Two Splice Variants of Protein Kinase By Have Different Regulatory Capacity Depending on the Presence or Absence of the Regulatory Phosphorylation Site Serine 472 in the Carboxy-terminal Hydrophobic Domain*

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We have reported previously the cloning and characterization of human and mouse protein kinase Bγ (PKBγ), the third member of the PKB family of second messenger-regulated serine/threonine kinases (Brodbeck, D., Cron, P., and Hemmings, B. A. (1999) J. Biol. Chem. 274, 9133–9136). Here we report the isolation of human and mouse PKBγ1, a splice variant lacking the second regulatory phosphorylation site Ser-472 in the hydrophobic C-terminal domain. Expression of PKBγ1 is low compared with PKBγ2, and it is regulated in different human tissues. We show that PKBγ and PKBγ1 differ in their response to stimulation by insulin, pervanadate, peroxide, or okadaic acid. Activation of PKBγ requires phosphorylation at a single regulatory site Thr-305. Interestingly, this site is phosphorylated to a higher extent in PKBγ compared with PKBγ1 upon maximal stimulation by pervanadate, and this is reflected in the respective specific kinase activities. Furthermore, upon insulin stimulation of transfected cells, PKBγ1 translocates to the plasma membrane to a lesser extent than PKBγ. Taken together, these results suggest that phosphorylation of the hydrophobic motif at the extreme C terminus of PKBγ may facilitate translocation of the kinase to the membrane and/or its phosphorylation on the activation loop site by phosphoinositide-dependent protein kinase-1.

Protein kinase B (PKB)† is implicated in a wide variety of cellular responses to insulin and growth factor signaling (for review, see Refs. 1 and 2). The three PKB isoforms identified so far, PKBα, β, and γ, constitute a subfamily among the second messenger-regulated serine/threonine protein kinases (3–9). The human enzymes are 73% identical in amino acid sequence, and all contain an N-terminal pleckstrin homology domain, a central kinase domain, and a C-terminal regulatory hydrophobic domain. Activation occurs in response to signaling via phosphoinositide 3-kinase (10–13). PtdIns(3,4,5)P3, the membrane-bound active second messenger (for review, see Refs. 2 and 14), is thought to recruit PKB to the membrane, promoting a conformational change that allows phosphorylation on two regulatory sites by upstream kinases that are either located at the membrane or brought there through interaction with PtdIns(3,4,5)P3 themselves (15–17). In support of this model, it has been shown that PtdIns(3,4,5)P3 and its metabolite PtdIns(3,4)P2 bind to the pleckstrin homology domain (18, 19) and that PKBα containing an N-terminal membrane-targeting signal is constitutively active, independent of the presence of a pleckstrin homology domain (20, 21). The two phosphorylation sites critical for regulation of the activity of all three enzymes have been identified (7, 22, 23); one 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).

Of the upstream kinases, the one that phosphorylates Thr-308 in the activation loop of PKBα in a PtdIns(3,4,5)P3-dependent manner has been identified and termed 3-phosphoinositide-dependent kinase-1 (24–27). In addition to PKBα, this kinase also phosphorylates equivalent sites in other second messenger-regulated kinases, such as p70S6 kinase (28, 29), protein kinase A (30), protein kinase C (31), serum- and glucocorticoid-regulated kinase (32, 33), and p90 ribosomal S6 kinase-2 (34). For the second regulatory site in the hydrophobic C-terminal domain of PKBα, several candidate upstream kinases have been identified. MAP kinase activated protein kinase-2 (MAPKAP-K2) is able to phosphorylate Ser-473 in vitro, but in vivo it is activated by stress and other stimuli that fail to activate PKB (35). The integrin-linked kinase ILK-1 is activated by phosphoinositide 3-kinase signaling and can phosphorylate Ser-473 in vitro (35). It has also been reported that 3-phosphoinositide-dependent kinase-1 might acquire the capability to phosphorylate Ser-473 by interaction with a region of protein kinase C-related kinase-2 (PRK2; 36), and a recent report claims that PKBα phosphorylated on Thr-308 is able to autophosphorylate on the Ser-473 site (37).

Activated PKB mediates a range of cellular responses to insulin and growth factors, such as phosphorylation and inactivation of glycosyn synthase kinase-3 (38), up-regulation of protein translation through activation of the mammalian target of rapamycin mTOR (39), inactivation of the translational repressor 4E-BP1 (40), and stimulation of ribosomal p70S6 kinase, as well as activation of phosphofructokinase-2 (41). Constitutively active PKB stimulates glucose uptake through

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recruitment of glucose transporters to the plasma membrane of adipocytes (11). Furthermore, PKB is linked to phosphorylation and activation of endothelial nitric-oxide synthase induced by shear stress on endothelial cells in the vasculature (42, 43). Stimulated PKB translocates to the nucleus in human embryonic kidney (HEK)-293 cells (21, 23), activates cAMP-responsive element-binding protein by phosphorylation (44), and mediates insulin response sequence-specific effects on hepatic gene expression (45).

Many reports show that PKB is a critical regulator of cell survival (46). Phosphorylation by PKB inactivates both the apoptosis-promoting Bcl-2 family member BAD (47, 48) and the protease caspase 9 (49, 50). Nuclear targets of PKB promoting cell survival are the Forkhead family of transcription factors, where the human homologs FKHR (51), FKHR1L (52, 53), and AFX (54) contain three consensus sites for phosphorylation by PKB (55). Accordingly, PKB phosphorylates and inhibits FKHR1L and FKHR, promoting their export from the nucleus or leading to retention in the cytoplasm, as part of an evolutionarily conserved mechanism by which insulin and growth factors regulate gene expression (56–59).

In this paper, we report the cloning and characterization of a novel variant of human and mouse PKBγ. Human PKBγ-1 is 14 amino acids shorter, and mouse PKBγ-1 9 amino acids, than PKBγ2, and both lack the second regulatory phosphorylation site, Ser-472. Both variants arise from the same gene through differential splicing of the C-terminal exons, and the respective mRNAs are expressed in a tissue-specific manner. Both kinases can be activated by a range of stimuli, but they vary considerably in the extent of phosphorylation on the regulatory site Thr-305 in their specific kinase activities and with respect to membrane localization.

**EXPERIMENTAL PROCEDURES**

**Cloning and Construction of Expression Vectors HA-PKBγ, HA-PKBγ-1, and Phosphorylation Site Mutants**—The cloning of human and mouse HA-PKBγ has been described previously (7). Two additional human PKBγ cDNA clones were isolated (encoding amino acids 16–451) which contained sequences for a further 14 amino acids and 3′-noncoding region. These sequences have been submitted to GenBank under the accession number AY005799. To generate HA-PKBγ-1 using the QuikChange kit (Stratagene) was used to generate the activation loop site mutants HA-PKBγ-1T305A and HA-PKBγ-1T305D and the Thr-447 site mutations HA-PKBγ-1T474A and HA-PKBγ-1T474D. All PCR-cloned constructs were verified by DNA sequencing.

**Generation of a Mouse BAC Sublibrary and Screening**—The full-length mouse PKBγ cDNA (7) was used to isolate a BAC clone (Incyte Genomics) containing the PKBγ gene. This clone was digested with different restriction enzymes, and the fragments were ligated into pBluescript II plasmids which were screened with a probe spanning exons 12 and 13 of mouse PKBγ, or about 400 base pairs of putative intronic sequence upstream of exon 14, by standard procedures.

**Amplification of Large PCR Fragments**—Long range PCR was performed with the Expand Long Template PCR System (Roche Molecular Biochemicals) according to the manufacturer's instructions, using 200 ng of human genomic DNA (Roche Molecular Biochemicals) as template. The PCR protocol consisted of 10 cycles of 94 °C for 10 s, 55 °C for 30 s, 68 °C for 4 min. This was followed by 30 cycles of 94 °C for 10 s, 55 °C for 30 s, 68 °C for 4 min, and an extension of the elongation step by 20 s/cycle. The reaction was reamplified if necessary, and the products were analyzed on a 0.6% agarose gel, extracted, and sequenced with the same primers used for amplification (primer 20909, 5′-CCT-CAAGTACATGACAGACAG-3′; primer 20429, see above; primer 28759, 5′-GGGTCTAGATTACTTTTATATCATGTTCCGAGGATTGG-3′; and primer 28144, 5′-GGGCTAGATTACTTTTATATCATGTTCCGAGGATTGG-3′).

**Isolation of Total RNA from Mouse Brain and Embryo**—Mouse brain or embryos (E15–E20) frozen in liquid N2 were extracted with Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions and the amount of total RNA quantified by absorbance at 260 nm.

**Reverse Transcription and Semi-quantitative PCR**—Reverse transcription was performed with the GeneAmp RNA PCR kit (PE Biosystems), essentially according to the manufacturer’s instructions, using random primers and 1 μg of total RNA from mouse brain or embryos as template in a total volume of 20 μl, and a reaction protocol of 10 min at 25 °C, 60 min at 42 °C and 5 min at 94 °C. 5 μl of the reaction was used as template for PCR in a total volume of 20 μl with a protocol of 35 cycles of 95 °C for 15 s, 54 °C for 30 s, and 72 °C for 30 s, and the reaction was reamplified if necessary. For amplification, the common primer 20091 (see above) was used in combination with the mouse splice variant-specific primers 32212 (5′-GGTGAAAGACCTTGGCTG-3′), resulting in a band of 798 base pairs, and 32210 (5′-GGGCTAGATTACCTTTTATATCATGTTCCGAGGACCTTT-3′), resulting in a band of 636 base pairs. For semi-quantitative RT-PCR of human tissue RNA, this protocol was modified as follows. To 1 μg of total RNA (CLONTECH) 104 copies of synthetic pAW109 RNA were added as normalization control. Serial dilutions were made of the reverse transcriptase reaction, and 10 μl of four consecutive dilutions was employed as the template for PCR. Primer 20901 was employed in combination with primers 26839 (5′-GTGGCCAGGGGGTCGGACCC-3′), resulting in a band of 509 base pairs, and 20429 (see above), resulting in a band of 621 base pairs, and annealing was raised to 56 °C. As a control for the reverse transcription, a 308-base pair fragment of pAW109 RNA was amplified with the primers DM151 and DM152 supplied in the kit. The reactions were electrophoresed on a 1% agarose gel, photographed under UV light, and the bands quantitated using Molecular Dynamics software.

**Cell Culture, Transfections, Stimulation, Collection of Cell Extracts, and Immunofluorescence**—HEK-293 cells were maintained and transfected by a modified calcium phosphate method as described previously (12). Stimulation was for 15 min with 500 nM insulin (Roche Molecular Biochemicals), 5 min with 0.2 mM pervanadate (12), 10 min with 10 mM H2O2 (Merek), or 1 h with 1 μM okadaic acid (Alexis). After stimulation, cells were washed once with 20 mM Hepes-NaOH, pH 7.0, 120 mM NaCl and extracted in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 40 mM β-glycerophosphate, 25 mM NaF, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 2 μM Microcystin-LR; Alexis). The cell extracts were centrifuged for 10 min at 10,000×g, and the protein concentration was determined by Bradford assay using bovine serum albumin as a standard.

For immunofluorescence, the cells were washed once with phosphate-buffered saline, fixed in 4% paraformaldehyde (Merek) in phosphate-buffered saline for 30 min at room temperature, and permeabilized with 0.2% Triton X-100 (Sigma) for 5 min. They were then stained with the monoclonal anti-HA antibody 12CA5 and a fluorescein isothiocyanate-coupled anti-mouse IgG secondary antibody (1:500) and observed by confocal microscopy.

**Immunoprecipitation, Kinase Assays, and Immunoblot Analysis**—HA-PKBγ protein was immunoprecipitated from 50–100 μg of cell lysate, with the 12CA5 monoclonal antibody bound to protein A-Sepharose beads. The immunoprecipitates were washed as described previously (21) and incubated in 50 μl of in vitro kinase assay mix (150 mM Tris, pH 7.5, 15 mM β-mercaptoethanol, 10 mM MgCl2, 1 μM protein kinase A inhibitor peptide (Bachem, Switzerland), 30 μM Crossstide substrate (GRIPPESSFAEG; 38), and 50 μM ATP (Amersham Pharmacia Biotech, 2500 cpm/pmol) for 30–60 min at 30 °C. The reaction was stopped and processed as described previously (21). Peptides utilized in the substrate specificity assay were obtained from NeoSystems or synthesized at the institute facility.

Western blot analysis was performed as described (22) and developed with either the monoclonal anti-HA antibody 12CA5, a polyclonal antibody raised against a pan-phospho-Thr PKB peptide (DAATTMTKPPFCGTP), or a polyclonal antibody raised against a pan-phospho-Ser PKB peptide (RHPFFQFSYPYSA). Alkaline phosphatase-coupled anti-mouse IgG secondary antibody (1:500) and observed by confocal microscopy.

**RESULTS**

**Two Variants of PKBγ Arise from a Single Gene through Differential Splicing of C-terminal Exons**—In the process of cloning human PKBγ (7), we isolated a cDNA clone, termed

AAAATTTG-3′; and primer 28144, 5′-GCAGGGGCACCTTGGCAGC-3′.
PKBγ, which was identical to PKBγ until amino acid 451 but differed in sequence at the C terminus. The human PKBγ sequence extended for a further 14 amino acids only, showed no homology to the C-terminal 28 residues of PKBγ, and did not contain the Ser-472 regulatory phosphorylation site (Fig. 1). With the novel amino acid sequence, we performed a search of the EST data base. The arrowhead indicates the site where the exon boundary had been mapped for PKBa (Ref. 61 and Footnote 2). The sequence of human PKBγ has been submitted to GenBank under the accession number AY005799.

Significantly, the starting point of the sequence divergence coincided with the location where an exon boundary had been mapped for the mouse PKBa gene (61). Taking into account the high degree of conservation among individual PKB isoforms, we assumed that the exon boundaries would be conserved and that the diversity at the C terminus could be caused by alternative splicing. With the two cDNA clones encoding PKBγ and PKBγ1 we screened the NCBI human genome data base and obtained homology with the same contig NT_004480, a chromosome 1 working draft sequence segment. The exon sizes, alignments with the contig, and analysis of the splice acceptor and splice donor sites are listed in Table I; the exons were numbered according to the mouse PKBa gene (61). The contig comprised exons 2–15 of the human PKBγ gene. Exons 2–12 were shared by both splice variants, exon 13 encoded the PKBγ-specific C terminus and 3′-untranslated region, exon 14 encoded the PKBγ1-specific C terminus and 6 nucleotides past the stop codon, and exon 15 contained the remaining 3′-untranslated region of PKBγ1. A graphic depiction of the exon structure of the two human PKBγ splice variants is given in Fig. 2A. The sequences borders the exons fit well to the consensus for splice acceptor and splice donor sites (Table I). Because this contig was only a working draft sequence segment, the sizes of the intervening introns could not be derived reliably.

To determine the gene structure and intron size in the region where alternative splicing occurred, we designed antisense primers specific for the two C-terminal coding exons (exon 13-primer 26759 for PKBγ; exon 14-primer 20429 for PKBγ1) and a common sense primer (20090) located in exon 8 and antisense primers specific for the individual isoforms (32212 for mPKBγ, 32210 for mPKBγ1). The products were purified over a 1% agarose gel and sequenced to confirm their identity (Fig. 3, B and C), establishing that both C-terminal splice variants of PKBγ were expressed in the mouse.

Analysis of the Abundance of mRNA Transcripts of Human PKBγ and PKBγ1—A more detailed analysis of the relative abundance of the two mRNA transcripts was done for the human PKBγ splice variants. Among the 12 human PKBγ cDNA clones isolated, 9 encoded the C terminus, and of these, two contained the PKBγ1-specific sequences. Furthermore, 15 clones obtained by performing 3′-RACE on brain cDNA-encoded PKBγ. Northern blot analysis had revealed the presence of two transcripts of 8.5 and 6.5 kb with comparable relative expression levels in several human adult and fetal tissues (7), but when the same blots were hybridized with a probe specific for PKBγ1 we obtained no signal. These results implied that PKBγ1 was less abundant than PKBγ, prompting us to quantitate relative abundance and possibly tissue-specific expression of PKBγ and PKBγ1 transcripts by semiquantitative RT-PCR. As templates for the RT reaction, we used total RNA samples derived from human prostate, testis, uterus, kidney, mammary gland, skeletal muscle, brain, trachea, lung, heart, and liver. Four consecutive 2-fold dilutions of these reactions were used as templates for the PCRs, chosen to en-

2 Z. Yang and B. A. Hemmings, unpublished results.
### Table I

Exon boundaries of the human PKBγ gene

| Exon | Base pairs (cDNA) | Amino acids (cDNA) | Cosmid NT_004480 | Splice acceptor site | Splice donor site |
|------|-----------------|------------------|------------------|---------------------|------------------|
| 1    | (−67)−46        | 1–16             | (not present)    | ACATGGCTGTTCTCTCATGAG | GAAATGAGT        |
| 2    | 47–172          | 16–58            | 519,865–519,740  | GAAATTTTTTTTTTATTCTAGA | AGGGTGAAG        |
| 3    | 173–284         | 58–95            | 448,099–447,988  | GGAACATTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 4    | 285–429         | 95–143           | 441,697–441,553  | GGAATTTTTTCTCTCTTTTTA | AGGGTGAAG        |
| 5    | 430–561         | 144–187          | 433,408–433,277  | AGTTTTTTTTTTTCTCTTTA | AGGGTGAAG        |
| 6    | 562–627         | 189–209          | 411,076–411,011  | CTCTTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 7    | 626–866         | 219–232          | 409,654–409,586  | TTCTTTTTCTTTTTTTTTA | AGGGTGAAG        |
| 8    | 697–819         | 233–273          | 404,133–404,011  | TTCTTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 9    | 820–948         | 274–316          | 397,960–397,832  | AGTCTTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 10   | 949–1,163       | 317–388          | 387,056–386,842  | CTCTTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 11   | 1,164–1,251     | 388–417          | 379,633–379,546  | AAGTTTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 12   | 1,252–1,354     | 418–452          | 376,097–375,995  | ACTCTTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 13   | 1,355–1,696     | 452–479          | 370,209–369,867  | TTCTTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 14   | 1,355–1,404     | 452–465          | 364,660–364,611  | TTCTTTTTTTTTTTTTTTA | AGGGTGAAG        |
| 15   | 1,405–1,622     | 358,866–358,669  | CTAA C/T-rich NCAGG | CCATTATCTCTGTCTCACAAGT | AAGGTGAAG        |

Consensus splice acceptor/donor sequences

| Exon | Consensus splice acceptor/donor sequences |
|------|-----------------------------------------|
| 1    | ACATGGCTGTTCTCTCATGAG                   |
| 2    | GAAATTTTTTTTTTATTCTAGA                  |
| 3    | GGAACATTTTTTTTTTTTTTTA                  |
| 4    | GGAATTTTTTCTCTCTTTTTA                   |
| 5    | AGTTTTTTTTTTTCTCTTTA                    |
| 6    | CTCTTTTTTTTTTTTTTTA                     |
| 7    | TTCTTTTTCTTTTTTTTTA                     |
| 8    | TTCTTTTTTTTTTTTTTTA                     |
| 9    | AGTCTTTTTTTTTTTTTTTA                    |
| 10   | CTCTTTTTTTTTTTTTTTA                     |
| 11   | AAGTTTTTTTTTTTTTTTTA                    |
| 12   | ACTCTTTTTTTTTTTTTTTA                    |
| 13   | TTCTTTTTTTTTTTTTTTA                     |
| 14   | TTCTTTTTTTTTTTTTTTA                     |
| 15   | CTAA C/T-rich NCAGG                     |

**Fig. 2. Genomic organization of the C-terminal region of the human PKBγ gene.** Panel A, exon structures of the human PKBγ and PKB-1 cDNAs. The amino acid sequences of the different C termini and the regulatory phosphorylation sites are indicated. Panel B, schematic representation of the genomic region encompassing exons 12–14. The 3′-border of exon 13 has not been determined. The primers used for long range PCR are indicated (not to scale). Panel C, long range PCR products amplified with the indicated primer combinations and human genomic DNA as template were visualized on a 0.6% agarose gel. Molecular weight (MW) markers are indicated in kb.

The expression of PKBγ varied among different tissues, ranging from undetectable levels in liver and low levels in heart and lung to highest expression in the brain, testis, uterus, and prostate. PKBγ1 was also detected in all tissues except liver, heart, and lung, and its abundance varied considerably but at significantly lower levels. Highest levels were detected in prostate, testis, and mammary gland. Thus, in tissues of the genitourinary tract, such as prostate, testis, uterus, and kidney, in the mammary gland and in skeletal muscle, PKBγ1 accounted for 2–8% of total PKBγ transcripts, whereas in trachea and brain, levels made up less than 1% (Table II).

**Regulation of HA-PKBγ Activity**—We have reported previously that mutation of Thr-305 of HA-PKBγ, the regulatory phosphorylation site in the activation loop, resulted in complete inactivation of the protein (7). Wild-type HA-PKBγ1, which lacks the second regulatory site in the hydrophobic domain, could be stimulated by pervanadate treatment when transiently expressed in HEK-293 cells (Fig. 4A). When Thr-305 of HA-PKBγ1 was mutated to an alanine that cannot be phosphorylated (HA-PKBγ1T305A), the activation was abolished (Fig. 4A). When we mutated Thr-305 to an aspartate (HA-PKBγ1T305D) to mimic the activated state, the activity of the kinase did not increase significantly above the levels of unstimulated wild-type protein and could not be activated by pervanadate treatment (Fig. 4A), again confirming the observations made with HA-PKBγ1 (7). This is in contrast to PKBαs, where mutation of the two regulatory phosphorylation sites to acidic residues produced a constitutively active protein (22).

Because PKBγ1 lacked the Ser-472 residue, we tested whether the phosphorylation status of Thr-447, shown to be a site of constitutive phosphorylation in PKBαs (22), influenced the activation of the kinase, in effect taking over the role of the second regulatory phosphorylation site. Mutants carrying either an alanine or an aspartate residue at Thr-447 (HA-PKBγ1T447A and HA-PKBγ1T447D) were transiently transfected and kinase activity measured in response to pervanadate. We observed an activation profile comparable to the wild-type protein with both constructs, indicating that the phosphorylation status of Thr-447 had no influence on the activation of HA-PKBγ1 (Fig. 4A). Furthermore, a double mutant HAPKBγ1T305D/T447D was also not active above basal levels and could not be stimulated (data not shown).

Western blot analysis of cell lysates revealed that HA-PKBγ1 wild-type and mutated at Thr-305 migrated as a doublet (Fig. 4B). In contrast, the Thr-447 mutants ran as single bands, with HA-PKBγ1T447A co-migrating with the faster, and HA-PKBγ1T447D migrating slightly slower (Fig. 4B). This is consistent with the notion that phospho-Thr-447 has a greater impact on the migration of the protein and suggests that Thr-447 is phosphorylated in vivo under wild-type conditions. Wild-type HA-PKBγ1 was also not active above basal levels, and the Thr-305 mutants were not affected by pervanadate treatment (Fig. 4B).
Table II

| mRNA source          | Relative mRNA abundance | Ratio PKBγ/PKBγ1 |
|----------------------|-------------------------|-----------------|
|                      | arbitrary units         |                 |
| Prostate             | 4.49 ± 0.25             | 3.8 ± 0.23      |
| Testis               | 7.84 ± 0.32             | 6.0 ± 0.10      |
| Uterus               | 5.83 ± 0.60             | 4.0 ± 0.05      |
| Kidney               | 2.10 ± 0.22             | 1.8 ± 0.13      |
| Mammary gland        | 3.96 ± 1.64             | 1.5 ± 0.14      |
| Skeletal muscle      | 1.13 ± 0.18             | 0.5 ± 0.03      |
| Brain                | 6.96 ± 2.02             | 1.7 ± 0.70      |
| Trachea              | 2.35 ± 0.50             | 0.5 ± 0.01      |
| Lung                 | 0.18 ± 0.11             | 0.0 ± 0.00      |
| Heart                | 0.10 ± 0.03             | 0.0 ± 0.00      |
| Liver                | 0.01 ± 0.01             | 0.0 ± 0.00      |

PKBγ1T447D with the slower conformation of HA-PKBγ1. Thus it was possible that the double band observed with HA-PKBγ1 was caused by differential phosphorylation at Thr-447.

Comparison of the Activities of HA-PKBγ and HA-PKBγ1 Elicited by Different Stimuli—We compared kinase activities of transiently transfected HA-PKBγ and HA-PKBγ1 under basal conditions and in response to a range of stimuli, including 500 nM insulin, 0.2 mM pervanadate, 10 mM peroxide, and 1 mM okadaic acid. The results shown in Fig. 5A demonstrated a robust activation of HA-PKBγ upon treatment of the cells with insulin or the insulin-mimetic compound pervanadate, but less potent stimulation of kinase activity when the cells were treated with peroxide or okadaic acid. In contrast, the most potent activation of HA-PKBγ1 was achieved in response to pervanadate stimulation, whereas only a weak activation was observed upon treatment with insulin or peroxide and none in response to okadaic acid treatment (Fig. 5A). Other stimuli tested included 12-O-tetradecanoylphorbol 13-acetate, forskolin, or the calcium ionophore A23187, none of which resulted in activation of either splice variant.

Overall, HA-PKBγ1 had lower basal and stimulated relative kinase activities compared with HA-PKBγ. The immunoprecipitates prepared for activity assays were analyzed by Western blot, showing that HA-PKBγ was expressed at a slightly lower level than HA-PKBγ1 (Fig. 5B). Relative kinase activities of both splice variants were reflected in the phosphorylation status of Thr-305, the lower level of phosphorylation of HA-PKBγ1 correlating with lesser activation (Fig. 5C). Finally, by monitoring the phosphorylation status of Ser-472 of HA-PKBγ under the different conditions we found that okadaic acid treatment led to phosphorylation at Ser-472 but not Thr-305 and to low levels of kinase activation (Fig. 5D).

Influence of Osmotic Stress on Basal and Stimulated Activities of HA-PKBγ and HA-PKBγ1—To test if HA-PKBγ and HA-PKBγ1 kinase activity could be regulated differentially under cellular stress, we examined possible effects of ceramide or sorbitol. Pretreatment of transiently transfected cells with

The text continues with further experimental details and analysis.
stimulated activity of either splice variant. This was in contrast to PKB\alpha, reflected by the phosphorylation status of Thr-305. HA-PKB\alpha was not the slight activation elicited by insulin (Fig. 6A). This again suggested that in contrast to HA-PKB\alpha, the average (±S.D.) of four experiments assayed in duplicate. Panels B–D, aliquots of the immunoprecipitates used for kinase assay were analyzed by Western blot developed with the monoclonal anti-HA antibody 12CA5 (B) and with polyclonal antibodies raised against pan-phospho-Thr (C) and pan-phospho-Ser (D) PKB peptides.

50 μM C2-ceramide for 2 h did not have an effect on basal or stimulated activity of either splice variant. This was in contrast to PKB\alpha, where ceramide treatment led to inhibition of kinase activity concomitant with a reduction in Ser-473 phosphorylation (62). However, we observed a differential effect on kinase activity concomitant with a reduction in Ser-473 phosphorylation of HA-PKB\gamma, which was higher than that of HA-PKB\alpha, and was slightly more sensitive to variations in peptide sequence, but overall no major divergence in substrate preferences could be determined.

Comparison of the Substrate Specificities of HA-PKBγ and HA-PKBγ1—We compared specific kinase activities of per-vanadate-stimulated HA-PKB\gamma and HA-PKB\gamma1 assayed with 15 different peptides, which were derived from possible kinase substrates. For this experiment, equal amounts of HA-tagged protein were immunoprecipitated from lysates of transiently transfected cells; the results are shown in Table III, with specific kinase activity expressed in pmol/min. For both splice variants, the most activity could be measured using Crosstide as the substrate, followed by a number of peptides conforming to the consensus motif RXXXXXST. A minimal peptide of the sequence RPRAATF elicited about half-maximal phosphorylation, but any variation in the position of the upstream arginines immediately led to a substantial decrease in activity. Surprisingly, a peptide with the motif of the phosphorylation site Ser-136 of BAD, a known target of PKB\alpha (47), was also a rather weak substrate for both kinases. Likewise, peptides corresponding to the phosphorylation site Ser-259 of the Raf isoforms or to Ser-112 of BAD were not phosphorylated, even though they conformed to the consensus motif. The Raf peptides were also tested as substrates for HA-PKB\alpha but elicited no kinase activity (data not shown). Specific kinase activity of HA-PKB\gamma was higher than that of HA-PKB\gamma1 and was slightly more sensitive to variations in peptide sequence, but overall no major divergence in substrate preferences could be determined.

Localization of HA-PKB\gamma and HA-PKB\gamma1 in Transiently Transfected Insulin-stimulated Cells—We have shown here that in contrast to HA-PKB\gamma, HA-PKB\gamma1 kinase activity was stimulated only weakly in response to insulin treatment of transiently transfected cells. This observation led us to investigate the localization of the two splice variants in response to insulin stimulation. It had been shown previously that insulin-like growth factor I or insulin treatment led to translocation of PKB\alpha and PKB\beta to the membrane, where the activation by
C-terminal Splice Variants of PKBγ

We have characterized the expression pattern of the two splice variants PKBγ and PKBγ1 by RT-PCR, showing that both are expressed in adult mouse brain and in E15–E20 embryos (Fig. 3). Furthermore, an extensive analysis of several different human tissue RNA samples revealed co-expression of both transcripts. PKBγ1 was expressed at relatively high levels in RNA samples from prostate, testis, uterus, and the mammary gland; in these tissues, and also in kidney and in skeletal muscle, it accounted for more than 2–8% of total PKBγ expression (Table II). PKBγ, the more abundant message, additionally showed high expression in brain and trachea, reflecting the Northern blot results published previously (6, 7). Also, neither of the splice variants were detected in liver and only very low levels of PKBγ in heart and lung, whereas analysis of expression of PKBα and β by human multiple tissue Northern blots revealed that both of the latter isoforms were highly expressed in these tissues.3 These results indicated that expression and alternative splicing of the PKBγ gene are under tissue-dependent regulation. Alternatively, the low abundance of mRNA encoding the minor variant PKBγ1 could be the result of high, or even exclusive, expression in only a small subset of cells of a given tissue.

Many examples of alternate splicing of protein kinases to generate multiple variants have been documented in the literature. However, only rarely did alternate C-terminal exon usage produce variants of the same kinase with different regulatory potential. Examples include the myotonic dystrophy protein kinase gene, where the primary mRNA transcripts were spliced to generate six major isoforms that differed in the presence or absence of an internal motif and in their C-terminal ends. Thus the mRNA transcript could be spliced to result in a protein with a strongly hydrophobic C-terminal domain, but usage of a cryptic splice acceptor site led to a frameshift and to translation of a less hydrophobic C terminus. This seemingly occurred as a stochastic event, whereas the skipping of two exons, resulting in a C-terminally truncated isoform, was cell type-dependent (64). In L6 skeletal muscle cells, stimulation by insulin shifted C-terminal alternate exon usage to enhanced inclusion of the PKCβII-specific, rather than the PKCβI-specific, exon into the mature mRNA transcript, a process likely regulated by phosphorylation of SR proteins (65). The two PKCβ splice variants differed in their binding to F-actin, implicating PKCβII in the process of insulin-stimulated actin rearrangements (66). The mechanism by which C-terminal splicing of the PKBγ gene is regulated remains to be established.

When we compared the kinase activities of the two PKBγ splice variants, we observed that PKBγ1 could not be activated to the same extent as PKBγ with any of the stimuli tested. This was in part due to the absence of the Ser-472 phosphorylation site and because the activation loop site Thr-305 was also phosphorylated to a lesser extent in PKBγ1 compared with PKBγ (Figs. 5 and 6). Kinase activity and Thr-305 phosphorylation of PKBγ1 were more sensitive to osmotic stress (Fig. 6) and less sensitive to stimulation by insulin (Figs. 5 and 6). Furthermore, less PKBγ1 than PKBγ was localized at the membrane of transfected cells after insulin stimulation (Fig. 7).

Together, these results implied that the presence of the second phosphorylation site Ser-472 positively influenced the induction or the maintenance of phosphorylation at Thr-305 and thus the activity of the kinase. This may occur through interactions of the unique termini of PKBγ and PKBγ1 with different modulators of PKB activity, which may regulate intracellular localization and facilitate translocation of the kinase to the intracellular localization of HA-tagged proteins. The data show (Fig. 7B) the fraction of cells where HA-tagged protein was localized in the cytosol, in cytosol and membrane, or at the membrane only, expressed as percentage of a total of about 1,200 cells counted for each condition. These data suggested that either HA-PKBγ1 translocated to the membrane less readily than HA-PKBγ upon insulin stimulation or that it dissociated more rapidly from the membrane and then became quickly dephosphorylated.

DISCUSSION

Previous studies on the regulation of the three PKB isoforms α, β, and γ revealed the central importance of the two regulatory phosphorylation sites, Thr-308/309/305 and Ser-473/474/472, in controlling kinase activity in response to insulin or growth factor signaling via the phosphoinositide 3-kinase pathway, with both sites required for full activation (for review, see Refs. 1 and 2). Rat PKBγ lacked the regulatory phosphorylation site in the C-terminal domain, and yet transiently transfected protein could be activated in vitro (6, 63). The rat PKBγ sequence differed significantly only at the C terminus; thus, it seemed likely to be a splice variant. We now provide compelling evidence that the human and mouse PKBγ genes contain additional exons that are utilized in a splice variant, leading to translation of proteins with different C termini (Table I and Fig. 2A). This splice variant PKBγ1 also lacked the Ser-472 phosphorylation site, and yet kinase activity of human PKBγ1 could be stimulated through a single regulatory phosphorylation site in the activation loop, Thr-305 (Fig. 4).

3 B. A. Hemmings, unpublished results.
the membrane, or the phosphorylation event of the kinase itself. Alternatively, an altered sensitivity to inactivation of the two splice variants may be dependent on differing interactions with phosphatases that down-regulate kinase activity. In support of this hypothesis, it has been suggested that phosphoinositide 3-kinase activates a signal transduction pathway promoting rapid dephosphorylation and inactivation of PKB and furthermore that membrane localization led to partial protection from inactivation (67).

The potential implications for downstream signaling offered by the presence of two splice variants of PKB are manifold. Although PKB-y and PKB-y1 possess identical kinase domains, the variable C-terminal sequences could impose different constraints on access of the substrate to the kinase pocket. To investigate this possibility, we examined the relative activities of the splice variants toward a range of peptides. However, even though specific kinase activities of the splice variants toward a range of peptides. However, downstream signaling of PKB-y1 after insulin stimulation, indicating a role for the C-terminal regulatory phosphorylation site Ser-472 in mediating the translocation of the kinase or the phosphorylation of Thr-305.

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REFERENCES
1. Galeti, I., Andjelkovic, M., Meier, R., Brodieck, D., Park, J., and Hemmings, B. A. (1999) Pharmacol. Ther. 82, 409–425
2. Chen, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999) Annu. Rev. Biochem. 68, 905–1014
3. Jones, P. F., Jakubowicz, T., and Hemmings, B. A. (1999) Cell Regul. 10, 1091–1099
4. Jones, P. F., Jakubowicz, T., Pintossi, F. J., Maurer, F., and Hemmings, B. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 98, 4171–4175
5. Cheng, J. G., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamil- lont, T. C., Tsichlis, P. N., and Testa, J. N. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9267–9271
6. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1999) Mol. Cell 39, 803–810
7. Frodin, M. (1999) Biochem. J. 339, 589–601
8. Nakatani, K., Sakaue, H., Thompson, D. A., Fujin, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) Nature 398, 579–601
9. Dimmeler, S., Fleming, I., Fischli, H.,和 Renz, R., and Zeiher, A. M. (1999) Circ. Res. 85, 1718–1725
10. Zundel, W., and Giaccia, A. (1998) Cancer Res. 58, 2485–2490
11. Galili, N., Davis, R. J., Fredericks, W. J., Mukhopadhyay, S., Rauscher, F. J. D., Emanuel, B. S., Rovera, G., and Barr, F. G. (1993) Oncogene 8, 261–269
62. Schubert, K. M., Scheid, M. P., and Duronio, V. (2000) *J. Biol. Chem.* **275**, 13330–13335
63. Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P., and Alessi, D. R. (1998) *Biochem. J.* **331**, 299–308
64. Groenen, P. J. T. A., Wansink, D. G., Coerwinkel, M., van den Broek, W., Jansen, G., and Wieringa, B. (2000) *Hum. Mol. Genet.* **9**, 605–616
65. Chalfant, C. E., Watson, J. E., Bisnauth, L. D., Kang, J. B., Patel, N., Obeid, L. M., Eichler, D. C., and Cooper, D. R. (1998) *J. Biol. Chem.* **273**, 910–916
66. Blobe, G. C., Strohling, D. S., Fabbro, D., Stabel, S., and Hannun, Y. A. (1996) *J. Biol. Chem.* **271**, 15823–15830
67. Andjelkovic, M., Maira, S.-M., Cron, P., Parker, P. J., and Hemmings, B. A. (1999) *Mol. Cell. Biol.* **19**, 5061–5072
Two Splice Variants of Protein Kinase Bγ Have Different Regulatory Capacity Depending on the Presence or Absence of the Regulatory Phosphorylation Site Serine 472 in the Carboxyl-terminal Hydrophobic Domain
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