DNA-dependent Protein Kinase-mediated Phosphorylation of Protein Kinase B Requires a Specific Recognition Sequence in the C-terminal Hydrophobic Motif

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DNA-dependent protein kinase (DNA-PK) has been implicated in a variety of nuclear processes including DNA double strand break repair, V(D)J recombination, and transcription. A recent study showed that DNA-PK is responsible for Ser-473 phosphorylation in the hydrophobic motif of protein kinase B (PKB/Akt) in genotoxic-stressed cells, suggesting a novel role for DNA-PK in cell signaling. Here, we report that DNA-PK activity toward PKB peptides is impaired in DNA-PK knock-out mouse embryonic fibroblast cells when compared with wild type. In addition, human glioblastoma cells expressing a mutant form of DNA-PK (M059) displayed a lower DNA-PK activity when compared with glioblastoma cells expressing wild-type DNA-PK (M059K) when PKB peptide substrates were tested. DNA-PK preferentially phosphorylated PKB on Ser-473 when compared with its known in vitro substrate, p53. A consensus hydrophobic amino acid surrounding the Ser-473 phospho-acceptor site in PKB containing amino acids Phe at position +1 and +4 and Tyr at position –1 are critical for DNA-PK activity. Thus, these data define the specificity of DNA-PK action as a Ser-473 kinase for PKB in DNA repair signaling.

Protein kinase B (PKB/Akt) is an important regulator of cell proliferation and survival (1). Amplification of genes encoding PKB isoforms has been found in several types of human cancer (2). The discovery that PKB activation is dependent on 3-phosphoinositides expanded much of the interest into the role of a phosphatidylinositol 3-kinase (PI3K) in cellular signaling (3). Both phosphatidyl-inositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate, the products of PI3K, bind with high affinity to the pleckstrin homology domain of PKB, thus recruiting the kinase to the plasma membrane (4, 5). However, additional events are required to fully activate PKB. One well defined regulatory mechanism for PKB activation is phosphorylation of Thr-308 in the activation loop of PKB by 3-kinase (6, 7). The unphosphorylated form of PKB is virtually inactive, and PKD1 phosphorylation stimulates its activity by at least 100-fold (6, 8–10). Furthermore, phosphorylation on a second regulatory site, Ser-473 at the C terminus, termed the hydrophobic motif, responsible for further 7–10-fold full activation of PKB (10). Based on the crystal structure of PKB, the role of Ser-473 phosphorylation is important not only for kinase activation but also for stabilization of the active conformation of PKB (11, 12). Recently, our laboratory showed that DNA-dependent protein kinase (DNA-PK) is a candidate kinase, responsible for Ser-473 phosphorylation during PKB activation in genotoxic-stressed cells such as HEK293 (13). Sarbassov et al. (14) identified that mammalian target of rapamycin (mTOR)/Rictor complex also phosphorylated PKB on Ser-473 in a range of human cancer cells. Furthermore, Dragoi et al. (15) reported that the catalytic subunit DNA-PKcs is an important intermediate in CpG-DNA-triggered PKB signaling pathway.

The core protein of the DNA-PK multiprotein complex is represented by the M₉ ~ 460,000 DNA-PKcs, which, in itself, is a serine/threonine kinase similar to proteins of the PI3K family (16, 17). In vitro, DNA-PK is activated by free DNA ends (18), is capable of phosphorylating several protein substrates, including transcription factors replication protein A, p53, c-Jun, HMG1, and RNA helicase A, and is also capable of autophosphorylation (17, 19). Although the precise function of DNA-PK in vivo is not well defined, its importance in non-homologous end-joining (NHEJ) and V(D)J recombination events has been...
clearly demonstrated (18, 19). NHEJ is one of the major pathways for the repair of DNA double strand breaks in human cells. Biochemical and genetic studies have shown that DNA-PK, XRCC4, DNA ligase IV, and Artemis are essential components of the NHEJ pathway (19, 20).

In the current study, we provide further evidence that DNA-PK is a bona fide Ser-473 kinase for PKB. We define the specificity of DNA-PK phosphorylation of PKB at the Ser-473 site and show that DNA-PK activity toward peptides containing the Ser-473 consensus site (FSYtide) is impaired in DNA-PK−/− MEFs. Together, these data support that DNA-PK acts upstream of PKB in the DNA repair signaling pathway.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Antibodies were purchased from the following companies: anti-phospho-Thr-308 (PKB) and anti-phospho-Ser-473 (PKB) (Cell Signaling); anti-pFoxO4, anti-FoxO4, and anti-DNA-PKcs (Santa Cruz Biotechnology). Fetal calf thymus dsDNA and DNA-cellulose beads were from Sigma. Purified DNA-PKcs and Ku70/80 were prepared from human placenta (21) as described previously. DNA-PK substrate peptide (22) was purchased from Promega. Inositol hexakisphosphate, spermine, poly(l-lysine), heparin, and poly(Glu-Na, Tyr) 4:1 were purchased from Sigma.

Construction of Expression Vectors—HA-tagged (HA-PKBα) were as reported previously (13, 23). Mutants at Tyr-474 (HA-PKBα Y474R and HA-PKBα Y474F) were created by using the QuikChange kit (Stratagene) with pCMV4 HA-PKBα as a template. Mutants at Phe-469 (HA-PKBα F469R), Phe-472 (HA-PKBα F472R), Phe-476 (HA-PKBα F476R), and Phe-480 (HA-PKBα F480R) were also created. All constructs were confirmed by automated DNA sequencing. Sequences of the mutagenic oligonucleotides are available upon request.
Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen) at 37 °C in an atmosphere containing 5% CO₂. DNA-PKcs (DNA-PKcat) MEFs, DNA-PKcs (DNA-PKcat) MEFs, and M059K and M059J cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 mm non-essential amino acid (Invitrogen), and 20 mm L-glutamine (Invitrogen). HEK293 cells were transfected with PKB plasmids by a modified calcium phosphate method (24) with 1–2 μg/ml plasmid DNA. Transfection mixtures were removed after 16 h incubation, and cells were serum-starved for 24 h before stimulation with 5 μM doxorubicin (Sigma) for the indicated times.

**Immunoprecipitation and in Vitro Kinase Assays**—Cells were placed on ice and extracted with lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1% w/v Nonidet P-40, 120 mM NaCl, 25 mM NaF, 40 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 2 μM microcystin-LR. For the immunoprecipitated DNA-PK assays, DNA-PKcs protein was immunoprecipitated from 1 mg of cell-free extracts with the anti-DNA-PK G4 monoclonal antibody to protein A/G-Sepharose (Amersham Biosciences) in a total volume of 500 μl of Z’0.05 buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 20% glycerol, 1% w/v Nonidet P-40, and 1 mM dithiothreitol) (25). Immune complexes were washed twice with 1 ml of Z’0.05 buffer and were finally resuspended in the same buffer. In vitro DNA-PK assays were performed for 15 min at 30 °C in 50 μl of reaction volume containing 30 μl of immunoprecipitate in Z’0.05 buffer, 1.0 mg/ml substrate peptide, 1 μM protein kinase A inhibitor peptide (Bachem), and 50 μM [γ-32P]ATP (Amersham Biosciences; 1,000–2,000 cpm/pmol). The sequences of A inhibitor peptide (Bachem), and 50 μM [γ-32P]ATP were determined from Lineweaver-Burk plots. The data are derived from duplicates, two separate experiments.

**RESULTS**

DNA-PK Effectively Phosphorylates FSYtide When Compared with p53tide in Vitro—DNA-PK has been shown to phosphorylate two types of consensus sequence: an SQ-motif present in p53, Ku70, SRF, c-Jun, RPA32, H2AX, and DNA-PK itself (17, 19) and a non-SQ motif found in PRA34, Ku70, Ku80, c-Fos, DNA-PK itself, and XRCC4 (17, 19) (Fig. 1A). PKB has a potential non-SQ at its C-terminal hydrophobic motif (Fig. 1A). To analyze the substrate specificity of DNA-PK, we measured the apparent Km and kcat toward short peptides derived from PKB and p53 representing the two types of DNA-PK substrate (Table 1). The synthetic peptide corresponding to the sequence surrounding Ser-473 of PKB (FSYtide; PHFPQFYSASSTA) with a kcat/Km of 5.4 × 10⁶ M⁻¹ s⁻¹ was a much more efficient DNA-PK substrate than the peptide containing the Ser-15 phosphorylation site of p53 (p53tide; EPPLSQEAFADLWKK) (kcat/Km = 2.9 × 10⁶ M⁻¹ s⁻¹). This result suggests that DNA-PK can phosphorylate PKB on Ser-473 and may potentially play an additional role in PKB signaling.

**Molecular Basis for the Substrate Specificity of DNA-PK**—Members of the AGC family of protein kinases such as PKB, p70 ribosomal protein S6 kinase (p70S6K), serum and glucocorticoid-inducible kinase (SGK), and p90 ribosomal protein S6 kinase (RSK) are activated and phosphorylated by PDK1 (6, 9, 29, 30). The C-terminal hydrophobic motif of these kinases is well conserved between these PDK1 substrates (Fig. 1B). To further characterize the substrate specificity of DNA-PK, hydrophobic motif peptides from the AGC family were tested as DNA-PK substrates in vitro. Peptides derived from PKB isoforms were preferentially phosphorylated by DNA-PK in this system (Fig. 1B). The PKBβ peptide exhibited the highest apparent activity, but this was likely due to an additional Thr residue in the N terminus of the peptide because the activity of a Thr—Pro mutant peptide (PKBβ-M) was dramatically reduced (Fig. 1B). These results suggest that DNA-PK can phosphorylate some AGC family members in the order PKBα > PKBβ/γ > SGK1. These data therefore confirmed that

**TABLE 1**

**Kinetic constants of peptide phosphorylation by DNA-PK**

The kinase reaction was carried out as described under “Material and Methods.” Kinetic constants were determined from Lineweaver-Burk plots. The data are derived from duplicates, two separate experiments.

| Substrate | Km (M) | kcat (s⁻¹) | kcat/Km (M⁻¹ s⁻¹) |
|-----------|--------|------------|-------------------|
| FSYtide   | 6.6    | 3471.5     | 5.4 × 10⁶         |
| p53tide   | 2.8    | 8368.1     | 2.9 × 10⁶         |

The molecular size used was 469 kDa for DNA-PKcs.

**FIGURE 1.** Characterization of the DNA-PK recognition motif in PKB. A, sequence comparison of non-SQ motifs for known DNA-PK substrates and hydrophobic motifs for Ser-473 kinase substrates. The original source proteins for the sequence are indicated. The serine residue phosphor-acceptor site is underlined. B, purified DNA-PKcs together with purified Ku70/80 was incubated with each peptide derived from the corresponding AGC kinase. The position of the phospho-Ser acceptor site is indicated. Data are expressed as relative DNA-PK activity when compared with that measured with the PKBα peptide control (in percent). C, each α-Scaning peptide derived from PKBα was assayed with purified DNA-PKcs. All reactions were performed in the presence of dsDNA and Ku70/80 and then processed as described. DNA-PK activity with PKBα peptide was taken as 100%. D, HEK293 cells overexpressing each PKBα mutant were treated with 5 μM doxorubicin (Dox) for 15 min after 18 h of serum starvation. PKB activity was measured with Crosside as substrate followed by immunoblot analysis with phospho-Ser-473 (pS473) or phospho-Thr-308 (pT308) phospho-specific antibodies and anti-PKB antibodies. Kinase activity is the average (± S.D.) of three independent experiments. Wt, wild type; KD, kinase-deficient. E and G, for all conditions except doxorubicin treatment for 4 h, changes in the induction of p21 (E) and phosphorylation of Foxo4 (G) were detected by Western blot analysis. F and H, statistical differences were determined by normalizing values for actin or total Foxo4 at each lane. The results are mean ± S.D. of three independent experiments. Asterisk, p < 0.05.
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the hydrophobic motif of PKB is specifically phosphorylated on Ser-473 by DNA-PK and suggest that PKBo may be the preferred substrate.

To study the influence of amino acid residues adjacent to the minimal consensus phosphorylation site for DNA-PK (FXFXFYYY), we generated variant Ala-scanning peptides. By comparing the phosphorylation of FSYtide with the variant Ala-scanning peptides, we identified that phenylalanine at position +1 and +4 and tyrosine at position −1 from the phosphoacceptor site were key residues for DNA-PK activity (Fig. 1C).

The mutant peptides (F469R, F472R, and Y474R, arrows), where a hydrophobic amino acid (Phe or Tyr) was substituted with a basic amino acid (Arg), showed a significant decrease in DNA-PK activity (Fig. 1D). These inhibitory effects were reversed by the hydrophobic “re-substitution” mutant (Y474F, Fig. 1D).

In addition, immunoblot analysis with anti-pSer-473 antibody revealed that PKB activation correlated well with Ser-473 phosphorylation of PKB, indicating that the hydrophobic motif of PKB plays a key role in determining the substrate specificity of DNA-PK.

To further evaluate these findings, we extended our analysis to the protein level by overexpression of mutant proteins in cultured cells treated with the DNA damage agent doxorubicin, a topoisomerase II inhibitor widely used as an anticancer drug. Doxorubicin-induced PKB activation was impaired in cells transfected with F469R, F472R, and Y474R mutants of PKBo in HEK293 cells (Fig. 1D). These inhibitory effects were reversed by the hydrophobic “re-substitution” mutant (Y474F, Fig. 1D).

Impairment of Ser-473 Kinase Activity in DNA-PK−/− and DNA-PK-deficient Cells—Recently, we have purified and identified DNA-PK as a candidate Ser-473 kinase for PKB in HEK293 cells (13). To further characterize this novel mechanism, DNA-PK was immunoprecipitated from DNA-PK+/+ and DNA-PK−/− mouse embryo fibroblast cell lines. These immunoprecipitates were incubated with peptides containing the consensus Ser-473 phosphorylation site (FSYtide), a Ser-473→Ala mutant peptide (FAYtide), or p53tide in vitro. DNA-stimulated DNA-PK activity toward both the FSYtide and p53tide was observed in DNA-PK+/+, but not in DNA-PK−/− MEFs, suggesting that DNA-PK is indeed capable of phosphorylating FSYtide and p53tide in a DNA-dependent manner (supplemental Fig. 1A). In contrast, no activation was observed when peptides containing a mutation in the Ser-473 amino acid (FAYtide) were used. Similar results were also obtained (supplemental Fig. 1B) when these peptides were incubated with DNA-PK immunoprecipitated from human glioblastoma cells containing wild-type, active DNA-PK (M059K) or DNA-PK inactive mutant cells (M059I). These data suggest that DNA-PK acts as a Ser-473 kinase for PKB and requires specific amino acids in the hydrophobic motif for phosphorylation.

DISCUSSION

DNA double strand breaks play a central role in the activation of damage response pathways in cells upon irradiation whereby the PI3K-like kinases, DNA-PK and ATM, function as damage sensors in downstream signaling (19, 33) and in double strand break repair (34, 35). Numerous studies have shown that the activation of the PI3K/PKB pathway is associated with resistance to radiation in many cell lines (36–38). Therefore, it has been suggested that radiation-induced DNA damage will lead to the activation of the PI3K/PKB signaling pathway through DNA-PK and ATM.

In the present study, we have characterized the phosphorylation of PKB by DNA-PK, a critical component of DNA double strand break repair and V(D)J recombination. The biochemical kinetics of DNA-PK suggests that PKB is a much more efficient substrate than p53 in vitro (Table 1). Furthermore, the results from in vitro DNA-PK assays with hydrophobic motif peptide substrates from the AGC kinase family (FXFXS/S/T; Fig. 1B) indicated that PKBo is preferentially phosphorylated by DNA-PK. By using DNA-PK assays with variant Ala-scanning peptides of FSYtide, we propose that phenylalanine at position +1 and +4 and tyrosine at position −1 from the phosphoacceptor site are required for DNA-PK activity in proteins. The resubstitution mutant peptide (Y474F) clearly showed the importance of hydrophobic amino acid in this position. Similar data were obtained from cells transfected with full-length PKBo mutants F469R, F472R, and Y474R (Fig. 1, D–H), indicating that the hydrophobic motif of PKB play a key role in defining the substrate specificity of DNA-PK.

In DNA-PK−/− MEFs and human glioblastoma cells expressing mutant, inactive DNA-PK, immunoprecipitated DNA-PK activity toward FSYtide (PKB peptide) was impaired when compared with that of control cells expressing wild-type DNA-PK (supplemental Fig. 1). Consistent with previous reports (39, 40), treatment of cells with doxorubicin, a DNA damage agent, increased Ser-473 phosphorylation of PKB in DNA-PK+/+ MEFs, but not DNA-PK−/− MEFs (31, 32), indicating that DNA-PK is required Ser-473 phosphorylation and that PKB activation also occurred during DNA repair signaling.

We have previously shown that insulin-stimulated PKB phosphorylation on Ser-473 did not occur through DNA-PK and that γ-irradiation triggered DNA-PK mediated phosphorylation on this residue (32). We now extend these findings to define the amino acids in the hydrophobic motif of PKB required for DNA-PK mediated Ser-473 phosphorylation.

We attempted to identify potential activators of DNA-PK that would stimulate PKB phosphorylation of Ser-473. Inositol hexakisphosphate (IP6) is a novel factor that is bound by
DNA-PK and stimulates DNA end joining in vitro (41, 42). Therefore, we used IP6 to stimulate DNA-PK activity in vitro in the presence/absence of Ku70/Ku86 subunits with FSYtide as substrate. In our hands, dsDNA induced DNA-PK activity toward FSYtide, whereas different concentrations of IP6 failed to activate DNA-PK (data not shown). The DNA end-joining activity of DNA-PK is triggered by binding of IP6 to the Ku subunit, where it induces a conformational change and a corresponding increase in DNA-PK end-joining activity (42). However, this conformational change appears not to be involved in DNA-PK activity toward FSYtide. We also tested charged molecules such as spermine, poly(L-lysine), poly(Glu-Tyr), and heparin, which mimic the negative charge of dsDNA to activate DNA-PK activity. However, none of these molecules activated DNA-PK activity toward FSYtide in the presence of Ku70/Ku86 (data not shown). It has been suggested that DNA-PK activity toward a degenerative non-SQ peptide library can be increased in the presence of Mn²⁺ instead of Mg²⁺ in vitro (43). In addition, Chan et al. (44) found that DNA-PK activity was independent on dsDNA ends and the Ku70/80 subunit in the presence of Mn²⁺, whereas DNA-PK activity was dependent on dsDNA ends and the Ku70/80 subunit in the presence of Mg²⁺, suggesting the possibility of differential regulation of DNA-PK activity by Mg²⁺ and Mn²⁺. We did not detect significant differences in DNA-PK activity in the presence of Mg²⁺ or Mn²⁺ (data not shown).

Recent observations from ATM⁻/⁻ MEFs or ATM⁻/⁻, ATR⁻/⁻ double MEFs (31) contrast with the conclusion of Viniegra et al. (45), who argued that ATM promotes PKB phosphorylation on Ser-473. It should be noted that ATM⁻/⁻ MEFs in their study were produced from a p53⁻/⁻ background. Therefore, it is possible to speculate that p53 has an influence on Ser-473 phosphorylation of PKB in ATM⁻/⁻ cells through DNA-PK in insulin signaling as well as DNA repair signaling (31). In addition, mTOR and its associated protein Rictor have been shown to phosphorylate PKB on Ser-473 in Drosophila and human cells (14, 46). Recently, it has also been reported that ATR, another member of the PI3K-like kinase, may act as a novel upstream activator of PKB in response to DNA damage induced by O⁶-guanine methylating agents (47). These data suggest that phosphorylation of PKB at Ser-473 is a complex and important regulatory process in cells and further underscore the key role of PKB activation in many physiological processes.

In conclusion, we provide additional evidence for the role of DNA-PK as a Ser-473 kinase for PKB in DNA repair mechanisms and have defined key amino acids in the hydrophobic motif of PKB that control DNA-PK substrate specificity. Further analysis is likely to elucidate the specific role of each putative Ser-473 kinase of PKB under different conditions and in different cellular environments, which will provide a better understanding of the role of PKB in signaling and its potential in therapeutic applications.

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Note Added in Proof—A recent report demonstrated that DNA-PK could be regulated by exchange protein-activated cAMP (EPAC), leading to phosphorylation of PKB on Ser-473 and repair of double strand breaks (48).

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