A Novel Protein Kinase B (PKB)/AKT-binding Protein Enhances PKB Kinase Activity and Regulates DNA Synthesis*

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Protein kinase B (PKB)/Akt reportedly plays a role in the survival and/or proliferation of cells. We identified a novel protein, which binds to PKB, using a yeast two-hybrid screening system. This association was demonstrated not only in vivo by overexpressing both proteins or by communoprecipitation of the endogenous proteins, but also in vitro using glutathione S-transferase fusion proteins. Importantly, this protein specifically associates with the C terminus of PKB but not with other AGC kinases and enhances PKB phosphorylation and kinase activation without growth factor stimulation. Thus, we termed this Akt-specific binding protein APE (Akt-phosphorylation enhancer). Since APE-induced phosphorylation of PKB did not occur in cells treated with wortmannin or LY294002, APE itself is not a kinase but seems to enhance or prolong the phosphoinositide 3-kinase-dependent phosphorylation of PKB. In cells in which APE was suppressed by small interfering RNA, DNA synthesis was significantly reduced with suppression of PKB phosphorylation, suggesting a synergistic role of APE in PKB-induced proliferation. On the other hand, in cells overexpressing both PKB and APE, despite markedly increased basal phosphorylation of PKB, both DNA rereplication and subsequent Chk2 phosphorylation and apoptosis were seen, suggesting the involvement of APE in the regulation of cell cycling replication licensing. Taking these observations together, APE appears to be a novel regulator of PKB phosphorylation. Furthermore, the interaction between APE and PKB, possibly dependent on the expression levels of both proteins, may be a novel molecular mechanism leading to proliferation and/or apoptosis.

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a v-Akt transformed lung epithelial cell line to wild type (11). TRB3 was also identified as a negative modulator of the PKB type (12), although a contradictory report was very recently published (13). These results indicate that understanding PKB modulation is important for elucidating the mechanism of PKB activation and its regulation of cellular functions.

In this study, we identified a novel protein that interacts with PKB (in vivo and in vitro). Without growth factor stimulation, overexpression of this protein markedly enhances phosphorylation of Thr208 and Ser473 in PKB, leading to its kinase activation and phosphorylation of its downstream substrates such as GSK-3 and FKHR. In addition, suppression of APE using RNA interference significantly reduces PKB phosphorylation and PKB kinase activity. Therefore, we termed this protein APE (Akt-phosphorylation enhancer) and herein demonstrate the possible role of APE in DNA synthesis and apoptosis in cooperation with PKB.

MATERIALS AND METHODS

Yeast Two-hybrid System—The DupLEX-A two-hybrid system (OriGene) was used for screening. We inserted cDNA into the yeast expression library with the pG4-5 vector with a bait protein corresponding to the full length of mouse PKBα using a pEG202 vector and yeast strain EGY48. The positive clones were selected and assayed for β-galactosidase activity. Plasmid DNAs were isolated from positive clones and co-transformed with bait cDNA or negative control cDNA back into yeast to reconfirm the interaction. A yeast β-galactosidase assay kit (Pierce) was used to measure the protein-protein interaction according to the manufacturer’s instructions.

Northern Blotting—Mouse Multiple Tissue Northern blot (Clontech) was used for Northern blotting. APE cDNA corresponding to the 600 bp of the coding region of the C-term and 400 bp of the untranslated region was used as a probe.

Antibody against APE—Fragments of the cDNA clone were subcloned into pGEX-6P-1 glutathione-S-transferase (GST) expression vector (Amerham Biosciences), expressed in BL21 and purified using glutathione-coupled Sepharose beads. Purified GST fusion proteins were injected into rabbits, and antisera were affinity-purified using the respective antigens. APE-C was generated to a fragment of APE encompassing amino acids 1464–1845. APE-N1 corresponds to amino acids 172–372, and APE-N3 to 501–601. Polyclonal antibodies to each antigen were affinity-purified, using each GST fusion protein, after removal of GST-specific antibody.

Gene Constructions and Expression System in Yeast and Mammalian Cells—Full-length APE cDNAs were cloned into the pShuttle vector to express these proteins with an adenovirus expression system (Clontech). The expression cassette was excited and subcloned into pAdeno-X Gene Constructions and Expression System in Yeast and Mammalian Cells. The expression cassette was then subcloned into a fragment of APE encompassing amino acids 1464–1845. APE-N1 corresponded to amino acids 172–372, and APE-N3 to 501–601. Polyclonal antibodies to each antigen were affinity-purified, using each GST fusion protein, after removal of GST-specific antibody.

Gene transduction and in vivo phosphorylation of PKB—Mamalian cell lines were infected with adenovirus the day after plating. Purified virus was added directly to the culture medium. Titers of adenovirus for protein overexpression were adjusted so that the exposure levels of the Myc-tagged PKBs were similar, irrespective of APE co-expression. Likewise, APE expression levels were adjusted so as to be similar, irrespective of Myc-tagged PKB co-expression. Experiments were performed 36 h later for in vivo phosphorylation. Cells were starved for 12 h with KRB-Hepes buffer (118.5 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 24.9 mM NaHCO3, 30 mM HEPES, pH 7.4) containing 20 mg/ml bovine serum albumin. Cells were stimulated by the indicated stimulus in each experiment and, whenever indicated, 1 μM wortmannin or 10 μM LY294002 1 h prior to stimulation.

Gene Silencing by siRNA—Gene silencing was performed by an adenovirus-mediated siRNA method. For silencing of endogenous APE gene expression in HepG2 cells, a sequence fragment (GAATTCTCTAGAGTTTGTGAACGGACGCAGCTTCTCGAGACAAAAACATTAACACCCACCCGCTCTCTTGAAGAGCGGGTGGCTTTTTCTAGAGAATTC) and an antisense fragment (GAATTCTCTAGAGTTTGTGAACGGACGCAGCTTCTCGAGACAAAAACATTAACACCCACCCGCTCTCTTGAAGAGCGGGTGGCTTTTTCTAGAGAATTC) were used for human APE. These two oligonucleotides were annealed in vitro, and the resultant double-stranded DNA fragments were subcloned into the BamHI-EcoRI site of a pSIREN- Shuttle vector. A negative control vector was supplied by Clontech. The expression cassette containing siRNA of APE or the negative control was excited and subcloned into pAdenov-X vector.
DNA Synthesis—HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum for 36 h and then transfected with an adenovirus encoding siRNA of APE or the negative control. Seventy-two hours after transfection, culture media were changed to Dulbecco’s modified Eagle’s medium supplemented with 0.2% bovine serum albumin, and cells were incubated for an additional 24 h. The cells were then incubated with BrdUrd labeling solution for 4 h. Incorporated BrdUrd was detected by cell proliferation ELISA, BrdUrd (colorimetric) (Roche Applied Science).

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT) Assay—Cellular proliferation was measured by reduction of MTT, which corresponds to the living cell number and metabolic activity (15). Cells were plated at 5 x 10^4 cells/well in 24-well plates and transfected with adenovirus. After incubation for the indicated time, MTT solution was added to each well. After 1 h of incubation, the reaction was stopped by adding 1 ml of isopropyl alcohol with 0.04 N HCl. The absorbance of each well was measured at 492 and 630 nm using a microplate reader.

Cell Viability Analysis—HepG2 cells and HeLa cells were infected with control GFP, PKB, APE, or both PKB and APE adenoviruses and incubated for the indicated times. Floating cells were recovered from culture medium by centrifugation at 1200 g for 1 min, and adherent cells were harvested by trypsinization. Both the floating and adherent cells were observed for morphologic changes with a light microscope at 200 magnification. We combined the adherent and floating cells and measured their viability by using a trypan blue dye exclusion assay.
Cell Cycle Analysis by Flow Cytometry—For cell cycle synchronization, cells were arrested at the G1-S phase transition separated by two subsequent thymidine blocks (2 mM thymidine) for 14 h, separated by a period of 10 h without thymidine. Both adherent and nonadherent cells were harvested by trypsinization, and an aliquot of $2 \times 10^6$ cells was fixed in ice-cold ethanol for at least 1 h at 4 °C. The cells were collected by centrifugation and resuspended in propidium iodine (10 μg/ml) solution containing RNase for analysis of DNA content. Data were then collected on a BD Biosciences FACScan, 20,000 events/sample, using Cellquest software. DNA content analysis was performed with Verity ModFit software for the Macintosh computer.

RESULTS

Cloning of cDNAs Encoding the Protein Binding with PKB—Using full-length mouse PKB as bait in a yeast two-hybrid screen of an embryonic mouse complementary DNA library, we isolated 31 clones displaying β-galactosidase activity. Sequencing analysis revealed 10 of the clones with the strongest β-galactosidase activity to be identical. In all cases, 606 bp of the coding region were followed by a 3’-untranslated region. Isolation of the full-length cDNA by phage screening of the mouse embryonic cDNA library and a series of 5’ rapid amplifications of cDNA ends by PCR showed the largest open reading frame to be 5538 bp, which encodes a 1845-amino acid protein with a predicted relative molecular mass ($M_r$) of 212,478 (Fig. 1A, accession number AB087827). By searching several databases, we found that some mouse clones (BC037020, BC079895, AK129310) and this cDNA to be identical to a mouse homologue of the Kazusa DNA Research Institute clone KIAA1212. This clone is located on mouse chromosome 11 and on human chromosome 2. Although some cDNAs in the data base are presented as “full-length,” it seems that they are not, judging from the size of the protein shown in this study. Our mouse cDNA is very likely to be full-length, and this protein was subsequently shown to enhance the phosphorylation of PKB, such that we designated the clone APE (Akt-phosphorylation enhancer). Protein analysis of APE revealed it to be a hydrophilic protein, and that its N terminus has a significant similarity with the putative coiled coil domain of the myosin heavy chain (Fig. 1B).

Tissue Distribution of APE—Northern blot analysis detected a 7.9-kb band of APE messenger RNA in the testis (Fig. 2A). Longer exposure revealed moderate expression in the brain, and low expressions in the spleen and lungs (Fig. 2A, upper panel). Anti-APE antibodies were generated against the three different portions of APE (Fig. 1B). Immunoblotting with antibodies against APE, irrespective of the differences in the
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epitopes of these antibodies (Fig. 1B), identified three bands of 220, 213, and 203 kDa in mouse tissues, whereas the control antibody against GST did not recognize any of these bands (Fig. 2B). The largest band of 220 kDa was observed in the lungs, testis, and fat. The 213-kDa band was detected in the brain, testis, heart, and fat. Finally, the smallest (203-kDa) band was detected in the lungs and spleen. These results were similarly obtained by immunoblotting of either immunoprecipitates of

FIG. 4. APE is a PKB-specific binding protein and has a domain responsible for the association. A, PKBα structural domains and pEG202-PKBα fusion proteins. B, in vivo interaction of APE and PKBα structural domain. Interactions of APE-200 amino acids with PKBα, the PH domain, kinase domain, kinase domain with the hydrophobic domain, and the hydrophobic domain of PKBα are indicated as arbitrary units. C, in vivo interactions of PKBα and APE fragments. pEG202-PKBα was expressed in EGY48 with the indicated portion of the C-terminal of APE. β-Galactosidase activity is indicated as arbitrary units. D, in vivo interactions of APE and AGC kinases. pEG202-PKBα was expressed in EGY48 with PKBα, PKBβ, SGK1, SGK2, PKCδ, or PKCε. These in vivo interactions were measured by β-galactosidase activity. E, physical association of APE and PKB in vitro. Extracts from E. coli BL21 cells expressing PKBα or APE with a pET system were used to test for APE or PKBα binding to the following bead matrices: GST beads coupled to either bacterially expressed GST or GST-PKB (amino acids 418–480) or GST-APE (amino acids 1646–1845). Extract bead complexes were washed three times to remove weakly bound protein prior to eluting off specifically bound proteins. The pulled down APE (FLAG-tagged) and PKBα (Myc-tagged) were resolved on an SDS-polyacrylamide gel and detected by α-FLAG or α-Myc antibody.

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tissue lysates (Fig. 2B, upper panel) or nonimmunoprecipitated lysate (Fig. 2B, lower panel). These results suggest the existence of alternatively spliced protein products from the APE gene because a search of human expressed sequence databases indicated the existence of alternatively spliced forms of APE (data not shown).

**In Vivo Association of PKB and APE—** Next, to demonstrate in vivo association between APE and PKB, full-length APE and c-Myc-tagged PKB-H9251 were overexpressed in COS-7 cells. As shown in the upper panel of Fig. 3A, APE was detected in the immunoprecipitate by the anti-Myc antibody (Fig. 3A, upper panel). Similarly, PKB was detected in the anti-APE immunoprecipitate (Fig. 3A, lower panel). This interaction between APE and PKB was demonstrated when both were overexpressed in SF-9 insect cells or HepG2 cells (data not shown).

We also demonstrated an endogenous interaction between PKB and APE by coimmunoprecipitation of the endogenous proteins using specific antibodies in HeLa cells, and mouse testis. As shown in Fig. 3B, APE was coimmunoprecipitated by anti-PKB antibody in HeLa cells. PKB was also coimmunoprecipitated by anti-APE-C antibody as shown in the lower panel. The PKB and APE interaction was confirmed by the same procedure using mouse testis homogenates (Fig. 3C), indicating that the PKB-APE interaction occurs under physiological conditions.

**APE Binds to the C-terminal Portion of PKB but Not to Other AGC Kinases—** To determine the region of PKB responsible for binding with APE, we generated four deletion mutants consisting...
\[ \text{PKB phosphorylation and kinase activity in HepG2 cells.} \]

**A**

HepG2 cells were transfected with adenovirus expressing negative control siRNA or APE siRNA for 60 h, starved for 12 h, and treated with 100 nM insulin for 15 min. A, endogenous PKB were immunoprecipitated with \( \alpha \)-PKB monovalent antibody, and PKB phosphorylation was analyzed by Western blotting using phosphospecific antibodies of Thr208 or Ser473. PKB and APE expression levels were analyzed using cell extracts. B, endogenous PKB immunoprecipitated by \( \alpha \)-PKB monovalent antibody from control or APE-depleted HepG2 cells was also used for an in vitro kinase assay.

**B**

To examine whether the association of APE and PKB occurs rather, a PKB-specific binding protein.

APE is not a common AGC kinase-binding protein but, since the deletion mutant amino acids 101–200 or 1–150 retain the ability to bind PKB, it is likely that the minimal portion necessary for the association with PKB is located within amino acid sequence 101–150 (Fig. 4C).

**C**

PKB belongs to a family of protein kinases, originally including protein kinase A, cGMP-dependent protein kinase and protein kinase C, termed the AGC family. Proteins in this family contain regions of high homology in their kinase domains (1). Since AGC kinases contain regions of high homology with the hydrophobic motif in PKB, we further examined whether APE interacted with AGC kinases other than PKB, using a yeast two-hybrid system. SGK1, SGK2, PKC\( \beta \), PKC\( \gamma \), and PKB\( \beta \)/Akt2 have a kinase domain and a hydrophobic motif highly homologous to those of PKB. As a result, PKB\( \beta \) and PKB\( \gamma \) bind efficiently to APE in yeast (Fig. 4D).

Conversely, very little interaction with APE was observed for SGK1, SGK2, PKC\( \beta \), or PKC\( \gamma \). These results indicate that APE is not a common AGC kinase-binding protein but, rather, a PKB-specific binding protein.

**D**

In vitro association between Amino Acids 418–480 of PKB and Amino Acids 1646–1845 of PKB—A PKB and PKB-\( \gamma \)-P308, PKB-pS473, and PKB-pS473 were expressed using \( \text{E. coli} \) and then purified. As shown in the left panel of Fig. 4E, GST-amino acids 418–480 of mouse PKBα fusion protein bound to His-tagged amino acids 1646–1845 of mouse APE fusion protein, but not GST alone, bound to His-tagged amino acids 418–480 of mouse PKBα (Fig. 4E, right panel). These results indicate that the interaction between APE and PKB is direct.

**E**

APE Markedly Enhances Basal Phosphorylation of PKB—PKBα is activated via phosphorylation of Thr208 in the activation loop of the kinase domain and of Ser473 in the hydrophobic motif of the carboxyl terminus (17–21). To test the effect of APE binding on phosphorylation of PKB, basal phosphorylation of endogenous PKBα cells transfected with GFP adenovirus or APE adenovirus was analyzed. As shown in Fig. 5A, there was no significant phosphorylation on Thr308 or Ser473 of PKBα after 12-h serum starvation (left lane of Fig. 5A), but Thr308 and Ser473 of endogenous PKB were apparently phosphorylated in cells overexpressing APE. A similar result was obtained for HepG2 cells (data not shown).

Pretreatment with pervanadate increased PKB phosphorylation, time-dependently, as reported previously (11). To explore the effect of APE on enhanced PKBα phosphorylation, we treated COS-7 cells with adenoviruses expressing PKBα and various amounts of APE. APE overexpression increased PKB phosphorylation, in a titr dependent manner, and the maximal phosphorylation of PKB obtained by APE overexpression was comparable with that achieved by long term pervanadate stimulation (Fig. 5B). These results suggest that APE overexpression can induce essentially maximal phosphorylation of PKB on Thr308 and Ser473, which indicates that APE is an enhancer of PKB in vivo.

**F**

Phosphorylation of PKB by APE Induces the Phosphorylation of GSK-3\( \beta \) and FKHR—PKBα reportedly phosphorylates several downstream molecules such as GSK-3\( \beta \) and FKHR (17, 22). As a positive control, we confirmed that insulin stimulation induced PKB phosphorylation as well as downstream phosphorylation of Ser256 of FKHR and Ser\( \beta \) of GSK-3\( \beta \) in HepG2 cells. Then we examined whether PKB phosphorylated by the overexpressed APE can induce the phosphorylations of GSK-3\( \beta \) and FKHR without growth factor stimulation. As shown in the right two lanes of Fig. 5C, overexpressed APE markedly enhanced phosphorylation of Ser256 of FKHR and Ser\( \beta \) of GSK-3\( \beta \), to degrees similar to those seen with insulin stimulation.
In Vitro Kinase Activity of PKB Enhanced by APE—To test the influence of APE binding on PKB kinase activity, we assayed kinase activity in immune complexes from transfected COS-7 cells treated with peroxanadate. Peroxanadate-stimulated PKB activity was time-dependently increased when COS-7 cells were transfected with PKB alone, and kinase activity paralleled the phosphorylations of Thr\(^{308}\) and Ser\(^{473}\). APE enhanced the basal phosphorylations of Thr\(^{308}\) and Ser\(^{473}\), and in vitro kinase activity was also maximally enhanced and paralleled these phosphorylations. These results indicate that APE induces maximal basal phosphorylation of PKB, thereby maximally enhancing its kinase activity (Fig. 5D).

PI 3-Kinase Activity Is Needed for APE-induced PKB Phosphorylation—To examine whether the APE-induced increase in PKB phosphorylation is mediated only by PI 3-kinase, we examined the effects of the PI 3-kinase specific inhibitors LY294002 and wortmannin on APE-induced PKB phosphorylation. As shown in Fig. 5E, both epidermal growth factor-induced and APE-induced phosphorylation of PKB were completely inhibited by LY294002 and wortmannin treatments. These results indicate PI 3-kinase activity to be essential for APE-induced phosphorylation of PKB.

APE siRNA Inhibits Insulin-stimulated PKB Phosphorylation and Activation—To verify the role of endogenous APE in PKB phosphorylation, HepG2 cells were transfected with the negative control or small interfering RNA (siRNA) mediated by the adenoviral expression system. Suppression of endogenous APE by APE siRNA overexpression markedly reduced the APE protein level (Fig. 6A, upper panel). Under these conditions, endogenous PKB phosphorylation of both Thr\(^{308}\) and Ser\(^{473}\) in response to insulin was apparently reduced (Fig. 6A, third and fourth panels). Consistent with the PKB phosphorylation results, insulin-induced PKB kinase activity measured by in vivo kinase assay was also reduced in APE-deficient cells (Fig. 6B).

Knockdown of APE Reduces DNA Synthesis—To explore the effect of APE depletion on proliferation, DNA synthesis in HepG2 cells was measured by BrdUrd incorporation. It was shown that suppressed expression of endogenous APE by siRNA led to decreased DNA synthesis in an APE siRNA titer-dependent manner (Fig. 7).

Cell Death Induced by Overexpression of Both APE and PKB—Recent investigations have shown that overexpression of constitutively activated PKB mutants in many cell types promotes cellular proliferation and inhibits apoptosis (23–25). On the contrary, several lines of evidence indicate that down-regulation of PI 3-kinase/PKB is required to execute the mitotic program efficiently (26). To explore the effect of prolonged PKB activation induced by APE, we next analyzed the effect of APE on cellular proliferation using COS-7 cells (Fig. 8A). The expression of GFP protein by adenovirus had no effect on COS-7 cell proliferation. COS-7 cells expressing PKBα proliferated slightly more slowly than the control GFP-expressing cells. However, COS-7 cells expressing both PKBα and APE showed no proliferation. Trypan blue exclusion was employed to assay cell viability in COS-7 cells and HepG2 cells overexpressing PKB and APE. COS-7 cells expressing both PKBα and APE showed reduced viability (i.e. these cells ultimately died) (Fig. 8C). Virtually the same observations were made in HepG2 cells (Fig. 8D).

Induction of Apoptosis by Overexpression of Both APE and PKB—To elucidate whether apoptosis is involved in the molecular mechanism of APE-induced inhibition of cellular proliferation and cell death, we analyzed the cleavage of caspase-3 and PARP (Fig. 8D). Caspase-3 and PARP are key mediators of apoptosis, and cleavage of these enzymes to their active form correlates with the onset of apoptosis (27, 28). When COS-7...
cells were treated with 1 μM staurosporine, cleaved caspase-3 and cleaved PARP were detectable after 3–6 h, in a time-dependent manner. In COS-7 cells expressing GFP, PKB, or APE, using an adenovirus expression system, no cleavage of caspase-3 or PARP was detectable. However, in cells expressing both PKB and APE, cleaved caspase-3 and PARP were

Fig. 9. Overexpressions of PKBα and APE result in cells with greater than 4n DNA content. A. PKB and PKB alone or in combination, were expressed with an adenovirus expression system, 18 h prior to thymidine release. Cells were collected at the indicated time points after thymidine release. As a control, cells expressing GFP were used. The DNA content was analyzed by fluorescence-activated cell sorting analysis. 2n (diploid) and 4n (tetraploid) represent the cells containing 2n and 4n DNA content, respectively. B. APE plus PKBα induces phosphorylation of Thr68 of Chk2. HeLa cells arrested in G1/S by a double-thymidine method were released into a synchronous cell cycle and sampled every 3 h. Phosphorylation of Chk2 was determined by immunoblotting with a specific antibody for phospho-Thr68 of Chk2.
detected. These findings indicate that apoptosis is not induced by PKB alone but rather by the interaction between PKB and APE accompanying prolonged activation of PKBα.

**Effect of APE-PKB Interaction on the Cell Cycle**—To study the effect of the APE-PKB interaction on cell cycle progression, cell cycle profiles were analyzed using flow cytometry (Fig. 9A). Overexpression of PKB or APE alone did not promote re-replication. However, co-expression of PKB and APE generated cells that had DNA contents greater than normal G2/M cells from 5 to 15 h after thymidine release, indicating that PKB and APE interact to induce DNA re-replication without mitosis.

**APE-PKB Interaction Induces Chk2 Phosphorylation**—Rereplication reportedly leads to DNA damage, and Vaziri et al. (29) demonstrated that APE phosphorylation in mammalian cells in which recombination had been induced by overexpression of the replication license factors CDT1 and CDC6. The amount of Chk2 protein was not altered by PKBα or APE expression (data not shown). However, in HeLa cells overexpressing both PKBα and APE, Chk2 was apparently phosphorylated starting 6 h after thymidine block release, and peak phosphorylation was observed 12–21 h thereafter (Fig. 9B). In contrast, overexpression of neither PKBα nor APE induced apparent Chk2 phosphorylation.

**DISCUSSION**

In this study, we identified a novel PKB-binding protein using a yeast two-hybrid screening system and named it APE. The APE protein was detectable in many tissues including the brain, spleen, lung, fat, and heart, although APE mRNA was most abundant in the testis. In addition, from the immunoblotting results obtained using antibodies against different portions of APE, the presence of alternatively spliced protein products is likely.

The in vivo interaction of APE with PKB was clearly demonstrated by the overexpression of both proteins as well as by communoprecipitation of the endogenous proteins. In vitro association was also demonstrated using bacterially expressed recombinant proteins. Notably, APE did not interact with any of the other AGC kinases tested in this study such as SGK1/2, PKCβ2, and PKCε, which have regions highly homologous to the hydrophobic motif of PKB. Thus, it is reasonable to consider APE a PKB-specific binding protein.

Subsequently, by overexpressing APE, we demonstrated that this protein markedly enhances the phosphorylation and kinase activity of PKB, whereas reducing endogenous APE expression using siRNA suppressed both. In addition, although APE binds to both phosphorylated and nonphosphorylated PKB, it seems that more PKB binds to APE when PKB is nonphosphorylated. Taking into consideration that APE-induced phosphorylation of PKB did not occur in cells treated with wortmannin or LY294002, APE itself is not a kinase and it is likely that APE enhances or prolongs the PI 3-kinase-dependent phosphorylation of PKB. In other words, we speculate that APE functions as a scaffold protein and facilitates Thr308-dependent phosphorylation of PKB. In other words, we speculate that APE functions as a scaffold protein and facilitates Thr308-dependent phosphorylation of PKB. In other words, we speculate that APE functions as a scaffold protein and facilitates Thr308-dependent phosphorylation of PKB. In other words, we speculate that APE functions as a scaffold protein and facilitates Thr308-dependent phosphorylation of PKB. In other words, we speculate that APE functions as a scaffold protein and facilitates Thr308-dependent phosphorylation of PKB. In other words, we speculate that APE functions as a scaffold protein and facilitates Thr308-dependent phosphorylation of PKB. In other words, we speculate that APE functions as a scaffold protein and facilitates Thr308-dependent phosphorylation of PKB. In other words, we speculate that APE functions as a scaffold protein and facilitates Thr308-dependent phosphorylation of PKB.

Recent evidence indicates that PI 3-kinase and PKB play important roles in regulating cell proliferation. In this study, it was demonstrated that suppression of APE by siRNA reduced DNA synthesis, with decreased phosphorylation and kinase activity of PKB. This result agrees with those of previous reports showing the important role of PKB in proliferation. Thus, when the level of PKB expression is limited, APE apparently enhances proliferation in cooperation with PKB.

On the other hand, interestingly, we demonstrated overexpressions of both APE and PKB to induce DNA re-replication rather than normal DNA synthesis, thereby proving that these overexpressions together increase the cellular DNA content more than 4n in the S phase within 10 h after initiation of the S phase. Similar re-replication was reported with overexpression of the DNA replication factors Cdt1 and Cdc6 in either yeast or mammalian cells (29). In such cell systems, re-replication induced DNA damage, and the checkpoint pathway including Chk2 was activated. Chk2 activation is involved in the p53-dependent apoptotic response observed with DNA damage (30). In good agreement with these previous reports, we observed Chk2 phosphorylation and subsequent apoptosis in PKBα-expressing cells, after DNA re-replication. Thus, although the overexpression of both APE and PKB observed in this study may not be physiological, it is likely that the prolonged PKB phosphorylation induced by the association with APE does not lead to normal cell proliferation but rather to re-replication and the ensuing apoptosis. In other words, in the cells with high PKB expression, increased APE expression could lead to apoptosis after DNA re-replication.

Although we cannot explain how PKB and APE induce re-replication in human cells, we observed APE-induced PKB phosphorylation to be markedly enhanced not only in the cytoplasm but also the nucleus (data not shown). Thus, we speculate that overexpressed PKB and APE might phosphorylate some unidentified proteins in the nucleus such that normal replication licensing is blunted. However, this phenomenon was observed only with the overexpression system, and further study is needed to clarify whether this phenomenon is physiological.

In summary, we identified a novel PKB-binding protein, which enhances the phosphorylation of PKB and termed it APE. APE plays a role in regulating the phosphorylation state of PKB and the resultant DNA synthesis. In addition, DNA re-replication and the resultant apoptosis might be a novel mechanism that is induced by the enhanced interaction between APE and PKB, the physiological significance of which merits further investigation.

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