Title
Cell cycle dependence of DNA-dependent protein kinase phosphorylation in response to DNA double strand breaks

Permalink
https://escholarship.org/uc/item/1bs799b7

Journal
Journal of Biological Chemistry, 280(15)

ISSN
0021-9258

Authors
Chen, BPC
Chan, DW
Kobayashi, J
et al.

Publication Date
2005-04-15

DOI
10.1074/jbc.M408827200

License
CC BY 4.0

Peer reviewed
DNA-dependent protein kinase (DNA-PK), consisting of the non-homologous end-joining (NHEJ) pathway of DNA double strand break (DSB) repair. Although the kinase activity of DNA-PKcs is essential for NHEJ, thus far, no in vivo substrate has been conclusively identified except for an autophosphorylation site on DNA-PKcs itself (threonine 2609). Here we report the ionizing radiation (IR)-induced autophosphorylation of DNA-PKcs at a novel site, serine 2056, the phosphorylation of which is required for the repair of DSBs by NHEJ. Interestingly, IR-induced DNA-PKcs autophosphorylation is regulated in a cell cycle-dependent manner with attenuated phosphorylation in the S phase. In contrast, DNA replication-associated DSBs resulted in DNA-PKcs auto-phosphorylation and localization to DNA damage sites. These results indicate that although IR-induced DNA-PKcs phosphorylation is attenuated in the S phase, DNA-PKcs is preferentially activated by the physiologically relevant DNA replication-associated DSBs at the sites of DNA synthesis.

Repair of DNA double strand breaks (DSBs) is critical for the maintenance of genome integrity, cell survival, and prevention of tumorigenesis (1, 2). In higher eukaryotes, non-homologous end joining (NHEJ) and homologous recombination (HR) are the two major pathways for DSB repair (3). HR requires the presence of a sister chromatid and is operational in the late S and G2 phases of the cell cycle because of the availability of an optimally positioned sister chromatid (4). NHEJ, on the other hand, does not depend on the presence of homologous DNA sequences and is the predominant pathway for DSB repair in mammalian cells (5). It was proposed that NHEJ is preferentially used in G1 and early S phases of the cell cycle (6, 7). However, a recent report indicating that NHEJ-deficient cell lines are radiation-sensitive in all phases of the cell cycle suggests that NHEJ is important throughout the cell cycle (8). Clearly, the exact contribution of NHEJ in different phases of the cell cycle needs to be defined further.

The NHEJ pathway of DSB repair requires both the DNA-dependent protein kinase (DNA-PK) complex and the XRCC4/DNA ligase IV complex, as well as possible additional accessory factors (5, 9, 10). DNA-PK, the key component of the NHEJ pathway, is composed of the Ku70/80 heterodimer and the catalytic subunit DNA-PKcs (11). Ku binds to DNA ends with very high affinity and is believed to function as the DNA-binding and regulatory subunit that recruits DNA-PKcs to breaks and stimulates its kinase activity (12, 13). DNA-PKcs is a member of the phosphatidylinositol-3-like kinase family that includes ATM (ataxia-telangiectasia mutated) and ATR-3-related (14, 15). Although the biochemical properties of DNA-PK have been extensively studied in vitro, it is still not clear how it functions in vivo in the context of NHEJ. Wild type DNA-PKcs, but not a kinase-dead mutant, is able to rescue the radiation sensitivity and DSB repair defect of DNA-PKcs-defective V3 cells demonstrating that the kinase activity of DNA-PKcs is essential for the NHEJ pathway (16).

To further elucidate the molecular mechanism(s) underlying NHEJ, it is critical to identify the in vivo substrates of DNA-PK and to understand exactly how its kinase activity is regulated. As a first step in this direction, we have previously reported that ionizing radiation (IR) induces DNA-PKcs phosphorylation at threonine 2609 and demonstrated that Thr-2609 phosphorylation is required for DSB repair (17). In addition to Thr-2609, several putative phosphorylation sites have been identified (18, 19) from analyses of purified DNA-PKcs autophosphorylated in vitro; however, none of these sites has been demonstrated to be phosphorylated in vivo in response to DSBs. Here we report the IR-induced autophosphorylation of DNA-PKcs at a novel site, serine 2056, a phosphorylation event that is required for NHEJ-mediated DSB repair. In addition, we also present evidence showing that the phosphorylation of DNA-PKcs is regulated in a cell cycle-dependent and DNA replication-associated manner. We demonstrate that the NHEJ
pathway is preferentially engaged to repair IR-induced breaks in the G1 phase of the cycle. Interestingly, although IR-induced breaks in the S phase do not elicit a DNA-PKcs phosphorylation response, replication-associated DSBs in the S phase appear to preferentially activate DNA-PK.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatments—HeLa, normal human skin fibroblasts (HSF), human glioma cell lines M059K (wild type) and M059J (DNA-PKcs-defective) (20), and Chinese hamster ovary (CHO) cell lines AA8 (wild type) and V3 (defective in both DSB repair and DNA-PKcs expression) (21) were maintained in a humidified atmosphere with 5% CO2 in α-minimum Eagle's medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Treatment with DNA-damaging agents was carried out at the indicated concentrations for 2 h: bleomycin (50 μg/ml), etoposide (30 μg/ml), methyl methanesulfonate (50 μg/ml), and hydroxyurea (1 mM). UV irradiation was carried out at the rate of 0.15 J/m²/sec to achieve a cumulative dose of 10 J/m² followed by a 2-h recovery period. DNA replication-associated DSBs were induced by camptothecin (1 μM) for 1 h, aphidicolin (2 μM/ml) for 1 h, or aphidicolin pretreatment for 30 min followed by camptothecin for 1 h.

Site-directed Mutagenesis and Generation of V3 Stable Cell Lines—Site-directed mutagenesis of the S2056A mutation was performed as described previously (17) using the forward (GGAGCTATTCATA-CAGTGCTCAAGATCGTAAACC) and the reverse (GTTTACGATCTTGAGGCTCATGTTGAAATGC) primers. The V3 cells complemented with wild type DNA-PKcs or kinase-dead mutant DNA-PKcs were described previously (16). V3 cell lines stably expressing S2056A or S2056A/T2609A mutant DNA-PKcs were generated by co-transfection of the S2056A or S2056A/T2609A mutant DNA-PKcs expression plasmids together with the pS2Vneo plasmid into the V3 cells. 48 h after transfection, cells were replaced with selection medium containing 400 μg/ml of G418 (Invitrogen). After 7–10 days selection, individual colonies were isolated and cultured further.

In Vitro Kinase Assay—Whole cell extracts from CHO cells were prepared previously (16). 1 mg of extract was incubated with 10 μM of anti-DNA-PKcs antibody for 5 h at 4 °C followed by the addition of 50 μl of slurry of sheep anti-mouse IgG antibody conjugated to Dynabeads (Dynal Biotech). After 2 h, the beads were washed repeatedly with TM buffer (50 mM Tris-Cl, pH 7.9, 12.5 mM MgCl2, 1 mM EDTA, and 20% glycerol) containing 100 mM KCl as described previously (16). The washed beads were resuspended in 20 μl of TM buffer containing 50 mg/ml Tris-Cl, 10 μM ATP, 10 μl of [γ-32P]ATP, 180 ng of sonicated salmon sperm DNA, and 50 ng of recombinant Phosphatase (Pharmingen) as substrate for 30 min at 30 °C. After electrophoresis in SDS-polyacrylamide gels, phosphorylated Phosphatase-I was visualized by autoradiography.

Microhomology-directed End-joining Assay—A microhomology-directed assay was performed as described previously (17, 22). In brief, the wild type CHO AA8, DNA-PKcs defective CHO V3, or V3 derivative cell lines were transfected with the linearized pDVG9 plasmid DNA. Plasmid DNA was recovered 48 h after transfection and was PCR-amplified across the joining region. Equal amounts of PCR products were digested with BanXI to determine NHEJ-directed (uncut) and microhomology-directed (cut) end joining.

Cell Cycle Synchronization and Colony Formation Assay—Human skin fibroblasts were subjected to serum starvation (0.5% fetal calf serum) for 48 h followed by 1 h of release (10% fetal calf serum) to synchronize in the G1 phase. Cells cultured in G1 were treated with aphidicolin overnight followed by 4 h of release to synchronize the cell cycle in the mid-S phase. V3 and AA8 cells were synchronized in G1, by isoleucine deprivation followed by release in complete medium (24). A colony formation assay was performed as described previously (16).

Fluorescent Immunostaining and Antibodies—Fluorescent immunostaining was performed as described previously (25). Anti-pS2056 polyclonal antibodies were prepared by immunizing New Zealand White rabbits with keyhole limpet hemocyanin-conjugated phosphopeptide QSYSSYSS(PO4)-QDRKPTC. Anti-pT2609 mouse monoclonal antibody was prepared by immunizing BALB/c mice with the keyhole limpet hemocyanin-conjugated phosphopeptide TPFMVFETIPO4/QASQGT according to standard procedures (26). Anti-pT2609 and anti-βH2AX polyclonal antibodies were prepared as described previously (17, 25). Anti-DNA-PKcs 25-4 mAb (NeoMarkers), anti-cyclin A mAb (Upstate Biotechnology), anti-Brc1 mAb (Oncogene), anti-BrdUrd mAb (Roche Applied Science), and anti-RPA2 mAb (Oncogene) are commercially available.

Laser Micro-irradiation—Laser micro-irradiation was conducted with a diode-pumped Spectra-Physics Vanguard laser coupled to a second harmonic generator providing 532 nm laser light pulsed at 76 MHz with a pulse duration of 12 ps as described previously (27). A modified dual camera adaptor with a short pass dichroic mirror substituted for its 50–50% image splitter was mounted on the microscope side port to passively combine green laser light with longer wavelength emissions from the microscope halogen light source used for phase contrast imaging. Laser light entered the straight-through port of the camera adaptor and passed through the dichroic mirror, whereas phase contrast light, having first passed through the specimen, was reflected upward by the dichroic mirror toward the charge-coupled device camera mounted on the right angle camera port of the camera adaptor. Laser light was focused parfocal to the image plane of a 60× oil immersion objective (Phase III, NA 1.4). A mechanical shutter in the laser light path was shuttered at 3–4 Hz with an open time of 3 ms allowing ~220,000 laser pulses/shutter opening. Live cells were imaged at video rate in phase contrast and scanned, using a motorized stage controlled with a joystick to position the nuclei of the cells at the focal point of the laser.

RESULTS

DNA-PKcs purified from IR-treated HeLa cells was separated on SDS-PAGE and digested with Asp-N. The extracted peptides before and after calf intestinal phosphatase treatment were analyzed with matrix-assisted laser desorption ionization time-of-flight (28). A putative phosphopeptide encompassing amino acids 2044–2072 was identified. The phosphopeptide was too large to be sequenced by liquid chromatography/tandem mass spectrometry to find the precise phosphorylation site. To identify the site, we generated a phospho-specific antibody (anti-pS2056, Fig. 1B) and confirmed that Ser-2056 is indeed phosphorylated in vivo in response to IR. Similar to Thr-2909, Ser-2056 is also conserved in all known DNA-PKcs homologues (Fig. 1A) suggesting that phosphorylation of DNA-PKcs at Ser-2056 upon IR may be an evolutionarily conserved response. Ser-2056 phosphorylation could be detected as early as 10 min post-irradiation in HeLa cells after 10 Gy of irradiation. At this dose, the phosphorylation of Ser-2056 reaches a maximum at 30 min and remains detectable even at 8 h after irradiation (Fig. 1C). Phosphorylation at Ser-2056 increases in a dosage-dependent manner; phosphorylation is detectable after 2 Gy of irradiation, and the level of phosphorylation continues to increase with the dose (up to 50 Gy) without reaching saturation (Fig. 1C). In addition to IR, other DSB-inducing agents such as bleomycin and etoposide can also induce Ser-2056 phosphorylation, whereas hydroxyurea, methyl methanesulfonate, and UV irradiation induce minimal phosphorylation at Ser-2056 (Fig. 1D) suggesting that Ser-2056 phosphorylation is specifically in response to DSBs.

The anti-pS2056 antibody is also able to recognize Ser-2056 phosphorylation from fluorescent immunostaining. It stains DNA-PKcs-proficient human glioma M059K cells after IR but not un-irradiated M059K or irradiated M059J cells (DNA-PKcs-defective) (Fig. 2A). Fluorescent immunostaining also revealed that DNA-PKcs phosphorylated at Ser-2056 (pS2056) is present at discrete nuclear foci that co-localize with βH2AX foci (Fig. 2B), presumably at the DNA damage sites (29). Using laser micro-irradiation, we further demonstrated that Ser-2056 phosphorylation of DNA-PKcs localizes directly at the DNA damage sites (Fig. 2C).

IR-induced Ser-2056 phosphorylation is abolished by low doses of wortmannin (Fig. 3A) that inhibit DNA-PKcs and ATM but not the related ATM-Rad5-related kinase (30). To determine whether DNA-PKcs itself or ATM is responsible for Ser-2056 phosphorylation, we examined IR-induced Ser-2056 phosphorylation in V3-WT (wild type) and V3-KD (kinase-dead) cell lines (16). In these cell lines, wild type (V3-WT) or kinase-dead mutant DNA-PKcs (V3-KD) were stably expressed in a CHO V3 cell line that is defective in both DSB repair and DNA-PKcs...
expression (21). In response to IR, Ser-2056 phosphorylation is markedly reduced in V3-KD cells as compared with that of V3-WT cells, whereas the DNA-PKcs protein levels in both cell lines are comparable (Fig. 3B). Moreover, Ser-2056 phosphorylation is unaffected in a panel of cell lines deficient in ATM (data not shown). Thus, IR-induced modification of Ser-2056 is

FIG. 1. Ser-2056 phosphorylation of DNA-PKcs in vivo in response to DSBs. A, alignment of human DNA-PKcs Ser-2056 with DNA-PKcs sequences available in the NCBI data base. B, the anti-pS2056 phospho-specific antibody recognizes IR-induced Ser-2056 phosphorylation specifically from CHO V3 cells (DNA-PKcs-defective) complemented with wild type but not S2056A mutant DNA-PKcs. C, HeLa cells were irradiated with 10 Gy and allowed to recover for the indicated times or irradiated at the indicated doses and allowed to recover for 30 min. Nuclear extracts were prepared for Western blot analysis using the anti-pS2056 or anti-DNA-PKcs antibody. D, HeLa cells were treated with DNA-damaging agents for 2 h (bleomycin (Ble, 50 μg/ml), etoposide (Eto, 30 μg/ml), methyl methanesulfonate (MMS, 50 μg/ml), hydroxyurea (HU, 1 mM)) or were subjected to UV irradiation (10 J/m²) and allowed to recover for 2 h. Nuclear extracts were prepared for Western blot analysis using the anti-pS2056 or anti-DNA-PKcs antibody. M, molecular marker.

FIG. 2. Localization of DNA-PKcs phosphorylated at Ser-2056 at DNA damage sites. A, human glioma cell lines M059K (wild type) and M059J (DNA-PKcs-defective) cells were subjected to IR (10 Gy, 30 min recovery) and were immunostained with the anti-pS2056 or anti-DNA-PKcs antibody. B, normal HSF were irradiated (10 Gy, 30 min recovery) and co-immunostained with anti-pS2056 and anti-H2AX antibodies. C, HSF cells were micro-irradiated with 532 nm pulsed Nd:YAG laser. Ten min after laser micro-irradiation, HSF cells were fixed and co-immunostained with anti-pS2056 and anti-H2AX antibodies. DAPI, 4',6-diamidino-2-phenylindole.

FIG. 3. Modification of Ser-2056 in vivo is due to autophosphorylation. A, HeLa cells were pretreated with increasing concentrations (1, 10, 30, and 100 μM) of wortmannin for 30 min followed by IR (10 Gy, 30 min recovery). Nuclear extracts were prepared and were Western blotted with anti-pS2056 or anti-DNA-PKcs antibody. B, DNA-PKcs-defective V3 cells stably expressing wild type DNA-PKcs (V3-WT), kinase-dead mutant (V3-KD), or T2609A mutation (V3-T2609A) were either mock-treated or irradiated (10 Gy, 30 min recovery). DNA-PKcs was immunoprecipitated and Western blotted with anti-pS2056 or anti-DNA-PKcs antibody. C, DNA-PKcs was immunoprecipitated from V3-vector (−), V3-WT, or V3-S2056A cell lines (upper panel, Western blot with anti-DNA-PKcs antibody). The kinase activity of DNA-PKcs was analyzed using recombinant PHAS-I as substrate (lower panel, autoradiograph of phosphorylated PHAS-I). D, DNA-PKcs immunoprecipitated from V3-S2056A or V3-KD cells were subjected to the in vitro kinase reaction either separately or in a mixture. The in vitro kinase reaction was then subjected to Western blotting to analyze the extent of Ser-2056 phosphorylation.
clearly an autophosphorylation event of DNA-PKcs. Phosphorylation at Ser-2056 is not affected if DNA-PKcs is mutated at the Thr-2609 phosphorylation site (17) indicating that phosphorylation at Thr-2609 does not influence Ser-2056 phosphorylation (Fig. 3B). Phosphorylation at Ser-2056 is also not required for further stimulation of DNA-PKcs kinase activity because alanine substitution at Ser-2056 (S2056A) does not affect the kinase activity of DNA-PKcs (Fig. 3C). In addition, S2056A mutant DNA-PKcs is able to phosphorylate kinase-dead mutant DNA-PKcs at Ser-2056 in vitro suggesting that autophosphorylation at Ser-2056 could occur in trans at least in vitro (Fig. 3D).

To investigate the biological significance of DNA-PKcs phosphorylation at Ser-2056, we examined the radiation sensitivities and DSB-rejoining capabilities of V3 cell lines stably expressing wild type or mutant DNA-PKcs. A colony formation assay was performed to compare the radiation sensitivities of the V3-vector, V3-WT, V3-S2056A, and V3-S2056A/T2609A cell lines. C, microhomology-directed end-joining assay. The parental CHO AA8, V3-vector, V3-WT, V3-S2056A, V3-S2056A/T2609A, and V3-T2609A cell lines were transfected with linearized pDVG9 plasmid DNA (22). Plasmid DNA was recovered 48 h after transfection and was PCR amplified across the joining region. Equal amounts of PCR products were digested with BstXI to determine NHEJ-directed (uncut) and microhomology-directed (cut) end joining.

Both assays (B and C) were repeated at least twice, and the error bars indicate the standard error derived from the independent experiments.
DNA-PKcs phosphorylation was analyzed. As shown in Fig. 5B, the protein level of DNA-PKcs remains constant throughout the cell cycle, whereas IR-induced Ser-2056 phosphorylation decreases from the G1 phase to the S phase. The decrease of IR-induced DNA-PKcs phosphorylation in the S phase was also evident by fluorescent immunostaining with anti-pS2056 antibody (Fig. 5C) and with anti-pT2609 antibody (Fig. 5D) suggesting an overall reduction of IR-induced DNA-PKcs phosphorylation in the S phase of the cell cycle. Note that the G1 cells stain poorly for Brca1, whereas the S phase cells stain strongly for Brca1 thereby confirming their cell cycle distribution (31). These results indicate that phosphorylation of DNA-PK in response to IR is regulated in a cell cycle-dependent manner. In HSF cells synchronized in the G1 phase, more than 90% of the HSF cells were positive for pS2056 foci upon IR. In contrast, less than 15% of the HSF cells synchronized in the S phase were positive for pS2056 foci (Fig. 5E).

Although phosphorylation of DNA-PKcs in response to IR is attenuated in the S phase, V3 cells are reported to be sensitive to agents (camptothecin and hydroxyurea) that disrupt the progression of the replication fork and induce replication-associated DSBs (32, 33). This suggests that although DNA-PKcs is less responsive to IR in the S phase, it may still play an important role in the resolution of DNA replication-associated DSBs in this phase of the cell cycle. We, therefore, examined DNA-PKcs phosphorylation in response to camptothecin, which inhibits topoisomerase I and induces replication-associated DSBs (34). Camptothecin treatment of HSF cells indeed induced DNA-PKcs phosphorylation at Ser-2056 (Fig. 6A). It was reported that DNA polymerase activity is required to convert camptothecin-induced lesions into DSBs and that pretreatment of cells with aphidicolin (DNA polymerase α and δ inhibitor) blocks the induction of DSBs by camptothecin (35). Camptothecin-induced Ser-2056 phosphorylation was significantly attenuated in the presence of aphidicolin (Fig. 6A) indicating that the observed phosphorylation of DNA-PKcs in response to camptothecin is associated with replication fork progression. In addition to the increase in Ser-2056 phosphorylation, fluorescent immunostaining revealed that camptothecin also induces pS2056 (Fig. 6B) and pT2609 (Fig. 6C) only in HSF cells synchronized in the S phase but not in the G1 phase.

To confirm that camptothecin-induced phospho-DNA-PKcs is associated with the sites of DNA replication, HSF cells were pulse-labeled with BrdUrd for 10 min followed by camptothecin treatment for 30 min. Fluorescent immunostaining demonstrated that camptothecin-induced pS2056, pT2609, and γH2AX foci co-localize with the sites of BrdUrd incorporation and that camptothecin-induced p Ser-2056 foci also co-localize with the p34 subunit of replication protein A RPA2 (Fig. 6D) (36). The observed co-localizations strongly suggest that DSBs generated at the sites of replication forks selectively activate DNA-PK. Thus, although IR-induced DSBs do not induce significant DNA-PKcs phosphorylation in the S phase, the DNA replication-associated DSBs selectively activate DNA-PKcs.

**DISCUSSION**

The kinase activity of DNA-PKcs is clearly essential for NHEJ-mediated DSB repair (16). To further elucidate the molecular mechanism(s) underlying NHEJ, it is critical to identify the in vivo substrates of DNA-PK and to understand exactly
how its kinase activity is regulated. Although DNA-PKcs promiscuously phosphorylates a plethora of substrates in vitro including itself, bona fide targets of DNA-PK have largely eluded identification (15). Recently, the locations of seven putative phosphorylation sites on DNA-PKcs were reported based upon analyses of DNA-PKcs autophosphorylated in vitro (17–19). Among the seven identified sites, only Thr-2609 has been conclusively demonstrated to be phosphorylated in vivo in response to IR (17). To directly identify sites that are important in the cellular response to IR, we analyzed DNA-PKcs from irradiated HeLa cells and successfully identified an in vivo autophosphorylation site, Ser-2056. The significance of Ser-2056 phosphorylation in DSB repair is demonstrated by its localization at the sites of DSBs and by the fact that serine to alanine mutation at this site compromises both radiation resistance and NHEJ.

IR-induced Ser-2056 phosphorylation is clearly because of DNA-PKcs autophosphorylation in vivo, as this is markedly reduced in V3 cells complemented with kinase-dead mutant DNA-PKcs (Fig. 3A). In addition, we were able to detect Ser-2056 phosphorylation when S2056A mutant DNA-PKcs and kinase-dead mutant DNA-PKcs were mixed together in an in vitro kinase reaction (Fig. 3D) clearly indicating that the autophosphorylation at Ser-2056 occurs in "trans" at least in vitro. It has been postulated based upon in vitro results that a single DSB may be synapsed by two DNA-PKcs molecules (37, 38). The presence of two active DNA-PKcs molecules in close approximation at a break might make trans autophosphorylation possible with one DNA-PKcs molecule at a break phosphorylating its partner and vice versa (37, 38). Our results suggest that trans or intermolecular autophosphorylation certainly occurs in vitro though we cannot entirely rule out the possibility of "cis" or intramolecular autophosphorylation also occurring in vivo.

Although Ser-2056 and Thr-2609 are both phosphorylated in response to IR and are both required for DNA-PKcs-mediated NHEJ, Ser-2056 and Thr-2609 phosphorylations may be distinctly regulated. First, the physical location of Ser-2056 is far removed from Thr-2609, which is part of a cluster of six putative SQ phosphorylation sites (17–19). Second, the level of Ser-2056 phosphorylation increases with IR dose increases up to 50 Gy without reaching saturation, whereas IR-induced Thr-2609 phosphorylation saturates at 10 Gy (17). Third, IR-induced Ser-2056 phosphorylation is unaffected in a panel of paired ATM-deficient and proficient cell lines (2), whereas IR-induced Thr-2609 phosphorylation is reduced in ATM-deficient cells (Chan et al. (17)). We speculate, therefore, that although Ser-2056 phosphorylation is purely an autophosphorylation event, both DNA-PK and ATM might contribute to Thr-2609 phosphorylation. It is not yet clear whether the difference between Ser-2056 and Thr-2609 phosphorylations reflects their different roles in the DNA-PKcs-mediated NHEJ pathway, but it certainly merits further investigation.

Another important finding from this study is that DNA-PKcs phosphorylation and focus formation in response to IR, although quite robust in G1 phase cells, is significantly attenuated in S phase cells without an apparent decrease in protein levels. This lends credence to the notion that DNA-PK functions primarily in the G1 phase, whereas HR takes over during S/G2 (6, 7). The mechanistic basis for the preferential use of NHEJ versus HR in different phases of the cell cycle is not yet clear. It is also not clear whether this is because of a simple competition between these two pathways or whether this involves active regulatory mechanisms. DNA-PKcs appears to have the ability to suppress the DSB-induced and spontaneous HR (39). It is possible that the decreased DNA-PKcs phosphorylation or activation might account for the increased HR activity in the late S and G2 phases and that regulatory mechanisms might serve to balance NHEJ and HR activities in different phases of the cell cycle. Although IR-induced DNA-PKcs phosphorylation is significantly attenuated in the S phase, we find that the physiologically relevant DSBs generated when a DNA replication fork encounters a damaged template result in robust DNA-PKcs phosphorylation and focus formation. Consistent with this finding, cells deficient in DNA-PKcs show radiation sensitivity in the S phase, although to a lesser degree than cells in the G1 phase (Fig. 5A) indicating that the activity of DNA-PKcs is still required in the S phase, perhaps to repair DSBs associated with DNA replication. Therefore, although DSBs generated by IR do not elicit a DNA-PK activation response in S phase cells, the more physiologically relevant replication-associated breaks may perhaps be preferentially repaired by DNA-PK. It is tempting to speculate that the quick response of DNA-PK to such breaks may be due to an intimate association with the replication machinery.

2 B. P. C. Chen and D. J. Chen, unpublished results.
mediated perhaps by replication protein A, with which DNA-PK is known to associate and phosphorylate (40).

The exact molecular mechanism by which Ser-2056 phosphorylation regulates DNA-PKcs still remains to be elucidated. Lack of Ser-2056 phosphorylation does not affect the kinase activity of DNA-PKcs (Fig. 3C). The localization of DNA-PKcs to DSBs is also not dependent on Ser-2056 phosphorylation, as S2056A mutant DNA-PKcs is able to localize to the DNA damage sites as visualized by immunofluorescent staining with the anti-pT2069 antibody. It is plausible that Ser-2056 phosphorylation could create a docking site for other damage-responsive proteins especially in light of recent evidence indicating that the Brc1 carboxyl-terminal domain interacts with phosphorylated "SQ" or "TQ" motifs (41, 42). Identification of potential pS2056-binding proteins would certainly help to unveil the molecular mechanisms underlying the DNA-PKcs-mediated NHEJ repair pathway.

In summary, we have identified Ser-2056 as a DSB-induced DNA-PKcs autophosphorylation site in vivo and demonstrated that Ser-2056 phosphorylation plays a significant role in NHEJ-mediated DSB repair. In addition, we have also presented evidence showing that the phosphorylation of DNA-PKcs is regulated in a cell cycle- and DNA replication-dependent manner. Although IR-induced DNA-PKcs phosphorylation is attenuated in the S phase, DNA replication-associated DSBs result in DNA-PKcs autophosphorylation and localization at the sites of DNA synthesis.

REFERENCES