Activation Mechanism of Protein Kinase B by DNA-dependent Protein Kinase Involved in the DNA Repair System

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DNA-dependent protein kinase (DNA-PK) is involved in joining DNA double-strand breaks induced by ionizing radiation or V(D)J recombination and is activated by DNA ends and composed of a DNA binding subunit, Ku, and a catalytic subunit, DNA-PKcs. It has been suggested that DNA-PK might be 2nd upstream kinase for protein kinase B (PKB). In this report, we showed that Ser473 phosphorylation in the hydrophobic-motif of PKB is blocked in DNA-PK knockout mouse embryonic fibroblast cells (MEFs) following insulin stimulation, while there is no effect on Ser473 phosphorylation in DNA-PK wild type MEF cells. The observation is further confirmed in human glioblastoma cells expressing a mutant form of DNA-PK (M059J) and a wild-type of DNA-PK (M059K), indicating that DNA-PK is indeed important for PKB activation. Furthermore, the treatment of cells with doxorubicin, DNA-damage inducing agent, leads to PKB phosphorylation on Ser473 in control MEF cells while there is no response in DNA-PK knockout MEF cells. Together, these results proposed that DNA-PK has a potential role in insulin signaling as well as DNA-repair signaling pathway.

Key words: DNA-PK, DNA damage, Protein kinase B, Insulin signaling, Cell Signaling

INTRODUCTION

Over time, DNA accumulates changes that activate proto-oncogenes and inactivate tumor-suppressor genes. The genetic instability driving tumorigenesis is fuelled by DNA damage and by errors made by the DNA machinery. However, ‘spontaneous’ mutations are insufficient to explain the lifetime cancer risk (Loeb, 1991). Indeed, numerous links have been identified between oncogenesis and acquired or inherited faulty genome guardians that cause a ‘mutator’ phenotype, highlighting the key role of DNA protection systems in tumor prevention (Hoeijmakers, 2001). To prevent or counteract the time-dependent erosion of our genes due to undesired but irresistible accumulation of DNA injury one of the most important systems is DNA repair. Because there are so many different types of damage, it is simply impossible that one mechanism is able to cope with all of them. So, every repair system has its own tasks, its own spectrum of damage that it can recognize and fix and other repair systems will deal with other types of damage. But, as a whole, a network of repair systems will be able to cope with almost every possible lesion in the DNA (Citterio et al., 2000).

DNA double-strand breaks (DSBs) can be accidentally introduced in cells by the action of ionizing radiation or certain reactive radicals. These agents have the ability to initiate a series of chemical reactions that ultimately sever the DNA backbone, resulting in chromosome breakage and fragmentation of genes (Hoeijmakers, 2001). Because such corruption of genetic material inevitably leads to problems with replication and cell division, it is of the utmost importance that cells have a mechanism to counteract DSBs (van Gent et
The serine/threonine kinase PKB (protein kinase B, also called Akt) constitutes an important node in diverse signaling cascades and acts as a major signal transducer downstream of activated PI3K (phosphoinositide 3-kinase) (Dummler and Hemmings, 2007). Mammalian cells contain three genes that encode three closely related and highly conserved isoforms of PKB, termed PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3 (Yang et al., 2004). Stimulation by numerous growth factors, cytokines, hormones and neurotransmitters can activate PKB/Akt in a phosphoinositide 3-kinase-dependent manner (Brazil et al., 2004). Through receptor tyrosine kinases, these stimuli cause phosphoinositide 3-kinase activation, and generation of the membrane phospholipid PtdIns(3,4,5)P3. PtdIns(3,4,5)P3 then recruits PKBI/Akt to the membrane, where it becomes phosphorylated at Thr308 and Ser473 (for PKBα/Akt1) by two upstream kinases, phosphoinositide-dependent kinase 1 (PDK1) and PDK2 (PDK1). Phosphorylated form of PKB is virtually inactive, and PDK1 phosphorylation stimulates its activity by at least 100-fold (Alessi et al., 1996, 1997). Anti-phospho specific Thr308 or Ser473 (PKB) antibodies are from Cell Signaling. Anti-DNA-PKcs (G4) antibody is obtained from Santa Cruz Bio-tech. Double-stranded fetal calf thymus DNA was from Sigma. The purified DNA-PKcs, Ku70/80 and DNA-PK substrate peptide were purchased from Promega. Inositol hexakisphosphate, Spermine, poly-L-lysine, Heparine, and poly (Glu-Na, Tyr) 4:1 were purchased from Sigma.

In vitro kinase assays. In vitro DNA-PK assays were performed for 15 min at 30°C. Briefly, 1.25 µg/ml of DNA-PKcs was assayed with or without 100 µmol/l of DNA and 1.75 µmol/l of Ku subunits, 1.0 mg/ml substate peptide, 1 µmol/l protein kinase A inhibitor peptide (PKI; Bachem), and 50 µmol [γ-32P]ATP (Amersham; 1,000–2,000 cpm/pmol) in 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) (Beamish et al., 2000) as indicated in each experiment. All reactions were stopped by adding 50 µmol EDTA and processed as described previously (Park et al., 2001). Protein concentrations were determined by the method of Bradford (Bradford, 1976) using BSA as a standard.

Immunoblot analysis. Cell extracts were resolved by 6% or 12% SDS/PAGE and transferred to Immobilon P membranes (Millipore). The membranes were blocked for 30 min in 1 × PBS containing 5% skimmed milk, and 0.1% Triton X-100, followed by 2 h incubation with the first antibody diluted 1000-fold in the same blocking solution. The secondary antibody was a goat anti-rabbit or anti-mouse IgG HRP-conjugated antibody (Amersham Biosciences) diluted 2500-fold in the blocking buffer. The detection and quantitation of protein expression were developed by using the ECL development reagents (Pharmacia) and exposure to film.

Data analysis. The results from western blot analysis are a representative of three independent experiments. Kinase activity is the average (± SD) of three independent experiments.
RESULTS

The absence of Ser473 PKB kinase activity from DNA-PK knockout MEF cells. It has been suggested that DNA-PK act as an upstream kinase for PKB through Ser473 phosphorylation (Feng et al., 2004). Numerous studies have proposed that DNA-PK is involved in DNA-repair as well as V(D)J recombination (Hoeijmakers, 2001a). During last decade, several substrates for DNA-PK have been suggested in vitro as well as in vivo (Burma and Chen, 2004; Meek et al., 2004). However, the significance of those substrates is not well established. During the purification of Ser473 kinase from the membrane fraction of HEK 293 cells, in which the mechanism of PKB activation is well established (Andjelkovic et al., 1997, 1999), it has been found that DNA-PK as a Ser473 kinase (Feng et al., 2004). Therefore, we monitored the status of Ser473 phosphorylation of PKB in DNA-PK knockout MEFs (Taccioli et al., 1998) following the treatment of cells with insulin. As shown in Fig. 1, DNA-PK knock-out MEFs didn’t properly respond to insulin stimulation in Ser473 phosphorylation of PKB whereas DNA-PK wild type control MEFs show a nice increase in Ser473 phosphorylation in a time-dependent manner. Thus these results indicate that DNA-PK is essential for PKB phosphorylation on Ser 473 and activation.

Lack of detection in Ser473 phosphorylation of PKB in human glioblastoma cells expressing an inactive mutant form of DNA-PK, M059J cells. In order to further develop the previous finding that DNA-PK is responsible for Ser473 phosphorylation, we take the advantage of human glioblastoma cells expressing an inactive mutant form of DNA-PK (M059J) or an active wild type of DNA-PK (M059K) (Allalunis-Turner et al., 1993). As expected, Ser473 phosphorylation of PKB was impaired in M059J cells, but not in wild type control cells, M059K cells (Fig. 2), confirming that Ser473 phosphorylation of PKB is mediated by DNA-PK.

PKB activation is normal in ATM knockout MEF cells as well as ATM wild type MEF cells. Recently, Viniegra and colleagues provide new clues in the search of the unknown Ser473 kinase that full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM (Viniegra et al., 2005). Therefore we also monitored the status of Ser473 phosphorylation of PKB in ATM knockout MEFs together with ATM wild type MEFs. Contrast with the previous results, PKB activation in response to insulin treatment was increased in ATM knockout MEFs as well as ATM wild type control MEFs (Fig. 3).

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**Fig. 1.** The status of PKB activation in DNA-PK knockout mouse embryonic fibroblast (MEF) cells. DNA-PKcs wild type (+/+) and knockout (-/-) MEFs were treated with 100 nM insulin in the indicated time after 18 hour serum-starvation. Then 50 μg of cell lysate were subjected to 6% or 12% SDS-PAGE and immunoblotted with pS473 or pT308 phospho-specific antibodies, anti-PKB antibodies or anti-DNA-PK antibodies. Each result is a representative of three independent experiments.

**Fig. 2.** Inhibition of insulin-mediated PKB activation in DNA-PK-deficient human glioblastoma cell lines. Human glioblastoma cells expressing a wild type DNA-PK, M059K or an inactive form of DNA-PK, M059J were treated with 100 nM insulin in the indicated time after 18 hour serum-starvation. Then 50 μg of cell lysate were subjected to 6% or 12% SDS-PAGE and immunoblotted with pS473 or pT308 phospho-specific antibodies, anti-PKB antibodies or anti-DNA-PK antibodies. Each result is a representative of three independent experiments.
ATM +/+ ATM −/−

Insulin(min)  5 15 30 45 5 15 30 45

pS473

pT308

PKB

Fig. 3. Insulin-induced PKB activation in ATM knockout MEF cells. ATM wild type (+/+) and knockout (−/−) MEFs were treated with 100 nM insulin in the indicated time and then analyzed by immunoblot analysis. Each result is a representative of three independent experiments.

Doxorubicin, a DNA-damage inducing agent, mediated Ser473 phosphorylation of PKB is inhibited in DNA-PK knockout MEF cells. Since DNA-PK play a major role in DNA-repairing system (Meek et al., 2004), we further examined the effects of doxorubicin, DNA-damage inducing agent, on Ser473 phosphorylation of PKB. Consistent with previous reports (Li et al., 2005; Quevedo et al., 2007), Ser473 phosphorylation of PKB is diminished in DNA-PK knockout MEFs whereas there is a gradual increase in Ser473 phosphorylation in a time-dependent manner in DNA-PK wild type control MEFs (Fig. 4A). This effect was not observed in ATM knockout MEFs (Fig. 4B), indicating that PKB activation by DNA-PK could also occur in DNA-damage condition.

Inositol hexakisphosphate do not activate DNA-PK in presence of Ku70/Ku86. To further evaluate the effect of DNA-PK on PKB activation, we have screened the potential activator of DNA-PK. Inositol hexakisphosphate (IP6) has been found as a novel factor that is bound by DNA-PK and stimulates DNA end-joining in vitro (Byrum et al., 2004; Hanakahi et al., 2000; Hanakahi and West, 2002; Ma and Lieber, 2002). Therefore, we use IP6 for modulating DNA-PK activity in vitro in presence/absence of Ku70/Ku86 subunit. As shown in Fig. 5A, double-strand (ds) DNA is nicely able to induce the DNA-PK activity toward FSYtide whereas different concentration of IP6 failed to activate DNA-PK. Additionally, we also tested some of charged molecule which mimic the negative charge of dsDNA to activate DNA-PK activity such as Spermine, Poly-L-lysine, Poly-Glu-Tyr, and Heparin. However, none of them success to activate DNA-PK activity toward FSYtide in presence of Ku70/Ku86 (Fig. 5B). Taken together, we were not able to find the modulator of DNA-PK activity addition to dsDNA.

Differential regulation of DNA-PK activity by Mg2+ and Mn2+. It has been reported that DNA-PK activity toward degenerative non-SQ peptide library can be increased in presence of Mn2+ instead of Mg2+ in in-vitro kinase assay (O’Neill et al., 2000). In addition, Chan et al found that DNA-PK activity is independent on double-strand (ds) DNA ends and Ku70/80 subunit in presence of Mn2+ whereas DNA-PK activity is dependent on dsDNA ends and Ku70/80 subunit in presence of Mg2+ (Chan et al., 2000), suggesting the possibility of differential regulation of DNA-PK activity by Mg2+ and Mn2+. Therefore we test the DNA-PK activity toward p53tide or p53tide with either Mg2+ or Mn2+ in in-vitro kinase reaction. Apparently, DNA-PK activity was not changed so much in these conditions (data not shown).

DISCUSSION

In higher eukaryotes, non-homologous end-joining

![Fig. 3](image-url)  

![Fig. 4](image-url)
(NHEJ) DNA is the primary pathway that repairs these breaks. NHEJ also functions in developing lymphocytes to repair strand breaks that occur during V(D)J recombination, the site-specific recombination process that provides for the assembly of functional antigen-receptor genes (Meek et al., 2004). In the last decade, an intensive research effort has focused on NHEJ resulting in a reasonable understanding of how double-strand breaks are resolved. Six distinct gene products have been identified that function in this pathway (Ku70, Ku86, XRCC4, DNA ligase IV, Artemis, and DNA-PKcs). Three of these comprise one complex, the DNA-dependent protein kinase (DNA-PK). This protein complex is central during NHEJ, because DNA-PK initially recognizes and binds to the damaged DNA and then targets the other repair activities to the site of DNA damage (Weterings and Chen, 2007). A large body of evidence suggested that activation of PI3K/PKB pathway is associated with resistance to radiation in many cell lines (Gottschalk et al., 2005; Grana et al., 2002; Gupta et al., 2001, 2003; Li et al., 2004). Therefore, it is tempting to speculate that radiation-induced DNA damage will result in activating PI3K/PKB signaling pathway through DNA-PK, ATM and ATR.

In current report, we have clearly demonstrated that DNA-PK is a key regulator to control PKB activation in insulin and DNA-repair signaling. Immunoblot analysis of DNA-PK knockout MEF cells suggested that PKB phosphorylation on Ser473 is significantly inhibited in DNA-PK knockout MEF cells following the treatment of cells with insulin (Fig. 1). These finding was further supported by the observation that PKB activation is blocked in human glioblastoma cells expressing an inactive mutant form of DNA-PK, M059J cells while PKB activation is normal in control M059K cells (Fig. 2). In addition, the treatment of cells with doxorubicin, a DNA-damage inducing agent, increased Ser473 phosphorylation of PKB in DNA-PK wild type MEFs, but not in DNA-PK knockout MEFs (Fig. 4A), suggesting that DNA-PK play a new role in PKB signaling.

Our results from ATM (-/-) MEFs (Fig. 3) contrast with the observation of Viniegra et al., who claimed that ATM is a major determinant of full PKB/Akt activation in response to insulin or gamma-radiation (Viniegra et al., 2005). However, ATM knockout MEFs in this report was generated from mice of p53 knockout background. Therefore, this discrepancy of observation is due to the fact that p53 somehow play a role in Ser473 phosphorylation of PKB in ATM knockout MEFs through DNA-PK in insulin signaling as well as DNA-repair signaling (Fig. 3 and 4B). Additionally, it has been proposed that mammalian target of rapamycin (mTOR) and its associated protein rictor are involved in Ser473 phosphorylation of PKB in Drosophila and human cells (Hresko and Mueckler, 2005; Sarbassov et al., 2005). Recently, Capeoral and colleagues also provided the evidence of a novel function of ATR as an upstream activator of PKB in response to DNA damage induced by O6-guanine.
methylating agents (Caporali et al., 2008).

Toker and colleagues suggested that phosphorylation of the hydrophobic motif on PKB requires catalytically competent PKB (Toker and Newton, 2000). To evaluate the possibility of PKB autophosphorylation on Ser473, we performed the in-vitro DNA-PK kinase assay with mono-phosphorylated PKB in presence of 1 μM staurosporine (in which PKB activity is completely blocked while Ser473 kinase activity is resistant (Hill et al., 2001)). PKB autophosphorylation on Ser473 is not occurred even though PKB get phosphorylate on The-308, which imply the active PKB (data not shown). In presence of 1 μM staurosporine where PKB activity is completely inhibited (Hill et al., 2001), DNA-PK still phosphorylates PKB on Ser473 (data not shown). These results perfectly match with the previous report (Hill et al., 2001), indicating that DNA-PK is the Ser473 kinase for PKB.

In the current study, we further provided the evidence that DNA-PK is a responsible Ser473 kinase for PKB by using DNA-PK knockout MEF cells. We show that Ser473 phosphorylation of PKB is impaired in DNA-PK knockout MEF cells following insulin stimulation. Furthermore, similar observation was also obtained from DNA-PK deficient cells (Hill et al., 2001), DNA-PK still phosphorylates PKB on Ser473 (data not shown). These results perfectly match with the previous report (Hill et al., 2001), indicating that DNA-PK is the Ser473 kinase for PKB.

In-vivo DNA-PK kinase assay with incorporation of [32P] ATP, using DNA-PK knockout MEF cells. We show that DNA-PK has a novel role in PKB signaling in insulin and DNA-repair signaling pathway. Furthermore, this report supports the role of DNA-PK in Ser473 phosphorylation for regulating PI3K/PKB signaling pathways. Thus it would provide better information for the clinical utilization of DNA-PK inhibitors.

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