A Human Protein Kinase Bγ with Regulatory Phosphorylation Sites in the Activation Loop and in the C-terminal Hydrophobic Domain

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We have cloned human protein kinase Bγ (PKBγ) and found that it contains two regulatory phosphorylation sites, Thr308 and Ser472, which correspond to Thr308 and Ser473 of PKBα. Thus it differs significantly from the previously published rat PKBγ. We have also isolated a similar clone from a mouse cDNA library. In human tissues, PKBγ is widely expressed as two transcripts. A mutational analysis of the two regulatory sites of human PKB showed that phosphorylation of both sites, occurring in a phosphoinositide 3-kinase-dependent manner, is required for full activity. Our results suggest that the two phosphorylation sites act in concert to produce full activation of PKBγ, similar to PKBα. This contrasts with rat PKBγ, which is thought to be regulated by 3-phosphoinositide-dependent protein kinase 1 alone.

Three members of the protein kinase B (PKB)γ subfamily of second-messenger regulated serine/threonine protein kinases have been identified and termed α, β, and γ (1–4). The isoforms are homologous, particularly in regions encoding the N-terminal pleckstrin homology (PH) and the catalytic domains. PKBs are activated by phosphorylation events occurring in response to phosphoinositide 3-kinase (PI3K) signaling (5–8). PI3K phosphorylates membrane inositol phospholipids, generating the second messengers phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (reviewed in Ref. 9), which have been shown to bind to the PH domain of PKB (10, 11). The current model of PKB activation proposes recruitment of the enzyme to the membrane by 3′-phosphorylated phosphoinositides, where phosphorylation of the regulatory sites of PKB by the upstream kinases occurs (12–14).

Phosphorylation of PKBα/β occurs on two regulatory sites, Thr308/309 in the activation loop in the catalytic domain and Ser473/474 in the C-terminal domain (15, 16). The upstream kinase, which phosphorylates PKBα at the activation loop site Thr308 has been cloned and termed 3-phosphoinositide-dependent protein kinase 1 (PDK1; Refs. 17–20). PDK1 phosphorylates not only PKBα, but also equivalent sites in the p70 ribosomal S6 kinase (21, 22), protein kinase A (23), and protein kinase C (24). The upstream kinase phosphorylating the second regulatory site of PKBα/β, Ser473/474, has not been identified yet, but a recent report implies a role for the integrin-linked kinase (ILK-1), a serine/threonine protein kinase (25).

Only a few studies have been reported on the third member of the PKB family, PKBγ, and these all involved a clone originating from a rat brain cDNA library (4, 26). A major feature distinguishing rat PKBγ from the otherwise very similar α and β isoforms is the C terminus, which is truncated by 23 amino acids and lacks Ser473/474, one of the two phosphorylation sites essential for activation of PKBα and β (15, 16). Consequently, it has been suggested that rat PKBγ activation depends solely on the upstream kinase PDK1. We now report the cloning and characterization of human PKBγ. This isoform differs significantly from the rat enzyme in that it contains a C-terminal domain similar to PKBα/β, with a putative second regulatory phosphorylation site at Ser472.

EXPERIMENTAL PROCEDURES

Cloning of Human HA-PKBγ and Mutant Isoforms—A 525-bp PCR product corresponding to nucleotides 815–1340 of the rat PKBγ sequence (4) was amplified from mouse brain cDNA and used to screen several different human cDNA libraries. Twelve overlapping clones were assembled into a cDNA encoding amino acids 16–479 of human PKBγ. This cDNA was repaired by PCR-mediated addition of a hemagglutinin (HA) tag and the missing N-terminal amino acids deduced from the rat PKBγ sequence and ligated as a KpnI/BamHI fragment into the pCMV5 eucaryotic expression vector (27). Subsequently, the 5′ end of PKBγ was amplified by 5′–rapid amplification of cDNA ends (RACE) primers (HA-PKBγ) and extended overlapping clones which could be assembled into a cDNA encoding the entire reading frame of mouse PKBγ. Mutations in HA-PKBγ (HA-PKBγ-T305A and HA-PKBγ-T305D) were done by Quikchange (Stratagene) or with mutagenizing 3′ primers (HA-PKBγ-T305A and HA-PKBγ-T305D). HA-ΔPHPKBγ was obtained by PCR with a primer encoding the HA-tag and amino acids 119–126. All PCR-cloned constructs were verified by DNA sequencing.

Northern Blot Analysis—Human adult and fetal multiple tissue Northern blots (CLONTECH) were hybridized with a 852-bp fragment encoding amino acids 110–384 of PKBγ according to the manufacturer’s instructions.

Cell Culture, Immunoprecipitation, in Vitro Kinase Assays, and Immunoblot Analysis—Human embryonic kidney (HEK) 293 cells were maintained and transfected by a modified calcium phosphate method as described previously (15, 28). Stimulation was for 5 min with 0.2 mM pervanadate (7) or for 15 min with 500 nM insulin (Boehringer Mannheim). Pretreatment with the PI3K inhibitor wortmannin (200 nM; gift of Dr. Markus Thelen, Theodor Kocher Institute, Bern, Switzerland) was for 15 min. Cells were extracted and HA-PKBγ activity determined exactly as described in Ref. 28. Western blot analysis was performed as described before (15) and developed with the polyclonal phospho-specific Ser473 antibody (1:1000, New England Biolabs), an alkaline phosphatase (AP)-coupled goat-anti mouse IgG secondary antibody (1:2000, Sigma), and alkaline phosphatase color development reagents (Boehringer Mannheim).

RESULTS AND DISCUSSION

Screening of several human cDNA libraries led to the isolation of 12 clones encoding partial and overlapping sections of the open reading frame of human PKBγ and the cDNA was

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Thr\textsuperscript{305} and Ser\textsuperscript{472} Regulate Human PKB\textgreek{g}

Fig. 1. Sequence alignment of human PKB\textgreek{a}, PKB\textgreek{b}, PKB\textgreek{c}, mouse PKB\textgreek{y}, and the C-term of rat PKB\textgreek{y}. Shown are the amino acid sequences of human PKB\textgreek{a} (1), PKB\textgreek{b} (3), and PKB\textgreek{c}, of mouse PKB\textgreek{y}, and the C-terminal domain of rat PKB\textgreek{y} (4). The numbering refers to PKB\textgreek{a}. The regulatory phosphorylation sites (Thr\textsuperscript{308} and Ser\textsuperscript{472} in PKB\textgreek{g}) are indicated with an asterisk.

Protein contained the Ser\textsuperscript{472} domain. However, human and rat PKB\textgreek{y} diverge in amino acid sequence precisely at a site where an exon boundary has been mapped for the mouse PKB\textgreek{a} gene (29). Thus, it is possible that the published rat cDNA sequence constitutes a minor splice variant of PKB\textgreek{y} or a partially processed mRNA.

To assess the tissue distribution of transcripts encoding PKB\textgreek{y}, we used an isoform-specific radiolabeled cDNA fragment to probe two human multiple tissue Northern blots. Two equally expressed transcripts of 8.5 and 6.5 kilobases were detected in all tissues tested, with highest levels found in adult brain, lung, and kidney and very low levels in heart and liver (Fig. 2A). Two transcripts of similar size were detected in fetal tissues (Fig. 2B), with high levels found in heart, brain, and liver, but none in the kidney. This observation, and the size of the transcripts, which are much larger than the 3.2–3.4-kilobase transcripts of PKB\textgreek{a} and PKB\textgreek{b}, indicated the presence of long untranslated regions, and thus the possibility of developmental regulation of expression or post-transcriptional modifications affecting mRNA stability.

In contrast to the rat enzyme, human PKB\textgreek{y} contains two predicted regulatory phosphorylation sites, Thr\textsuperscript{305} in the activation loop, and Ser\textsuperscript{472} in the C-terminal domain. Does PKB\textgreek{a}/\textgreek{b} to PKB\textgreek{c}, and the C-terminal domain of rat PKB\textgreek{y} to PKB\textgreek{a} diverge in amino acid sequence precisely at a site where an exon boundary has been mapped for the mouse PKB\textgreek{a} gene (29). Thus, it is possible that the published rat cDNA sequence constitutes a minor splice variant of PKB\textgreek{y} or a partially processed mRNA.

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In contrast to the rat enzyme, human PKB\textgreek{y} contains two predicted regulatory phosphorylation sites, Thr\textsuperscript{305} in the activation loop, and Ser\textsuperscript{472} in the C-terminal domain, as does PKB\textgreek{c}/\textgreek{b}. To determine the importance of these two residues, we mutated them to alanine which cannot be phosphorylated, or to aspartate to mimic the phosphorylated state, and assayed HA-PKB\textgreek{y} kinase activity following transient transfection and stimulation with the insulin mimetic compound pervanadate (Fig. 3). The results presented here show that wild type HA-PKB\textgreek{y}, which had a low basal activity, could be stimulated 67-fold by pervanadate treatment; furthermore, mutation of Thr\textsuperscript{305} to alanine completely ablated activation. No activity above basal levels was observed for HA-PKB\textgreek{a} Thr\textsuperscript{305} or a double mutant HA-PKB\textgreek{a} Thr\textsuperscript{305}, Ser\textsuperscript{472}. On the other hand, mutation of the C-terminal regulatory site to Ser\textsuperscript{472} (HA-PKB\textgreek{a} Ser\textsuperscript{472}) reduced but did not abolish activation by pervanadate (35-fold), albeit to a lesser extent. Thus the aspartic acid moiety could not substitute for the phosphorylated serine residue.

Furthermore, mutant HA-PKB\textgreek{a} Ser\textsuperscript{472} (HA-PKB\textgreek{α} Ser\textsuperscript{472}) reduced but did not abolish activation by pervanadate (35-fold), albeit to a lesser extent. Thus the aspartic acid moiety could not substitute for the phosphorylated serine residue.

Fig. 2. Northern blot analysis of PKB\textgreek{y} expression in human tissues. A, adult and B, fetal multiple tissue Northern blots were probed for expression of PKB\textgreek{y} with a [α-\textsuperscript{32}P]dATP-random-prime-labeled probe derived from a 825-bp fragment of the human PKB\textgreek{y} cDNA spanning amino acids 110–384 and exposed for 3 days at −70 °C. RNA molecular weight markers (in kilobases) are indicated.

1) M. C. Hoops, D. L. Zipke, M. G. Loewen, and E. D. Klee, unpublished results.
extent than the wild type. These results establish that the phosphorylation site Thr\textsuperscript{305} in the activation loop is absolutely necessary for activation of PKB\textgamma, with conformational constraints around the active site apparently so stringent that substitution by a negatively charged residue is not tolerated.

To determine the role of the C-terminal Ser\textsuperscript{472} in regulation of human PKB\textgamma, we tested whether activation of HA-PKB\textgamma, HA-PKB\textgamma:S472A, and HA-PKB\textgamma:S472D by insulin was sensitive to inhibition of PI3K. Fig. 4A depicts the results of a representative experiment, which show that insulin activated HA-PKB\textgamma, HA-PKB\textgamma:S472D, and, to a lesser extent, HA-PKB\textgamma:S472A. This stimulation was dependent on the activity of PI3K, since pretreatment of transfected cells with the PI3K inhibitor wortmannin inhibited activation by insulin. Furthermore, we subjected the immunoprecipitated proteins to Western blot analysis with an antibody generated specifically against the phosphorylated Ser\textsuperscript{473} peptide of PKB\textgamma (Fig. 4A, inset). The antibody cross-reacted with HA-PKB\textgamma only upon stimulation with insulin, and phosphorylation of Ser\textsuperscript{472} was prevented by wortmannin. Since Ser\textsuperscript{472} was mutated in HA-PKB\textgamma:S472A and HA-PKB\textgamma:S472D and could not be phosphorylated, we concluded that the wortmannin-sensitive, insulin-stimulated activity of these proteins was entirely due to phosphorylation at Thr\textsuperscript{305}, dependent on the presence of 3-phosphorylated phospholipids.

In this analysis, we also included PKB\textgamma constructs lacking the N-terminal PH domain. In the basal state, this domain is thought to restrict access to the phosphorylation site in the activation loop, thus leaving Thr\textsuperscript{305} more accessible to phosphorylation by upstream kinases when it is removed. The proteins lacking the PH domain now presented a different picture (Fig. 4B): HA-ΔPHPKB\textgamma was maximally activated under basal conditions and could not be stimulated further by insulin treatment. This contrasts with results obtained for ΔPHPKBs, shown to be activated by insulin (6). Furthermore, pretreatment of the cells with wortmannin led to a reduction in activity of HA-ΔPHPKB\textgamma, indicating that it was still a target for PI3K-dependent phosphorylation. HA-ΔPHPKB\textgamma:S472A activity was comparable with that of wortmannin-treated HA-ΔPHPKB\textgamma and was not responsive to insulin or wortmannin. In contrast, HA-ΔPHPKB\textgamma:S472D was again fully active in the absence of stimulation but, unlike HA-ΔPHPKB\textgamma, was not inhibited by wortmannin. The Western blot signals with the phospho-specific Ser\textsuperscript{473} antibody correlated with the activities observed (Fig. 4B, inset); HA-ΔPHPKB\textgamma was strongly phosphorylated in extracts of unstimulated and insulin-stimulated cells, but pretreatment with wortmannin reduced the signal. We found previously that transiently transfected PDK1, the upstream kinase phosphorylating Thr\textsuperscript{305} in PKB\textgamma, is active in serum-starved HEK-293 cells. The present results seem to indicate that removal of the PH domain causes a conformational change of PKB\textgamma favorable to phosphorylation at Thr\textsuperscript{305}, so as to make it independent of PI3K activity. Basal activity of PI3K in unstimulated cells allowed phosphorylation of HA-ΔPHPKB\textgamma by the Ser\textsuperscript{472} kinase, resulting in full activation. Conversely, inhibiting this basal PI3K activity by wortmannin treatment reduced HA-ΔPHPKB\textgamma activity, probably due to the rapid action of phosphatases on phosphorylated Ser\textsuperscript{472}. Thus, HA-ΔPHPKB\textgamma is a model for studying phosphorylation of Ser\textsuperscript{472}, the second regulatory site of human PKB\textgamma, almost independent of Thr\textsuperscript{305}.

In summary, we report the cloning and characterization of human PKB\textgamma, a PKB isofrom distinct from its rat counterpart in having two regulatory phosphorylation sites, Thr\textsuperscript{305} and Ser\textsuperscript{472}, both of which are required for full activation of the protein. Our results suggest markedly similar regulation mechanisms for PKB\textgamma and the α and β isofoms, with both upstream kinases phosphorylating the regulatory sites being sensitive to PI3K-derived signals. Furthermore, we found a high abundance of PKB\textgamma mRNAs encoding the C-terminal hydrophobic domain and have isolated a similar mouse PKB\textgamma, showing that this isoform is not restricted to humans. Taken together, we conclude that the truncated rat PKB\textgamma used in all studies so far (26) probably constitutes a minor splice variant of endogenous PKB\textgamma protein. The crucial question now emerging is that of the specific roles of the three different PKB isofoms.

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