Mitogenic Activation, Phosphorylation, and Nuclear Translocation of Protein Kinase Bβ*

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Roger Meier, Dario R. Alessi‡§, Peter Cron, Mirjana Andjelkovic, and Brian A. Hemmings†

From the Friedrich Miescher Institute, P. O. Box 2543, CH-4002 Basel, Switzerland and the MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, United Kingdom

Protein kinase B (PKB) is a member of the second messenger-dependent family of serine/threonine kinases that has been implicated in signaling pathways downstream of growth factor receptor tyrosine kinases and phosphatidylinositol 3-kinase. Here we report the characterization of the human β-isoform of PKB (PKBβ). PKBβ is ubiquitously expressed in a number of human tissues, with mRNA and protein levels elevated in heart, liver, skeletal muscle, and kidney. After transfection into HEK-293 or COS-1 cells, PKBβ is activated 2- to 12-fold by mitogens and survival factors. Activation was due to phosphorylation on Thr-309 and Ser-473, which correspond to Thr-308 and Ser-474 implicated in the regulation of PKBo. Both phosphorylation and activation were prevented by the phosphatidylinositol 3-kinase inhibitor wortmannin. Moreover, membrane-targeted PKBβ was constitutively activated when overexpressed in HEK-293 cells. Although the specific activity of PKBβ was lower than that of PKBo toward Crosstide as a substrate (23 nmol/min/mg compared with 178 nmol/min/mg for PKBo), both enzymes showed similar substrate specificities. Using confocal microscopy, we show that activation of PKBβ results in its nuclear translocation within 20 to 30 min after stimulation. These observations provide evidence that PKBβ undergoes nuclear translocation upon mitogenic activation and support a role for PKB in signaling from receptor tyrosine kinases to the nucleus through phosphatidylinositol 3-kinase.

A major effect resulting from activation of receptor tyrosine kinases by growth- and survival factors is the increase in the activity of PI3K (reviewed in Ref. 1). PI3K phosphorylates phosphoinositides at the D3 position of the inositol ring, thus producing molecules that are thought to function as second messengers. Recent data indicate that protein kinase B (PKB; also termed Akt) is a physiological target of PI3K (2, 3). Most of our current understanding of the regulation of PKB is derived from studies of the α-isoform (reviewed in Ref. 4).

PKBα is activated by many growth factors, including PDGF, EGF, FGF, insulin and IGF-1, and also by the protein phosphatase inhibitors pervanadate, okadaic acid, and calyculin A (2–8). Activation of PKBα is mediated by growth factor receptor tyrosine kinase stimulated PI3K, which is wortmannin-sensitive (2, 3, 5, 6). PKBα activation requires phosphorylation on both Thr-308 and Ser-473, and the phosphorylation of these residues is prevented by wortmannin (8). The phosphorylation of Thr-308 is apparently catalyzed by a 3-phosphoinositide-dependent protein kinase (PDK1), which is active only in the presence of phosphatidylinositol-3,4,5-triphosphate and phosphatidylinositol-3,4-biphosphate (9). However, PKBα may also be activated by direct interaction of phosphatidylinositol-3,4-biphosphate with the PH domain in vitro (10, 11) and in vivo (12).

Two further isoforms termed PKBβ and γ (13–15), which display an overall structure similar to that of PKBα, have also been cloned. Both isoforms consist of an amino-terminal PH domain (16) adjacent to the kinase catalytic domain and a short carboxyl-terminal Ser/Thr-rich regulatory domain. PKBα and β-are amplified and overexpressed in a number of ovarian (14), gastric (17), pancreatic (18) and breast carcinomas (19). Although the mechanism by which PKB contributes to neoplastic transformation and proliferation is still unclear, PKBβ has been implicated in a number of cellular responses. In particular, PKBα phosphorylates and inhibits GSK-3 (6), lies upstream of p70 ribosomal S6 protein kinase (2) and stimulates glucose uptake in adipocytes by promoting GLUT4 translocation to the plasma membrane (20). Furthermore, PKBα is involved in the promotion of cell survival through inhibition of apoptosis (21), plays a role in PI3K-mediated neuronal cell survival (22), suppression of c-Myc induced apoptosis (23) and IL-2 signaling (24). Its ability to promote cell survival was shown to be directly proportional to kinase activity that modulates Ced3/ICE-like protease activity in fibroblasts (25).

Here we report the characterization of human PKBβ. We show that it is ubiquitously expressed, activated by mitogens and survival factors in a PI3K-dependent manner, and that activation is dependent upon phosphorylation on Thr-308 and Ser-473 (the corresponding residues in PKBα are Thr-308 and Ser-471). Furthermore, we show that PKBβ and α display similar substrate specificities and present evidence that activation of PKBβ results in its translocation to the nucleus.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Stimulation Conditions—Monkey kidney COS-1, human embryonic kidney (HEK)-293, and rat embryo fibroblast (REF52) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS at 37 °C in a humidified incubator in 5% CO₂. For transient transfection of COS-1 cells, 3 μg of plasmid DNA was introduced using a DEAE-dextran method (26). Plasmid DNA (10 μg) was transfected into HEK-293 or REF52 using the calcium phosphate method (27). Transiently transfected cells were serum starved for 24 h, followed by stimulation with 100 nM insulin (Boehringer Mannheim), 50 ng/ml IGF-1 (Boehringer-Mannheim), 100 ng/ml EGF...
FIG. 1. Tissue distribution of PKBβ protein. A, comparison of COOH termini of PKBa and -β. Amino acids in boldface were used as peptide immunogens for the Ab C-511 (PKBβ) and for the PKBα-specific Ab C-520. B, characterization of the PKBβ-specific Ab C-511. HA-PKBβ and α were transiently expressed in HEK-293 cells, immunoprecipitated with the anti-HA mAb 12CA5, separated by 10% SDS-PAGE, and immunoblotted with either Ab C511 (β-specific) or Ab C520 (α-specific). C, total protein (20, 40, and 60 μg) of PKBα overexpressed in HEK-293 cells was immunoblotted with the PKBβ-specific Ab C511. D, total protein extracts (50 μg/lane) obtained from the indicated tissues were subjected to Western blot analysis using affinity-purified polyclonal Ab C511.

(Life Technologies, Inc.), 50 ng/ml PDGF-BB (Life Technologies, Inc.), 10% FCS, 0.1 mM pervanadate (7), or 100 ng/ml TPA (Life Technologies, Inc.). Pretreatment with 100 nM wortmannin was carried out as described previously (8).

Construction of Expression Vectors—The cytomegalovirus-based expression construct encoding the human myristoylation/palmitoylation (m/p)-HA-PKBα has been described (38). Wild-type HA-PKBβ was amplified by PCR, either from a human MCF-7 cell cDNA (13) or placenta cDNA and subcloned as a KpnI/XbaI fragment into the pECE or pCMV4 expression vectors. The primers were designed according to the human PKBβ/akt2 sequence (14). PKBβ from human placenta carried a polymorphism (Thr-20 to Ala substitution) that did not affect its kinase activity compared with the MCF-7 cell line-derived PKBβ (data not shown). The pCMV4 constructs encoding HA-T309A-PKBβ and HA-T474A-PKBβ, respectively, were made by two-stage PCR (28) and subcloned as KpnI/XbaI fragments into pCMV4. The membrane targeting pECE-m/p-HA-PKBβ construct was made by PCR to add 12 amino acids derived from the NH2 terminus of the Lck tyrosine kinase3 to the NH2-terminus of HA-PKBβ. The PCR fragment was subcloned as a KpnI/XbaI fragment into pECE. The correctness of the constructs was confirmed by restriction analysis and automated DNA sequencing. The sequences of all oligonucleotides used for modifications of PKBβ are available upon request.

Antibody Purification and Immunoblot Analysis—Synthetic peptides corresponding to the C-terminal domain of human PKBa (DQDDS-MECVDSSERR) or -β (DRYDSGLLELDQRTF) were coupled to key-hole limpet hemocyanin and used to immunize rabbits (HTI, Bio-Prod-

FIG. 2. Growth factor-induced and PI3K-dependent activation of PKBβ. HA-PKBβ was transiently expressed in COS-1 and HEK-293 cells. After serum starvation for 24 h, cells were stimulated, lysed, and assayed for PKB kinase activity as described under “Experimental Procedures.” Kinase activity is the average (mean ± S.D.) of two independent experiments assayed in duplicate and shows activities relative to PKBβ activity in unstimulated cells. A, serum-starved COS-1 cells expressing PKBβ were stimulated with 10% FCS (15 min), 0.1 mM pervanadate (15 min), 50 ng/ml PDGF (15 min), 100 ng/ml EGF (15 min), and 100 ng/ml TPA (10 min) or left untreated (Control). B, PKBβ kinase activity in quiescent transiently transfected HEK-293 cells (Control) or stimulation with 0.1 mM pervanadate (15 min), 50 ng/ml IGF-I (10 min), and 100 nM insulin (10 min). C, HEK-293 transiently transfected with HA-PKBβ were starved for 24 h and were left untreated (Control) or were stimulated for 10 min with either 50 ng/ml IGF-I or 100 nM insulin in the presence or absence on 100 nM wortmannin that was added 15 min prior to stimulation. D, activation of endogenous PKBβ is accompanied by electrophoretic mobility shift. HEK-293 cells were starved for 24 h and stimulated for 15 min with either 0.1 mM pervanadate or left untreated. After immunoprecipitation with the Ab C511, PKBβ was separated by 7.5% SDS-PAGE and detected with the same antibody.

*3B. A. Hemmings and P. Cron, unpublished results.
Immunofluorescence Staining and Laser Confocal Scanning Microscopy—HEK-293 cells were grown on glass coverslips to 80% confluency, washed with PBS, and fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. Permeabilization of the cells was achieved through incubation with PBS containing 0.2% Triton X-100 for 10 min followed by a PBS wash. For detection of HA-PKBβ, cells were incubated for 1 h at 37 °C with the 12CA5 antibody diluted 1:10 in PBS. After washing with PBS, cells were incubated for 30 min with goat anti-rabbit IgG-FITC conjugate (Sigma) diluted 1:50. The cells were washed with PBS followed by water and mounted in Moviol supplemented with 0.1% p-phenylenediamine. Laser confocal scanning images were obtained using a TCS 4D confocal system (Leica Instruments).

RESULTS

Expression of PKBβ in Human Tissue—To study the tissue distribution of PKB at the protein level, we developed peptide-specific antisera to both the PKBβ and -α isoforms (Abs C511 and C520, respectively, see Fig. 1A). Specificity of the antibodies was established by expressing epitope-tagged cDNAs corresponding to the β and α isoforms of PKB. HA-PKBβ and -α were transiently expressed in HEK-293 cells, immunoprecipitated with the HA-specific mAb 12CA5 and analyzed by 10% SDS-PAGE. Immunoblots probed with either Ab C511 or Ab C520 revealed a high degree of specificity for PKBβ and -α, respectively (Fig. 1B). The Ab C511 (β-specific) detected a polypeptide with an apparent molecular mass of 60 kDa on immunoblots derived from HEK-293 total cell extracts transfected with HA-PKBβ (Fig. 1C). This size was similar to that of the in vitro translated human PKBβ (14). Western blot analysis of PKBβ expression in human tissues (Fig. 1D) was consistent with the Northern blot data (data not shown): PKBβ and -α were expressed in all tissues analyzed but were most abundant in heart, liver, skeletal muscle, and kidney. Expression of PKBβ in brain, placenta, lung, and pancreas was significantly lower.

Regulation of PKBβ Activity—To investigate the regulation of PKBβ, we determined its activity in response to different stimuli. For the experiments outlined below, we used a PKBβ cDNA, isolated from a placenta cDNA library, which corresponds to the human PKBβ sequence described previously (14). Transiently transfected and serum starved HEK-293 or COS-1 cells were treated with insulin, IGF-1, FCS, pervanadate, PDGF-BB, EGF, or TPA. HA-PKBβ was immunoprecipitated and tested for in vitro kinase activity using Crosstide as substrate, which is a peptide derived from the NH₂-terminus of GSK-3 (6). These experiments (Fig. 2, A and B) revealed increased PKBβ activity in response to every stimuli tested except TPA treatment. Pervanadate was the most potent activator (12-fold activation) in both cells lines. PDGF and EGF each caused a 6-fold activation of PKBβ in COS-1 cells, while 5- and 7-fold activation occurred in response to insulin and IGF-1, respectively. FCS treatment caused a 2-fold increase above basal activity. To test whether PKββ activation was PI3K-de-
pendent, we treated transiently transfected HEK-293 cells with 100 nM wortmannin for 15 min prior to stimulation. Similar to its effects on PKBa, wortmannin abolished the activation of PKBb in response to insulin or IGF-1 stimulation (Fig. 2C).

Endogenous PKBb from pervanadate-treated HEK-293 cells recovered by immunoprecipitation with Ab C511 showed a 3-fold activation compared with unstimulated PKBb, and recovery of PKBb was blocked by addition of the antigenic peptide (data not shown). Similarly, using a different PKBb antibody (raised against the carboxyl-terminal peptide CRYDSLGSLELDQRT), IGF-1 stimulation of HEK-293 cells caused a 2–3-fold wortmannin-sensitive activation of PKBb.

The analysis of total cell extracts from quiescent and pervanadate-stimulated HEK-293 cells by Western blotting revealed a substantial decrease in the electrophoretic mobility of endogenous PKBb. Thus, the protein appeared as a single major band in quiescent cells and formed a second, slower migrating band after stimulation with pervanadate (Fig. 2D).

To compare the specific activities of PKBb and -a, we measured the activity of each enzyme toward various peptide substrates. Transiently expressed wild-type HA-PKBb and -a were immunoprecipitated with the 12CA5 antibody from pervanadate-stimulated HEK-293 cells and assayed using peptides related to the sequence surrounding the phosphorylation site on GSK-3. These experiments revealed no significant difference in the substrate specificity of PKBb compared with PKBa. Thus, both isoforms phosphorylated Cross tide (GRPRTSSFAEG) and RPRAATF most efficiently, followed by KKLNRRLSVA and KKLNRRLSVA. KKLNRRLSVA and KKLNRRLSVA proved to be poor substrates for both, PKBb and -a (data not shown). Although we did not observe major differences in substrate specificity, the specific activities of the two isoforms differed significantly. Using Cross tide, the specific activity of PKBb was about 8-fold lower (23 nmol/min/mg) compared with that of PKBa (178 nmol/min/mg) when HEK-293 cells were stimulated with pervanadate (see "Discussion").

Activation of HA-PKBb by Insulin and IGF-1 in HEK-293 Cells Is Accompanied by a PI3K-dependent Phosphorylation of Thr-309 and Ser-474—HEK-293 cells transiently expressing HA-PKBb were 32P-labeled and treated with and without 100 nM wortmannin for 15 min and then stimulated with buffer, insulin, or IGF-1 for 10 min prior to cell lysis. In the absence of wortmannin, HA-PKBb was activated about 7-fold with either insulin or IGF-1, whereas in the presence of wortmannin, no activation occurred in response to insulin or IGF-1 (data not shown). The 32P-labeled HA-PKBb was immunoprecipitated from cell lysates and digested with trypsin, and the resulting peptides were analyzed by C18 chromatography. Three prominent 32P-labeled peptides were present in unstimulated HEK-
Regulation of PKBβ

Overexpression of PKBβ is thought to contribute to the malignant phenotype of some pancreatic, ovarian, and breast carcinomas (30). To investigate the physiological role of PKBβ and how it might contribute to cellular transformation, we undertook a detailed characterization of the enzyme.

Recently, we identified Thr-308 and Ser-473 as positive regulatory phosphorylation sites in PKBα that cause activation of the kinase in response to insulin and IGF-1 (8). Since these regulatory phosphorylation sites are well conserved between the α- and β-isomers, we tested for a similar activation mechanism for PKBβ mediated by mitogenic stimulation. Indeed, like PKBα, PKBβ was activated by mitogens, survival factors, and perversanate but not by agents that activate protein kinase C (Fig. 2A and B). Similarity of function between PKBα and PKBβ is further supported by the following observations. First, the pattern of expression of PKBβ and -α is similar in various tissues although the different levels of expression of the two isoforms suggest that they perform specific functions. Second, like PKBα, PKBβ activation is PI3K-dependent since pretreatment of cells with wortmannin abolished the activation of overexpressed and endogenous PKBβ in response to insulin and IGF-1 treatment (Fig. 2C and data not shown). Wortmannin-sensitive phosphorylation on Thr-309 and Ser-474 was essential for PKBβ activation. In addition, PKBβ was constitutively phosphorylated at Ser-126 and Thr-451 in HEK-293 cells and that insulin and IGF-1 stimulation induces the phosphorylation on Thr-309 and Ser-474.

Membrane-targeting Promotes Constitutive Activation of PKBβ—The PH domain of PKBα is thought to mediate translocation of the enzyme to the plasma membrane where it becomes activated by phosphorylation (4, 38). To test whether a similar mechanism exists for PKBβ, we fused the Lck-derived NHL-terminal motif that promotes myristoylation and palmitoylation and tested whether this motif would promote membrane localization of PKBβ. Wild-type HA-PKBβ and m/p-HA-PKBβ were transiently expressed in HEK-293 cells, and after serum starvation, cytoplasmic localization of wild-type HA-PKBβ (data not shown) and membrane-localization of m/p-HA-PKBβ was confirmed by immunofluorescence (Fig. 6A). Immunoprecipitation of wild-type HA-PKBβ or m/p-HA-PKBβ from cells treated with or without IGF-1 followed by in vitro kinase assays revealed that membrane association led to a 15-fold activation of m/p-HA-PKBβ and the activity of m/p-HA-PKBβ was not further increased by IGF-1 treatment (Fig. 6B).

Translocation of PKBβ from the Cytoplasm to the Nucleus after Mitogenic Stimulation—Previous studies showed that PKB is primarily localized in the cytoplasm of unstimulated cells, but the oncogenic form of PKBα (v-Akt) is present at about equal levels in the plasma membrane, cytoplasm, and the nucleus (29, 30). To test whether activation of PKBβ changes its localization, we expressed HA-PKBβ in HEK-293 or REF-52 cells and examined kinase localization by confocal laser microscopy in serum-starved IGF-1 (HEK-293) or FCS plus okadaic acid (REF-52)-stimulated cells (data not shown and Fig. 7). In unstimulated cells, PKBβ was cytoplasmic in both cell lines. After mitogenic stimulation, the subcellular distribution changed in both HEK-293 and REF-52 cells within 20–30 min with most of the PKBβ being localized to the nucleus.

DISCUSSION

To confirm the in vivo labeling data, various PKBβ mutants (HA-PKBβ, HA-PKBβ-T309A, and HA-PKBβ-S474A) were transiently transfected into HEK-293 cells and assayed following IGF-1 treatment (Fig. 5). IGF-1 activated wild-type PKBβ about 7-fold but failed to activate either the T309A or the S474A mutant, thus demonstrating the importance of Thr-309 and Ser-474 as regulatory phosphorylation sites. Taken together, our data indicate that PKBβ is constitutively phosphorylated at both Ser-126 and Thr-451 in HEK-293 cells and that insulin and IGF-1 stimulation induces the phosphorylation on Thr-309 and Ser-474.
transfected with HA-PKB. Cells were transfected with wild-type or m/p-HA-PKB, were stimulated with 50 ng/ml IGF-1 for 10 min or left untreated. After cell lysis, PKB was immunoprecipitated from total cell lysates using mAb 12CA5 and assayed for in vitro kinase activity as described under “Experimental Procedures.” The results are shown in means ± S.D. for two experiments performed in duplicate.

Control

FCS/OA

REF52

Fig. 6. Membrane targeting causes constitutive activation of PKBβ. A, HEK-293 cells were transiently transfected with m/p-HA-PKBβ. Cells were stained with mAb 12CA5, followed by a FITC-conjugated secondary antibody and analyzed by laser confocal scanning microscopy (see “Experimental Procedures”). B, HEK-293 cells, transiently transfected with wild-type or m/p-HAPKBβ, were stimulated with 50 ng/ml IGF-1 for 10 min or left untreated. After cell lysis, PKBβ was immunoprecipitated from total cell lysates using mAb 12CA5 and assayed for in vitro kinase activity as described under “Experimental Procedures.” The results are shown in means ± S.D. for two experiments performed in duplicate.

Fig. 7. Immunolocalization of PKBβ in REF52 cells. Cells were transfected with HA-PKBβ as described under “Experimental Procedures.” Following starvation, cells were stimulated with 20% FCS supplemented with 1 μM okadaic acid (FCS/OA) for 30 min as indicated or left untreated (Control). After stimulation, cells were fixed, permeabilized, stained with mAb anti-HA 12CA5 followed by a FITC-conjugated secondary antibody, and prepared for analysis by Laser Confocal Scanning Microscopy. Scale bars, 6 μm.

wild-type HA-PKBα resulted in about a 6-fold higher level of phosphorylation of Thr-309 and Ser-474 than that which occurred in cells transiently expressing wild-type HA-PKBβ (Fig. 3 and data not shown). This difference in the degree of phosphorylation and activation of PKBα and -β could be due to the presence of an alternative PI3K-independent activation mechanism (31, 32), or tight control of PKBβ by phosphatases. It also remains to be elucidated whether PKBβ is a substrate for PDK1, a 3-phosphoinositide-dependent Thr-308 kinase of PKBα (9), and it will be important to understand the mechanism by which Ser-474, the second regulatory phosphorylation site in PKB, becomes phosphorylated. Furthermore, differences in enzyme activities could arise from interactions of the α- and β-isozymes with different phosphatidylinositol-phosphates via their PH domains (10, 12).

Data obtained from different cell lines suggest that a significant fraction of PKBβ is translocated from the cytoplasm to the nucleus after activation of the enzyme (Fig. 7 and data not shown). We recently made a similar observation with PKBβ, suggesting that both isoforms translocate to the nucleus. The finding that activated PKBβ, which lacks a nuclear localization signal, accumulates in the nucleus indicates a different mechanism by which translocation occurs. One possible mechanism to account for this result is the activation-dependent association of PKB with a second protein that provides the nuclear import signal. The existence of such a mechanism has been reported for stimulation-dependent MAP kinase translocation by MAP kinase kinase (33, 34). Partial nuclear translocation has also been observed with the constitutively activated v-Akt (2), a protein carrying a viral Gag sequence (29). The presence of one myristoylation signal in the Gag sequence is not sufficient to stably anchor the protein to the membrane (35). This may explain why (activated) v-Akt can translocate to the nucleus, but m/p-PKBβ, which contains two additional palmitoylation signals (36), is stably associated to the membrane and therefore cannot translocate (Fig. 6A).

In conclusion, the results presented here provide the basis for the understanding how PKBβ contributes to cellular transformation. The fact that PKBβ translocates to the nucleus following stimulation with mitogens or survival factors suggest that transcription factors could be important in this context. The identification of authentic substrates of the different isoforms of PKB will provide considerable insight into this signaling cascade.

3 M. Andjelkovic, unpublished observations.

4 N. Lamb, A. Fernandez, and B. A. Hemmings, unpublished data.
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