Identification of a PKB/Akt Hydrophobic Motif Ser-473 Kinase as DNA-dependent Protein Kinase* †

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Full activation of protein kinase B (PKB/Akt) requires phosphorylation on Thr-308 and Ser-473 by 3-phosphoinositide-dependent kinase-1 (PDK1) and Ser-473 kinase (S473K), respectively. Although PDK1 has been well characterized, the identification of the S473K remains controversial. A major PKB Ser-473 kinase activity was purified from the membrane fraction of HEK293 cells and found to be DNA-dependent protein kinase (DNA-PK). DNA-PK co-localized and associated with PKB at the plasma membrane. In vitro, DNA-PK phosphorylated PKB on Ser-473, resulting in a ~10-fold enhancement of PKB activity. Knockdown of DNA-PK by small interfering RNA inhibited Ser-473 phosphorylation induced by insulin and pervanadate. DNA-PK-deficient glioblastoma cells did not respond to insulin at the level of Ser-473 phosphorylation; this effect was restored by complementation with the human PRKDC gene. We conclude that DNA-PK is a long-sought after kinase responsible for the Ser-473 phosphorylation step in the activation of PKB.

The signaling pathway centered on protein kinase B (PKB, also called Akt) has emerged as a critical mediator of diverse cellular processes including metabolism, gene expression, migration, angiogenesis, proliferation, and cell survival (1, 2). PKB is tightly controlled and the consequences of its deregulation have been implicated in the development of cancers and diabetes (1, 2). The activity of PKB is markedly stimulated in a phosphatidylinositol 3-kinase (PI3K)-dependent manner. Upon stimulation, PKB is recruited to the plasma membrane through the binding of its N-terminal pleckstrin homology (PH) domain to phosphatidylinositol 3,4,5-trisphosphate (PIP₃), a lipid product of PI3K. PKBα is then activated by phosphorylation on two residues: Thr-308 in the activation loop and Ser-473 in the hydrophobic motif of the C-terminal tail (3). There is convincing evidence that Thr-308 is phosphorylated by 3-phosphoinositol-dependent kinase 1 (PDK1) (4, 5). In embryonic stem cells in which the PDK1 gene has been genetically disrupted, PKB is resistant to growth factor stimulation as consequence of loss of Thr-308 phosphorylation (5), but phosphorylation on Ser-473 still occurs. Like PKB, PDK1 also contains a PH domain that binds to PIP₃ (4, 6). Phosphorylation of Thr-308 in vivo is dependent on PDK1 activity, but it is unclear if this requirement is necessary for the unfolding of PKB to allow access of PDK1 to Thr-308 site or direct activation of PDK1 through its PH domain (6, 7). Other results indicate that PDK1 is important for PKB on Ser-473 because analysis of knock-in embryonic stem cells expressing PDK1 with a mutation in its PH domain revealed that PKB is not activated by insulin-like growth factor-1 (IGF-1), whereas ribosomal S6 kinase (RSK) is activated normally, indicating the importance of co-localization of PKB with PDK1 at the plasma membrane (8).

Identification of the kinase responsible for phosphorylating Ser-473 has been a major challenge for a number of years but remains elusive. Several kinases have been reported to possess Ser-473 phosphorylating activity, including mitogen-activated protein kinase-activated kinase-2 (MAPKAPK-2) (3, 9), integrin-linked kinase (10, 70), PDK1 (11), and PKB itself (12). However, there is evidence that these kinases are not the physiological PKB Ser-473 kinase (S473K) (3, 5, 13–15, 69). Activation of MAPKAPK-2 is PI3K-independent, whereas PKB Ser-473 phosphorylation is sensitive to PI 3-kinase inhibitors (5). PDK1-null cells undergo Ser-473 phosphorylation, suggesting that PDK1 is not required for Ser-473 phosphorylation (5). Furthermore, insulin-stimulated PKB Ser-473 phosphorylation does not require activation of PDK1 or PKB, as Ser-473 phosphorylation is not sensitive to staurosporine treatment, which inhibits both PDK1 and PKB (13). Overexpression of certain kinase inactive mutants can mimic wild type ILK in inducing Ser-473 phosphorylation, suggested that ILK influences PKB phosphorylation indirectly (69). Phosphorylation of PKB/Akt was unaffected in ILK-deficient fibroblasts (16) and chondrocytes (17), indicating that ILK is not required for the phosphorylation of PKB on Ser-473. Moreover, a physiological role of ILK in regulating PKB phosphorylation has been questioned, since ILK knock-out in Drosophila melanogaster shows a phenotype more similar to the integrin knock-out than to the PKB knock-out (14). However, ILK knock-out in mouse macrophages resulted in substantial inhibition of Ser-473 phosphorylation (70). We and other groups (13, 15, 18, 19) provided data that strongly argue against autophosphorylation mechanism. The recently solved crystal structure of PKBβ reveals the importance of Ser-473 phosphorylation for PKB activation and...
strongly argues that autophosphorylation cannot be the physiological mechanism of Ser-473 phosphorylation (20).

We have previously identified S473K activity present in lipid rafts of plasma membrane from HEK293 cells (15). In this paper we report the purification and identification of the Ser-473 kinase(s) from HEK293 cell membrane extracts. We show here that DNA-dependent protein kinase (DNA-PK) is a dominant upstream kinase of PKB that specifically targets PKB phosphorylation on Ser-473 in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—Antibodies specific for PKB phosphorylated on Thr-308 (pT308) and Ser-473 (pS473) were from Cell Signaling Technologies. Polyclonal anti-PKB antibody (Ab10) and monoclonal anti-PKB (A4D6) have been described previously (21, 22). Other antibodies were from the following sources: Ku80, Ku70, and β-tubulin (NeoMarkers); DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (G4), lamin A/C, and caveolin-1 (Santa Cruz Biotechnology Inc.); Texas Red-conjugated anti-rabbit IgG antibody and fluorescein isothio cyanate-conjugated anti-mouse IgG antibody (Sigma). Double-stranded fetal calf thymus DNA was from Sigma, and purified HeLa DNA-PK protein (23) and p53 substrate peptide EPPLSQEAFADLWKK (24) were from Promega. Purified human placental DNA-PKcs and Ku70/80 were from ATCC and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% non-essential amino acids, 4 mg/ml glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For RNA interference, HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% non-essential amino acids, and 4 mg/ml glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. M059J/Fas1 cells were grown in the same medium containing 250 μg/ml Genetin. For analytical experiments, cells were starved overnight prior to treatment and the cells were lysed in Nonidet P-40 lysis buffer (21).

Immunofluorescence—For immunofluorescence, cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. After blocking, cells were incubated with monoclonal anti-DNA-PK antibody (G4, 2 μg/ml diluted in phosphate-buffered saline) for 2 h, followed by polyclonal anti-PKB (Ab10) antibody (2 μg/ml diluted in phosphate-buffered saline) for a further 2 h. Cells were then extensively washed and incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG and TR-conjugated anti-rabbit IgG (both 1:100 dilution). After extensive washing, the antibodies were visualized using confocal microscopy.

Immunoprecipitation—Extracts (500 μg) were incubated in co-immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) with monoclonal DNA-PK (G4, 2 μg/ml) or monoclonal PKB (A4D6, 2 μg/ml) antibodies and Protein G beads (20 μg/ml; Amersham Biosciences). After 6 h of continuous gentle agitation at 4 °C, the beads were collected by centrifugation and washed three times in co-immunoprecipitation buffer, after they were resuspended in SDS sample buffer and heated at 95 °C for 3 min before analysis by 6% SDS-PAGE followed by Western blotting.

RESULTS

Purification and Identification of DNA-PKcs as a Ser-473 Kinase—We previously (15) identified a S473K activity in lipid rafts of plasma membrane and now report an efficient method for purification and identification of this kinase from HEK293 cells. The purification procedure was monitored using a peptide derived from the hydrophobic motif of PKBα containing Ser-473 (FSY) and a mutant peptide with Ser-473 mutated to Ala (FAY) as substrates as described in Ref. 15. In parallel, specific S473K activity was also monitored using GST-PKB1-480 (GST fusion protein containing the hydrophobic motif site) as substrate, followed by Western blotting with a Ser-473 phospho-specific antibody as described under “Experimental Procedures.” This combined assay approach allowed the identification of the enzymes specifically targeting Ser-473 of PKB. The procedures for purification of S473K, including membrane fractionation, chromatography on Q-Sepharose, Mono S, and Mono Q columns are described under “Experimental Procedures.” The elution profile from the final Mono Q step (Fig. 1A) shows two distinct kinase activities. S473K1 activity in fraction 27 specifically phosphorylated recombinant GST-PKB1-480, with only poor activity toward the peptides (inset in Fig. 1A). The second activity (S473K2) peaking at fraction 30 phosphorylated both recombinant PKB1-480 and Ser-473 peptides (Fig. 1A). SDS-PAGE analysis of Mono Q fractions revealed one major band of apparent molecular mass ~350-kDa whose elution profile paralleled S473K1 activity (Fig. 1, A and B). Furthermore, the 350-kDa bands co-eluted with S473K1 activity on gel filtration chromatography (Fig. 2). The band corresponding to the 350-kDa proteins was analyzed by mass spectrometry.
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FIG. 2. Analysis of S473K1 by Superdex 200 gel filtration chromatography. Aliquots (0.1 ml) of S473K1 (Mono Q fraction 27) were applied to a Superdex 200 gel filtration column (Amersham Biosciences, HR10/30) equilibrated with column buffer (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 300 mM NaCl, and protease inhibitors). The column was eluted with the same buffer, and 0.5-ml fractions were collected. S473K1 activity was determined with FSY/FAY peptides (A) or with GST-PKBβ419–440 as substrate (upper panel in B). Aliquots of each fraction were immunoblotted for DNA-PKcs (lower panel in B). The elution positions of blue dextran (2000 kDa), apoferritin (443 kDa), β-amylase (200 kDa), and bovine serum albumin (66 kDa) are indicated.

try, and the sequences of 65 peptides obtained all showed a perfect match to the human DNA-PKcs (data not shown). Western blot analysis of Mono Q fractions revealed that the 350-kDa bands corresponded to DNA-PKcs, which exactly paralleled S473K activity in the presence of FSY (solid symbols) or FAY peptides (open symbols) in DNA. Inset in A, fractions were assayed using GST-PKBβ142–440 as substrate, followed by Western blotting with pS473 phospho-specific antibody. B, a 10-μl aliquot of each fraction was subjected to 8% SDS-PAGE and proteins visualized by Coomassie Blue staining. C, Western blotting of fractions with monoclonal antibodies specific for DNA-PKcs and Ku70/Ku80 subunits.

Vested by DNA ends (29); we therefore tested the DNA dependence of S473K1 by assaying the kinase activity of Mono Q fractions in the presence of linear double-stranded fetal calf thymus DNA. As expected, the S473K1 activity toward the substrate FSY peptide was robustly stimulated by DNA and peaked at fraction 27, corresponding exactly to DNA-PKcs (Fig. 1, A–C). It is worth noting that DNA also stimulates S473K2 activity probably due to contamination of S473K2 fractions with DNA-PKcs (Fig. 1, A and B). We are currently characterizing the protein kinase activities in the S473K2 and will be reported elsewhere.

To test whether Ser-473 phosphorylation catalyzed by purified S473K1 is sufficient for activation of PKB activity, a monophosphorylated form of recombinant PKB (ΔPH-PKBβT309P prepared by in vitro phosphorylation with PDK1 (20)) was first phosphorylated by S473K1 and PKB assayed using RPRAAATF (R7Ftide) as substrate (26). As shown in Fig. 3A, ΔPH-PKBβT309P activity toward R7Ftide was increased 10-fold after incubation with S473K1. Phosphorylation of ΔPH-PKBβT309P on Ser-474 (identical to Ser-473 in PKB) was also increased in a time-dependent manner (Fig. 3A). Significantly we carried out in vitro phosphorylation reactions of DNA-PK with ΔPH-PKBβT309P in the presence of 5 μM staurosporine; this revealed that Ser-474 phosphorylation was unaffected by the inhibitor. This rules out the possibility that DNA-PK promotes PKB autophosphorylation because under these conditions PKB is fully inhibited by the inhibitor.

We further characterized the S473K1 fraction using PI3K inhibitors known to be effective inhibitors of DNA-PK activity (27, 28). As shown in Fig. 3, B and C, S473K1 was potently inhibited by 5 μM LY294002 and 2 μM wortmannin suggesting that DNA-PKcs is a major active component of S473K1. This result is consistent with previous cell culture data that showed Ser-473 phosphorylation is sensitive to these two inhibitors (3). Our previous studies revealed that S473K is staurosporine insensitive (13). Consistent with this, S473K1 activity was not
affected by 5 μM staurosporine (Fig. 3, B and C). Based on these results, we conclude that DNA-PK is S473K1. In contrast, the S473K2 fraction is sensitive to staurosporine (data not shown), suggesting that S473K2 is distinct from S473K1.

**DNA-PK Activity Is Required for Ser-473 Phosphorylation and Activation of PKB**—To confirm that DNA-PK is S473K1, purified DNA-PK (from HeLa cells and human placenta) was tested for their ability to phosphorylate PKB on Ser-473. As shown in Fig. 4, A and B, both the FSY peptide and GST-PKBa419–480 were efficiently phosphorylated by purified DNA-PK in the presence of DNA. Strikingly, DNA-PK significantly phosphorylated GST-PKBa419–480 in the absence of added DNA but did not significantly phosphorylate FSY peptide in the absence of DNA (Fig. 4B). This result is consistent with S473K1 activity observed in assays of chromatographic fractions (Fig. 1A). In addition, the phosphorylation of PKB catalyzed by purified DNA-PK was inhibited by LY294002 and wortmannin but was resistant to staurosporine (Fig. 4A). Next, we tested the effect of phosphorylation of ΔPH-PKBbT309P by purified DNA-PK under the conditions described above. As shown in Fig. 4C, PKB activity toward R7Ftide was enhanced more than 8-fold with increasing Ser-474 phosphorylation. This persistent but low Ser-473 phosphorylation was due to DNA-PK. The stoichiometry of GST-PKBa419–480 phosphorylation was ~0.3 mol of phosphate/mol of protein, comparable with phosphorylation of p53 by DNA-PK (Fig. 4D). Thus, the properties of nearly homogenous DNA-PK purified from either Hela cells or placenta coincide perfectly with the properties of our partially purified S473K1 activity, further supporting the concept that DNA-PK is a S473K1.

**Impairment of Ser-473 Phosphorylation in DNA-PKcs-deficient Cells and in DNA-PKcs-deficient Cells**—To confirm that the DNA-PK can function as a S473K1 in vivo we transfected HEK293 cells with specific siRNA leading to a marked loss of DNA-PKcs protein (Fig. 5A, upper panel). Stimulation of siRNA-transfected cells with insulin or pervanadate markedly reduced PKB phosphorylation on Ser-473 phosphorylation but did not markedly affect Thr-308 phosphorylation (Fig. 5A). Similar results were also obtained by knockdown of DNA-PKcs in human fetal lung MRC5 fibroblasts (data not shown). Thus, DNA-PK again appears to be required for Ser-473 phosphorylation. The human glioblastoma cell line M059J lacks DNA-PKcs and DNA-PK activity, while the related cell line M059K contains wild type DNA-PK (30). Treatment of cells with insulin increased phosphorylation of both Ser-473 and Thr-308 residues in M059K cells, but there was no significant increase in M059J cells (Fig. 5B). Surprisingly the level of phosphorylation of both sites was higher in starved M059J cells suggesting further alterations in PKB regulation in this transformed cell. To further determine whether the insulin-resistant phenotype of PKB phosphorylation is due to a defect in DNA-PK in M059J cells, rescue experiments were performed with M059J/Fus1 cells. M059J/Fus1 cells were generated from M059J by cell fusion leading to DNA-PK expression because a fragment of chromosome 8 with the PRKDC gene is integrated into these cells (31). The results in Fig. 5B clearly show that M059J/Fus1 cells regained an insulin-sensitive phenotype for Ser-473 phosphorylation similar to that of M059K. Thus Ser-473 phosphorylation can be restored by complementation of DNA-PKcs-defective cells with a fragment of chromosome 8 containing a copy of the PRKDC gene, again implicating DNA-PK as a determinant kinase responsible for Ser-473 phosphorylation. This persistent but low Ser-473 phosphorylation in DNA-PKcs-deficient cells (Fig. 5, A and B) indicates the existence of additional kinase(s) that can phosphorylate the hydrophobic motif site Ser-473. This could correspond to the S473K2 activity identified in the Mono Q fraction (Fig. 1A). Interestingly in the absence of DNA-PK, or because of cell transformation, this activity appears to be constitutively up-regulated.

**DNA-PK Localizes at the Plasma Membrane and Interacts with PKB**—It has been shown that efficient DNA repair re-
quires growth factor signaling (32) and that this effect may be due to the physical association of DNA-PK with epidermal growth factor receptor (33). DNA-PK can be detected in both the nucleus and cytoplasm (34, 35). Significantly inositol hexakisphosphate (IP6) was reported to bind to DNA-PK (36–38), suggesting that IP6 might play a pivotal role in modulating the localization and/or biological properties of DNA-PK. More recent data indicate a novel localization of the DNA-PK complex in lipid rafts (39) in line with our finding that PKB S473K is associated with plasma membrane lipid rafts (15). Subcellular fractionation of HEK293 cells revealed substantial DNA-PKcs in the membrane fraction as shown in Fig. 6A. Both the cytosolic marker protein β-tubulin and nuclear marker protein lamin A/C were absent in the membrane fraction indicating that no significant contamination of the membrane fraction from cytosol and nuclear fractions. Similarly the Ku subunits were also found in both membrane and nuclear fractions (data not shown).

We also tested whether DNA-PKcs and PKB co-localized in unstimulated 3T3L1, M059K, and M059J cells (Fig. 6B). DNA-PKcs protein with monoclonal G4 antibody. B, M059K, M059J, and Fus1 cells were starved for 24 h and the cells treated with insulin (Ins) for different times before harvest. Aliquots (30 μg) of cell extracts were analyzed by Western blotting using phospho-specific antibodies pS473, pT308, and total PKB protein with Ab10 and DNA-PKcs protein with monoclonal G4 antibody. B, M059K, M059J, and Fus1 cells were starved for 24 h and the cells treated with insulin (Ins) for different times before harvest. Aliquots (30 μg) of cell extracts were analyzed by Western blotting using phospho-specific antibodies pS473, pT308, and total PKB protein with Ab10.
was mainly cytosolic with a fraction associated with the membrane in 3T3L1 and M059K cells. Significantly DNA-PK co-localized with PKB at the plasma membrane in these cells. No DNA-PKcs staining was observed in the membrane and nucleus in the DNA-PKcs-deficient M059J cells (Fig. 6B). To determine whether DNA-PKcs associates with PKB, HEK293 and M059K cells were starved and treated with insulin for 30 min. Cells were lysed in Nonidet P-40 lysis buffer and immunoprecipitated with DNA-PK or PKB monoclonal specific antibodies (Fig. 6C). The results reveal that PKB is associated with DNA-PKcs and following insulin treatment was phosphorylated on Ser-473.

**DISCUSSION**

Here we report for the first time the identification of the DNA-PK as a PKB hydrophobic motif (Ser-473) kinase. We predicted, from a number of different experimental approaches, that the Ser-473 kinase should be located at the membrane. Following the development of appropriate assays (15) we have now identified the predicted kinase as DNA-PK, a member of the PIKK subfamily of protein kinases. This is a surprising result and probably explains why it has been so difficult to identify this kinase. This study now opens up many significant possibilities in terms of fully understanding PKB regulation and also extends the roles of the DNA-PK. Significantly mTOR, a close relative of DNA-PK in the PIKK family, phosphorylates p70S6 kinase on Thr-389 of hydrophobic motif (40). In addition, mTOR also plays a role in controlling phosphorylation of novel PKCδ and novel PKCe on the hydrophobic C-terminal site (41).

Our results would appear to rule out a number of earlier proposed mechanisms for the regulation of PKB Ser-473 hydrophobic motif phosphorylation. First, we found no evidence for autophosphorylation on this site using purified PKB (APH-PKBβ<sup>720SP</sup>) prepared for crystallographic studies (19).<sup>2</sup> Also DNA-PK phosphorylated Ser-473 under conditions where PKB activity is completely inhibited by staurosporine, thus ruling out the possibility that DNA-PK promotes autophosphorylation. A number of other kinases have been proposed (see Introduction) to phosphorylate the hydrophobic motif of PKB. None of these candidates were identified in the Mono Q fraction (Fig. 1) we extensively studied in this paper (all protein bands in the purified S473K fraction were analyzed by mass spectrometry yielding a total of about 150 proteins).

We found that a fraction of DNA-PK localized to the membrane, consistent with a previous observation that DNA-PK complex could be recovered from lipid rafts (39). The mechanism of how DNA-PK localizes to the membrane is not clear but DNA-PK has been reported to be associated with epidermal growth factor receptor (33). Recent findings revealed that the inositol phosphates, especially IP<sub>6</sub>, serve as a potent co-factor for DNA-PK activity in non-homologous end-joining via binding to the Ku subunits (36–38). IP<sub>6</sub> is an abundant molecule in cells, and it is reported that IP<sub>6</sub> can enhance Ku mobility (42, 43). It is likely that IP<sub>6</sub> might play a significant role in modulating the localization of DNA-PK.

DNA-PKcs is a large protein of about 4100 amino acids with the kinase domain located at the C-terminal of the polypeptide. A recent analysis predicts that DNA-PKcs contains several domains located N-terminally to the kinase domain, including two different type helical repeat motifs that specifically interact with some known and other as yet unidentified proteins (44, 45). These helical regions are thought to interact with Ku70/Ku80 subunits, c-Abl, and other factors to modulate DNA-PK activity. It will be important in the future to investigate how this large multifunctional enzyme is acutely regulated at membrane. It is established that DNA-PKcs is a phosphoprotein, and indeed DNA-PK activity is regulated by phosphorylation (27). Several of the phosphorylation sites are conserved in vertebrate species (46, 47) and become phosphorylated in vivo following okadaic acid treatment (46). Phosphorylation on Thr-2638/Thr-2647 is essential for radioresistance conferred by DNA-PKcs (48). Significantly, protein phosphatase 5 interacts with DNA-PKcs and preferentially dephosphorylates Thr-2609 and a lesser extent to Ser-2056 (49). Overall it will be necessary to reinvestigate the phosphorylation of DNA-PKcs in response to reinvestigate the phosphorylation of DNA-PKcs in response to DNA-PKcs and following insulin treatment was phosphorylated on Ser-473.

**FIG. 6.** Subcellular localization of DNA-PK and interaction with PKB at the plasma membrane. A, HEK293 cells were starved and then treated with insulin for 15 min, and cytosolic (Cyt), membrane (Mem), and nuclear (Nuc) fractions were prepared as described under “Experimental Procedures.” Fractions (each 40 µg) were immunoblotted with antibodies for DNA-PKcs, cytosolic maker β-tubulin, nuclear maker lamin A/C, and membrane maker caveolin-1. B, 3T3L1, M059K, and M059J cells were fixed in 3.7% formaldehyde, permeabilized in 2% Triton X-100, and subjected to immunostaining with anti-DNA-PKcs monoclonal G4 and Ab10 specific for PKB. C, HEK293 and M059K cell lysates were immunoprecipitated with normal IgG (lane 1) or anti-DNA-PKcs (G4) (lanes 2 and 3) or anti-PKB (A4D6) (lanes 4 and 5) and blotted with anti-DNA-PKcs (upper panel), Ab10 (middle panel), or pS473 PKB phospho-specific antibody (lower panel).

A. Feng, J. Park, and B. A. Hemmings, unpublished data.
to growth factors and DNA damage to establish the role of this modification in regulating kinase activity, localization, and interaction with other proteins. A recent study (50) showed that the ATM protein kinase is a dimer with the kinase domain bound to Ser-1981 contained in the FAT domain. Following cellular irradiation Ser-1981 becomes autophosphorylated and thus promotes the dissociation of the complex. Apparently this event leads to an activation of ATM activity. It has been reported that c-Abl and PKCδ interacts and phosphorylates DNA-PKcs, which leads to inhibition of the ability of DNA-PK to form a complex with DNA (51, 52). The fact that DNA can robustly activate DNA-PKcs suggests there could be several mechanisms for activating the kinase. It is possible that alternative mechanisms are utilized in different subcellular locations.

Although DNA was originally shown to be a potent activator of DNA-PKcs, recent studies (53–55) indicate that the multiple proteins are recruited by the Ku subunits that may also serve as activators to stimulate DNA-PK activity. For instance, several homeodomain proteins, including Oct-1, have recently been shown (53) to interact with Ku70 and enhance DNA-PK phosphorylation. Consistent with these findings, thyroid hormone receptor-binding protein (55), strongly indicates that DNA-PK can be fully activated in the absence of DNA. It appears that although DNA-PK can be stimulated by DNA ends (27), or by other kinases (51, 52), the large DNA-PK protein may have a scaffold function and the protein-protein interactions via the heterodimeric Ku subunits may be another important but previously less appreciated mechanism for DNA-PK activation.

PKB belongs to the AGC family of protein kinases that possess a highly conserved activation loop phosphorylation site in the central kinase domain and a hydrophobic motif phosphorylation site in the C terminus (reviewed in Ref. 58). The hydrophobic motif of most AGC kinases is characterized by a conserved motif: F-X-X-F/S-T-Y/P (the S/T residue is equivalent to Ser-473 of PKBα) (58). DNA-PK can phosphorylate many protein substrates on Ser/Thr residues followed by glutamine, i.e. the “XQT” motif (59, 60). However, DNA-PK also phosphorylates proteins at so-called “non-S/TQ” sites, with a preference for Ser/Thr residues followed by a hydrophobic amino acid (27, 59, 60). It is noteworthy that DNA-PK appears to have a predisposition for phosphorylation sites at the extreme termini of its substrates; this may indicate better accessibility of the substrate to the active site of this large kinase complex (60). The substrate specificity of DNA-PK warrants further investigation; our initial data indicate that PKB hydrophobic motif FSY peptide is about three times more effective than the p53 peptide (data not shown). We also tested DNA-PK with several other hydrophobic motif peptides from RSK1, RSK3, PCKα, NDR2, S6K-1, and S6K-1 modeled on the PKB site and found that only those from the three PKB isoforms served as substrate (data not shown). Furthermore, our in vitro results with PI3K inhibitors on DNA-PK activity suggest that at high concentrations in vitro these compounds can directly inhibit PKB phosphorylation on Ser-473. Interpretation of results obtained with LY294002 and wortmannin need to be re-evaluated because of their apparent direct inhibition of PKB phosphorylation.

DNA-PK is activated upon DNA damage by UV irradiation, as is PKB (61). Induction of apoptosis by cisplatin was ex-
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PDK1/mTOR. This leads to the suggestion that possibly other members of the PIKK family could function as hydrophobic motif kinases. It will of course be necessary to identify the kinase in the S473K2 fraction to fully elucidate the regulation of PKB. However, the identification of a second protein kinase with potential to phosphorylate for Ser-473 leads to the speculation that possibly different stimuli (growth factors, chemokines, DNA damage) activate specific protein kinase to phosphorylate the key hydrophobic motif regulatory phosphorylation site. Furthermore our data (Fig. 5B) with the transformed glioblastoma cell line M059J appears to indicate that this kinase is deregulated in these cells leading to constitutive phosphorylation of PKB.

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