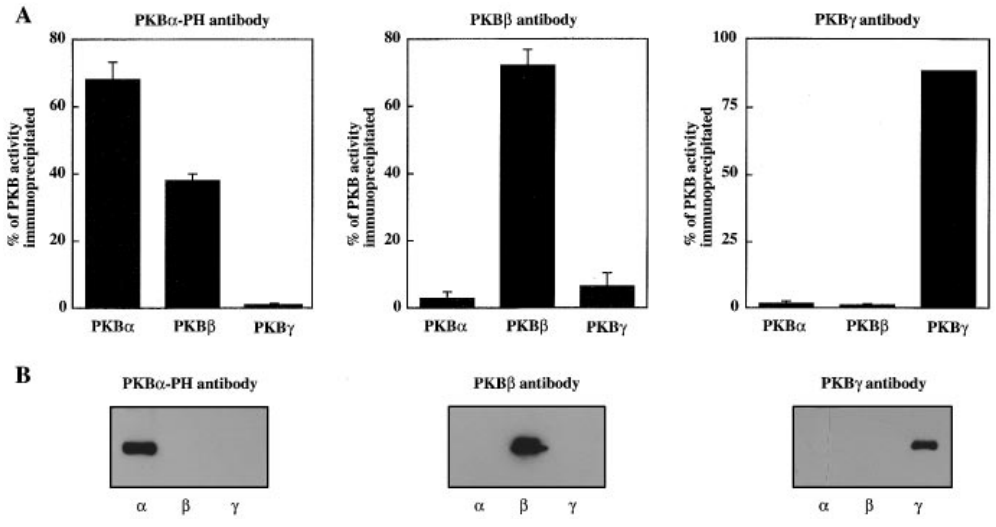

Figure 7 Development of isoform-specific PKB antibodies

(**A**) Portions (25 μl) of GST–PKBα, His–PKBβ and GST–PKBγ (all at an activity of 1 unit/ml) were immunoprecipitated with 5 μg of the anti-PKBα, anti-PKBβ or anti-PKBγ antibodies coupled to 5 μg of Protein G–Sepharose. The percentage of PKB activity immunoprecipitated in each case is shown. (**B**) A 200 ng portion of GST–PKBα, His–PKBβ and GST–PKBγ were subjected to SDS/PAGE, transferred to a nitrocellulose membrane and immunoblotted with the anti-PKBα, anti-PKBβ or anti-PKBγ antibodies, all at a concentration of 1 μg/ml. Immunoblots were revealed using the enhanced chemiluminescence system (ECL) (Amersham International, Amersham, Bucks., U.K.).

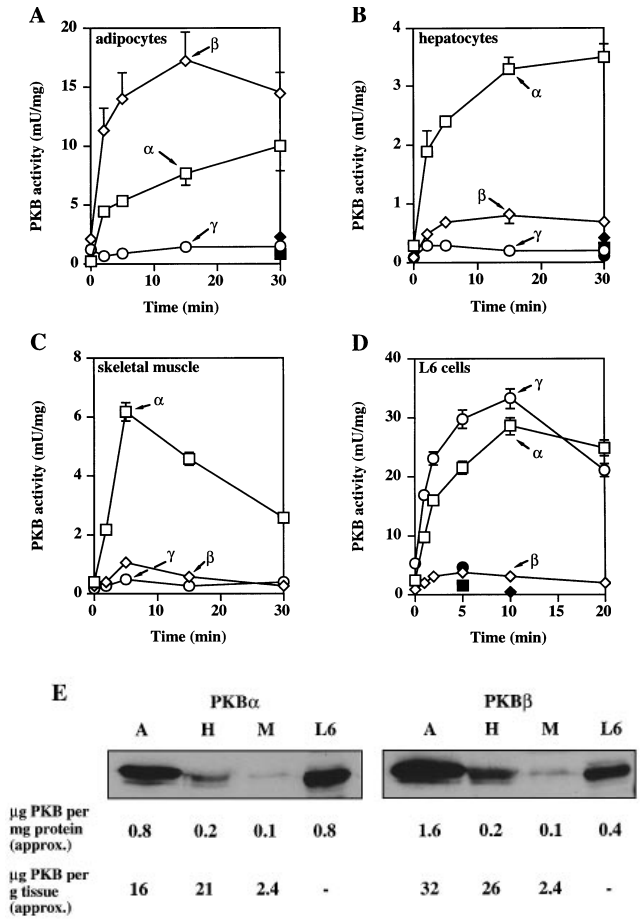

Figure 8 Activation of the three PKB isoforms in insulin-responsive tissues

(**A**) Primary rat adipocytes, (**B**) primary rat hepatocytes and (**D**) L6 myotubes were incubated for 10 min in the presence (closed symbols) or absence (open symbols) of 100 nM wortmannin, and then stimulated with 100 nM insulin for the times indicated. PKBα (squares), PKBβ (diamonds) and PKBγ (circles) were immunoprecipitated from the lysates and the immunopsrecipitates assayed for PKB activity. (**C**) Anaesthetized, food-deprived rats were injected with insulin and, at the times indicated, one hind-limb was freeze-clamped and extracts were prepared (see the Materials and methods section). The three PKB isoforms were immunoprecipitated individually from the extracts and assayed in triplicate. The data are given as means ± S.E.M. for two experiments (**C**) and three experiments (**A**, **B** and **D**). (**E**) A 25 μg portion of protein derived from adipocyte (A), hepatocyte (H), skeletal muscle (M) and L6 myotube (L6) extracts was subjected to SDS/PAGE, transferred to a nitrocellulose membrane and immunoblotted with the anti-PKBα antibody (3 μg/ml) or the anti-PKBβ antibody (2 μg/ml). Recombinant GST–PKBα and GST–PKBβ (1–50 ng of protein) were run in parallel on the blot, and the concentration of each PKB isoform in the lysates estimated 'by eye'. Identical results were obtained in four separate experiments.

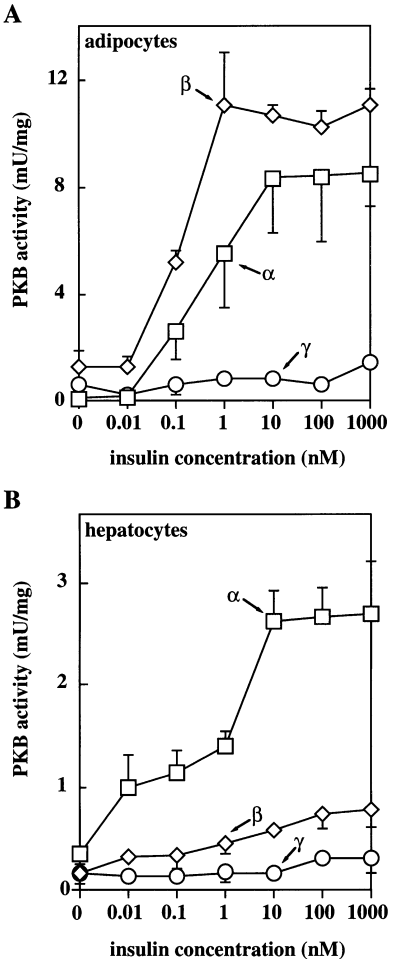

Figure 9 Activation of the three PKB isoforms in rat adipocytes and hepatocytes at various concentrations of insulin

Adipocytes (**A**) or hepatocytes (**B**) were incubated for 10 min with the indicated concentrations of insulin and then lysed. PKBα (squares), PKBβ (diamonds) and PKBγ (circles) were immunoprecipitated from the lysates and the immunoprecipitates assayed for PKB activity in triplicate. The data are given as means ± S.E.M. for two (**A**) or three (**B**) separate experiments.

degradation (Figure 6B). Since ^{32}P radioactivity was released at the same cycle when the tryptic phosphopeptide from PKBα containing Thr^{450} was subjected to solid-phase sequencing [23], peptide B is likely to be phosphorylated at Thr^{447} of PKBγ. Peptide C was co-eluted with the major tryptic peptide from PKBγ phosphorylated at Thr^{305} (compare Figures 4B and 5B). Peptide C contained phosphothreonine, and ^{32}P radioactivity was released after the first cycle of Edman degradation, strongly suggesting that the site of phosphorylation is Thr^{305} (Figure 6C). The degree of labelling of peptide C (Thr^{305}) is much lower than that of peptide A (Ser^{120}) and peptide B (Thr^{447}), indicating that only a small proportion (~ 10%) of the overexpressed PKBγ becomes activated in response to IGF1.

Development of specific antibodies against the different isoforms of PKB

Anti-peptide antibodies were raised in sheep against sequences unique to each isoform (see the Materials and methods section), and the affinity-purified PKBβ and PKBγ antibodies were shown to be specific in immunoprecipitation and immunoblotting experiments using the bacterially expressed fusion proteins (Figures 7A and 7B). The PKBα-PH antibody did not immunoprecipitate GST–PKBγ, but this antibody did immunoprecipitate a significant amount of His–PKBβ (Figure 7A). In order to assay PKBα specifically in cell lysates, PKBβ was therefore immunoprecipitated before immunoprecipitation of PKBα using the PKBα-PH antibody.

Activation of PKB isoforms by insulin in adipocytes, hepatocytes, skeletal muscle and L6 myotubes

The activation of the three isoforms of PKB by insulin was studied in three major insulin-responsive tissues (adipocytes,

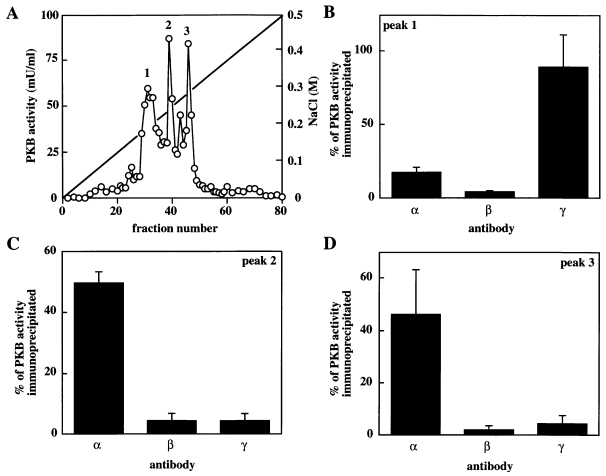

Figure 10 Identification of different PKB activities in L6 myotubes

(**A**) L6 myotubes were incubated for 50 min with PD 98059 (50 μM) and for a further 10 min in the presence of PD 98059 plus rapamycin (0.1 μM), stimulated for 5 min with 100 nM insulin and then lysed. The lysate (3 mg of protein) was chromatographed on a 5 cm $\times$ 0.5 cm Mono Q column equilibrated in 50 mM Mops (pH 7.0)/5% (v/v) glycerol/1 mM EDTA/1 mM EGTA/0.1 mM sodium orthovanadate/0.1% (v/v) β-mercaptoethanol/0.5% (w/w) Triton X-100. The column was developed with a linear NaCl gradient in the same buffer (diagonal line) and fractions (0.5 ml each) were collected and assayed for Crosstide kinase activity (circles). Fractions 30–33 (peak 1), 38–40 (peak 2) and 45–47 (peak 3) were pooled separately and 25 μl aliquots immunoprecipitated with specific antibodies raised against each isoform (see the Materials and methods section). (**B**), (**C**) and (**D**) show the percentage of PKB activity immunoprecipitated from peaks 1, 2 and 3 respectively by each PKB antibody, where peak 1 contained 54 munits/ml, peak 2 contained 57 munits/ml and peak 3 contained 55 munits/ml of Crosstide kinase activity. Assays were performed in triplicate, and the results are given as means $\pm$ S.E.M. for three separate experiments.

hepatocytes and skeletal muscle), as well as in the skeletal-muscle cell line L6.

Stimulation of primary adipocytes (Figure 8A) or hepatocytes (Figure 8B) with 100 nM insulin resulted in the rapid activation of PKBα and PKBβ. The activation was maximal after 10 min (adipocytes) or 20 min (hepatocytes) and abolished by wortmannin. The activity of PKBβ was twice that of PKBα in insulin-stimulated adipocytes, but PKBα activity was 3–4-fold higher than PKBβ in hepatocytes (Figure 8). The concentration of insulin required to achieve half-maximal activation of PKBα or PKBβ was between 0.1 nM and 1 nM in adipocytes or hepatocytes (Figure 9). Furthermore, decreasing the glucose concentrations of the hepatocyte incubation medium from 10 mM to 2 mM did not have any effect of insulin-mediated PKB activation (results not shown). In both cell types PKBγ was not activated significantly by up to 1 μM insulin (Figures 8 and 9). A single injection of insulin into food-deprived, anaesthetized rats induced the activation of PKBα in the hind-limb skeletal muscle. Activation occurred with a half-time of 1–2 min and was maximal after 5 min (Figure 8C). There was little activation of PKBβ and the activation of PKBγ was negligible. Interestingly, the pattern of PKB isoform activation in L6 myotubes was quite different from that in skeletal muscle; the activation of PKBγ in L6 myotubes was at least as high as PKBα. The activation of both isoforms was prevented by wortmannin (Figure 8D). Stimulation of undifferentiated L6 myoblasts with insulin also resulted in an activation of PKBα and PKBγ (and no activation of PKBβ) similar to that observed in myotubes (results not shown).

Adipocytes express 2-fold more PKBβ than PKBα (Figure 8E), consistent with the activation observed after stimulation with insulin (Figure 8A). However, in hepatocytes and skeletal muscle, which express similar levels of PKBα and PKBβ, the PKBα isoform appears to be activated preferentially (Figures 8B, 8C and 8E). This is also the case in L6 myotubes, where insulin-induced PKBα activity was 7-fold higher than PKBβ (Figure 8D), although the expression of PKBα was only 2-fold higher (Figure 8E). We were unable to estimate the level of PKBγ because the endogenous protein could not be detected with our antibody (results not shown).

We have reported previously that three major peaks of Crosstide kinase activity can be resolved by Mono Q chromatography of lysates from insulin-stimulated L6 myotubes that had been pre-treated with the compound PD 98059 to prevent activation of the mitogen-activated protein ('MAP') kinase cascade and with the immunosuppressant drug rapamycin to prevent activation of p70 S6 kinase [15]. The activity in the second and third peaks, but not the first peak, was immunoprecipitated by two anti-PKBα antibodies [15]. The immunoprecipitation data presented in Figure 10 establish that the first peak is largely PKBγ and confirm that the second and third peaks are largely PKBα. No activity in any of the three peaks could be immunoprecipitated by the anti-PKBβ antibody.

DISCUSSION

In the present study we have compared the regulatory and catalytic properties of the three isoforms of PKB. PKBα, PKBβ and PKBγ were phosphorylated and activated by PDK1 at similar rates and to similar extents, phosphorylation occurring at the residues analogous to Thr^{308} of PKBα. After activation, the specificities of each isoform towards a variety of synthetic peptide substrates were similar (Table 1; [32,41]).

The PDK1-mediated activation of each isoform only occurred in the presence of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, and no isoform was activated directly by either of these 3-phosphoinositides in the absence of PDK1. Earlier reports that PtdIns(3,4)P_2 can activate PKBα directly [42–44] may be explained by trace contamination with PDK1 of the PKBα preparation used in these experiments (discussed in [26]).

After activation by PDK1, the specific activity of PKBγ was severalfold higher than that of PKBα. This result might be explained by the C-terminal truncation of PKBγ, which, as a consequence, lacks any residue equivalent to Ser^{473} of PKBα. Thus PKBγ is likely to be fully activated by the phosphorylation of Thr^{305} alone, whereas PKBα and PKBβ require the phosphorylation of Ser^{473}/Ser^{474} as well as Thr^{308}/Thr^{309} to become activated fully.

After phosphorylation by PDK1, PKBβ (tagged at either the N-terminus or C-terminus) had a much lower activity than PKBα. However, whether this difference is real or an artefact of expression is unclear, because in adipocytes the insulin-induced activation of PKBα and PKBβ correlate with the relative level of expression of these isoforms (Figures 8A and 8E). If PKBβ has a lower specific activity than PKBα in adipocytes, this would suggest that PKBβ is activated preferentially. However, an alternative explanation is that phosphorylation of Ser^{474} makes a greater contribution to the activation of PKBβ than does the phosphorylation of Ser^{473} to the activation of PKBα. This would explain why PKBβ has a lower specific activity after phosphorylation of PDK1.

The mRNA encoding PKBγ is known to have a restricted tissue distribution in adult mammalian cells, being present mainly in the central nervous system and in the testis [22]. The low level

or absence of this isoform in other tissues is likely to explain why negligible PKBγ activity was induced by insulin in the major insulin-reponsive tissues (Figure 9). However, an unexpected finding made in the present study was the high level of PKBγ activity induced by insulin in the skeletal-muscle cell line L6. Moreover, we have also observed that PKBγ accounts for a major proportion of the total PKB activity induced by IGF1 stimulation of human embryonic kidney 293 cells [45] and Swiss 3T3 cells (M. Shaw, D. R. Alessi and P. Cohen, unpublished work). It has also been found that nerve-growth-factor stimulation of PC12 cells activates PKBγ [46]. These observations suggest that PKBγ is the major isoform expressed in many embryonic cells and/or the major isoform expressed in many transformed cell lines.

The overexpression of PKBβ in a significant percentage of ovarian [3] and pancreatic cancers [4], and its likely role in enhancing the survival of these tumour cells, has made PKBβ a potential target for the development of drugs to treat these diseases. The observations made in the present study, that PKBα accounts for most of the PKB activity after stimulation of rat skeletal muscle or rat hepatocytes with insulin and that PKBα accounts for 40 % of the total PKB activity induced by insulin in adipocytes, suggests that a specific inhibitor of PKBβ that does not inhibit PKBα may not cause diabetes.

We thank Gareth Browne and Morag Shaw, both of the MRC Protein Phosphorylation Unit, University of Dundee, for their help with the preparation of hepatocytes and adipocytes respectively. We also thank Dr. Peter Watt (Department of Anatomy and Physiology, University of Dundee) for help with preparation of skeletal-muscle extracts. We thank Dr. Richard Currie and Professor Peter Downes (Department of Biochemistry, University of Dundee), and Dr. Piers Gaffney and Professor Colin Reese (Department of Chemistry, King's College, University of London) for providing inositol lipids. We also thank Mr. Barry Caudwell and Dr. Nick Morrice (MRC Protein Phosphorylation Unit, University of Dundee) for synthesis and sequencing of peptides. We acknowledge the U.K. Medical Research Council for financial support and for the award of a postgraduate studentship (to K.S.W.). This work was also supported by the British Diabetic Association and the Royal Society of London (P. C.).

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Received 19 November 1997/9 February 1998; accepted 17 February 1998