Threonines 2638/2647 in DNA-PK Are Essential for Cellular Resistance to Ionizing Radiation

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Abstract

DNA-dependent protein kinase (DNA-PK) is required for the repair of double-stranded DNA breaks through the nonhomologous DNA end joining pathway. DNA-PK activity is required for DNA repair, but kinase activity also appears to be attenuated through an autoregulatory feedback loop. We show that autophosphorylation of DNA-PK catalytic subunit occurs in trans at least three sites NH₂ terminal to the catalytic domain and that two sites, threonine 2638 and 2647, determine DNA-PK autophosphorylation in vitro. Thr2638/2647ala substitution in DNA-PK catalytic subunit compromised cellular resistance to ionizing radiation without affecting DNA end joining, suggesting a requirement for DNA-PK inactivation for cell survival at a step after the rejoining of double-stranded DNA breaks.

Introduction

Cellular survival in response to the introduction of double-stranded DNA breaks is largely dependent on DNA repair through the NHEJ pathway. DNA-PK, comprised of DNA-PKcs and the Ku antigen (Ku70/Ku80) regulatory subunit, is an essential component of the NHEJ pathway. The requirement for DNA-PKcs is enzymatic rather than structural as kinase-dead DNA-PKcs mutants are compromised for NHEJ. However, it also appears that DNA-PK activity is regulated through an autoregulatory feedback mechanism involving autophosphorylation of DNA-PKcs (4). In the present study, we have identified at least three key sites for DNA-PKcs autophosphorylation. Our results show that alanine substitution at two of these sites results in a fully active DNA-PK that is unable to rescue the survival of DNA-PKcs-deficient cells after treatment with ionizing radiation while fully complementing NHEJ. These results identify a specific requirement for DNA-PK inactivation for cellular recovery from ionizing radiation at a step after DNA end joining.

Materials and Methods

Cloning and Expression of Recombinant DNA-PKcs. DNA-PKcs fragments for bacterial expression were amplified by PCR. Site-directed mutations in DNA-PKcs were prepared using QuikChange (Stratagene, La Jolla, CA). For expression in mammalian cells, the complete coding sequence DNA-PKcs was inserted into a modified pCI-neo (Promega) with an NH₂-terminal Flag tag. Site-directed mutagenesis within full-length DNA-PKcs was accomplished by shuffling of a BesEll fragment encompassing the substitution sites. Construct accuracy was confirmed by DNA sequencing as needed.

Phosphorylation Assays. For purified DNA-PKcs, assays were performed as described previously with 10 ng of DNA of ssM13 DNA (ssM13) or HindIII-IplBlue and 0.04 or 50 μM [γ-32P]ATP (3000 Ci/mmol) as indicated. Phosphorylation products were resolved by SDS-PAGE and quantified by phosphorimager (Typhoon; Molecular Dynamics). For stably transfected Si7 SCID cell lines and C.B-17 cells, analysis of p53 peptide phosphorylation by DNA-PK was performed using the double-stranded DNA pull-down procedure of Achari and Lees-Miller (5). All results shown are representative of a minimum of three independent trials with equivalent results.

Mapping of DNA-PK Autophosphorylation. 32P-labeled full-length DNA-PKcs or polypeptide fragments were excised from SDS-PAGE gels and digested at 37°C in situ in 400 μl of buffer D [50 mM NH₄HCO₃ (pH 8.0)] containing 0.050 μg/μl N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin (Worthington Biochemical Corp., Freehold, NJ), for 19 h, with trypsin added at 0 and 16 h. Tryptic phosphopeptides were resolved by 40% alkaline PAGE as described previously (6). Secondary digestion with endoprotease V8 (Glu-C; 25 ng/μl) or endoproteinase Asp-N (Asp-N; 5 μg/μl) was performed on tryptic peptide fragments before secondary alkaline PAGE. Phosphorylation was quantified by phosphorimager. All experiments were repeated two to five times with equivalent results. Phosphopeptide sequencing was performed by manual Edman degradation essentially as described previously (6). Peptide coupling efficiencies to the support matrix were typically 50–70%.

Stable Expression of WT and Mutant DNA-PK. Si7 cell and the parental Sf7 cell line C.B-17 were propagated in DMEM with 10% FCS. After transfections of SfiI-linearized DNA-PKcs, pCI-neoF constructs or empty plasmid (pCI-neoF) using Fugene 6 (Roche Diagnostics), colonies were selected in 300 μg/ml G418. DNA-PK expression was assessed by Western analysis.

Analysis of Radioresistance and DNA Repair. Colony formation assays were performed on 1000 cells irradiated at 2 or 4 Gy by a 137Cs source. Seven days later, colonies that contained >50 cells were scored visually after methanol fixation and trypan blue staining (0.02% in methanol). To monitor in vivo DNA repair, cells exposed to 40 Gy X-rays at 4°C were allowed to recover for 0–4 h postirradiation at 37°C. Subsequent to agarose embedding, pulse field electrophoresis was performed at 10°C in 40 msp Tris acetate and 1 mm EDTA at 24 h at an included angle of 90° (CHEF-DR III). DNA fragmentation was quantified by analysis of the ethidium bromide-stained gel (Typhoon).

Results and Discussion

DNA-PK is a ser/thr kinase with relaxed specificity, displaying a preference for SQ and TQ motifs (7). Indeed, substrate recognition may be primarily determined by substrate colocalization through DNA tethering and protein–protein interactions with Ku rather than by specific protein sequence (6–9). Recently, we showed that structured single-stranded DNA is a cofactor for DNA-PK that preferentially promotes DNA-PKcs autophosphorylation and autoactivation (8). To begin to assess the nature of DNA-PKcs autophosphorylation that leads to autoactivation, we performed a tryptic peptide analysis of the autophosphorylation of DNA-PKcs (Fig. 1A). In this analysis,
PKcs is localized to a peptide containing aa 2596–3075. In are identified I, II, III, and IV based on relative mobility. In B, tryptic phosphopeptide analysis of DNA-PKcs autophosphorylation in the presence of ssM13 and lpBlue DNA and 40 nM or 50 μM γ32P ATP. Approximately 1000 cpm of each sample was resolved by alkaline PAGE. Total DNA-PKcs phosphate incorporation (pmol) is indicated above each lane. The four major tryptic peptides are identified I, II, III, and IV based on relative mobility. In B, try�-autophosphorylation of DNA-PKcs is localized to a peptide containing aa 2596–3075. The schematic at the top outlines the eight GST-DNA-PKcs fusion peptides expressed. The SDS-PAGE gel below displays 32P incorporation into the recombinant DNA-PKcs fragments on phosphorylation by DNA-PK in the presence of lpBlue DNA, whereas the bottom panel is a Coomassie blue-stained gel of the protein preparations used for kinasing. C, alkaline PAGE resolution of tryptic phosphopeptides within DNA-PKcs protein fragment g. Left, phosphorylation of fragment g; right, phosphorylation of DNA-PKcs in the presence of lpBlue and 40 nM ATP. D, secondary proteolytic mapping of tryptic peptides I, II, and IV from peptide g with endoproteinase Asp-N (Asp-N) or Glu-C (Glu-C) as indicated. Control samples were mock digested.

we reasoned that limiting kinase activity might be most effective in distinguishing key regulatory modifications from secondary phosphorylation events of a broad specificity kinase, while recognizing the importance of visualizing the same events under the saturating ATP conditions likely to be prevalent in vivo.

Autophosphorylated DNA-PKcs resulted in at least four phosphopeptides (I-IV) resolved by alkaline PAGE that were differentially represented under the four incubation conditions (Fig. 1A). As described previously, ssM13 DNA was 10–20-fold more effective in stimulating DNA-PKcs autophosphorylation (8). At limiting (40 nm) ATP on ssM13, phosphorylation within phosphopeptide II occurred with an efficiency ∼5–10-fold higher than within the other peptides. By contrast, relative phosphorylation within peptide IV was higher in the presence of lpBlue. Similar relative ratios of phosphorylation were observed at saturating ATP (50 μM). However, in this instance, phosphorylation within an additional peptide (III) was enhanced relative to the phosphorylation within peptide IV.

To begin mapping the DNA-PKcs autophosphorylation sites (Fig. 1B), we compared the phosphorylation of eight GST-DNA-PKcs fusion proteins covering 90% of full-length DNA-PKcs (production of a peptide containing aa 3075–3484 was resistant to all cloning efforts). Only one DNA-PKcs fragment g (aa 2596–3075) was phosphorylated. Thus, DNA-PK exhibited the potential for autophosphorylation in trans within a region that is NH2-terminal to the catalytic domain and COOH-terminal to the site of interaction with Ku antigen. Trypsin digestion of fragment g yielded four phosphopeptides with mobility equivalent to the mobilities of peptides I-IV in full-length DNA-PKcs (Fig. 1C), strongly suggesting that g contained the major DNA-PKcs autophosphorylation sites.

Secondary cleavage of tryptic peptides I, II, and IV with glu-C and asp-N narrowed the search for the autophosphorylation sites by demonstrating the presence of glu in peptide IV and asp in peptides I and II (Fig. 1D). Furthermore, the closely matching mobilities of the asp-N fragments of peptides I and II suggested that these two peptides may have arisen from a common precursor that was differentially or incompletely cleaved by trypsin.

Manual Edman degradation showing the release of 32P from peptide IV at position 14 identified Ser2612, which occurs within an SQ motif, as one phosphorylation site within DNA-PKcs fragment g (Fig. 2, A and B). Repeated analysis of peptide IV suggested the possibility of additional phosphorylation at position 11 in some assays (data not shown), which would correspond to the secondary phosphate release point from peptide IV at thr2609, which occurs within a TQ motif.

Edman analysis of peptides I and II resulted in 32P release at cycle 2 (I and II) and 11 (II only; Fig. 2A). As these release points did not allow for definitive assignment of phosphorylation sites, we performed a tryptic analysis of a smaller DNA-PKcs peptide g fragment, g' (aa 2630–2800), with a single tryptic peptide with release points at positions 2 and 11. Trypsin digestion of g' released phosphopeptides with mobility equal to both peptides I and II (Fig. 2C). This placed phosphorylation within peptides I and II at Thr2638 and Thr2647, both within TQ motifs. It also confirmed that peptides I and II had resulted from directional trypsin cleavage at the COOH-terminal end of a DNA-PKcs peptide beginning at Ala2638.

To confirm the positions of phosphorylation suggested by our analysis, we prepared variants of DNA-PKcs peptide g with ala substitutions at T2609/S2612 and T2638/S2647, respectively (Fig. 2D). Substitutions T2609A/S2612A reduced total phosphate incorporation into DNA-PKcs fragment g at 50 μM ATP by ∼45%, providing strong evidence for significant phosphorylation at these sites. More strikingly, substitutions T2638/S2647A reduced total phosphate incorporation into a fragment g by 85%. Thus, in addition to a strong indication that T2638/S2647 are major DNA-PK phosphorylation sites in DNA-PKcs, our data indicate that phosphorylation at these sites directly facilitates phosphorylation at T2609/S2612.

Tryptic phosphopeptide analysis (Fig. 2E) confirmed that substitu-
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Fig. 2. Identification of the DNA-PKcs autophosphorylation sites. A. 32P release from peptide g tryptic peptides I, II, and IV by Edman degradation. Peptide g was phosphorylated in the presence of ssM13 DNA and 40 nM ATP. 32P release is expressed as a percentage of radioactivity on the sequencing disk. B. sequence of DNA-PKcs from 2599 to 2552 highlighting the positions of phosphorylation (boldfacing) and showing the position of predicted trypsin (closed arrowhead), Glu-C (open arrowhead), and Asp-N (arrow) cleavage. In C, phosphopeptides I and II occur between 2630 and 2800. Alkaline PAGE analysis of tryptic phosphopeptides generated on phosphorylation of DNA-PKcs fragment g (lane 1). Lane 2 and 3 compared with similar analysis of peptide g (Lane 1). In D and E, threonine 2638 and 2647 determine phosphorylation of protein fragment g by DNA-PK. D. 32P labeling of equimolar amounts of WT protein fragment g (Lane 1), T2638/2647A (Lane 2), and T2638A/S2612A (Lane 3) by DNA-PK in the presence of lPBlue and 50 μM ATP. Quantification of 32P incorporation (pmol) is summarized at the top of the gel. E, trypsin cleavage analysis of protein fragments g from D.

Significantly, a report in press appears to confirm that Thr2609/2638/2647 and Ser2612 of DNA-PKcs are phosphorylated on DNA-PKcs in vivo, because DNA-PKcs was recognized by specific phosphopeptide antibodies to these sites in human cells treated with the protein phosphatase inhibitor okadaic acid to increase total cellular phosphoprotein levels (10). Furthermore, it is noteworthy that these four potential DNA-PK autophosphorylation sites are conserved in all six of the vertebrate DNA-PKcs sequences T2638/2647A strongly reduced 32P-incorporation into peptides III and IV in addition to eliminating phosphate incorporation into peptides I and II. By contrast, substitutions T2609A/S2612A reduced but did not eliminate 32P-incorporation into peptide IV. This latter result is consistent with the presence of three additional ser/thr residues within peptide IV that would have the potential to serve as alternative DNA-PK phosphorylation sites on ala substitution of the primary sites.

Fig. 3. Substitutions T2638A/T2647A abrogate the ability of DNA-PKcs to complement radiosensitivity of murine SCID cells. A. Western analysis of the expression of WT DNA-PKcs (Sf7-PKWt) and DNA-PKcs T2638/2647A (Sf7-PK38/47) in stably transfected, clonally selected murine Sf7 SCID fibroblasts. Expression levels are shown relative to DNA-PKcs levels in the parental C.B-17 cell line and purified DNA-PKcs, and represent the signals from 20 μg of whole cell extract. B, mutant DNA-PKcs are kinetically active. Phosphorylation of p53 peptide by DNA-PK partially purified from whole cell extracts by DNA-agarose precipitation, in the presence of lPBlue and 50 μM ATP. In C, DNA-PKcs T2638/2647A-expressing cells are radiosensitive. Cells (1000) from the indicated cell lines were irradiated with γ radiation as indicated. Cell survival was determined by recording colony growth 7 days after irradiation and expressed as a percentage of untreated cells. Results show the mean of duplicate determinations (+/−SD). D, normal DNA repair in DNA-PKcs T2638/2647A-expressing cells. DNA repair in irradiated cells was assessed by pulsed-field gel electrophoresis. Data are expressed as the percentage of breaks remaining 4-h postirradiation. The error bars indicate the SD of three independent experiments. For the difference between Sf7 and C.B-17, Sf7-PKWt and Sf7-PK38/47 P < 0.05 in each instance, whereas the difference between C.B-17, Sf7-PKWt, and Sf7-PK38/47 is not statistically significant.
a 5-h period in response to exposure to 5 Gy of ionizing radiation (data not shown).

Similar to previous results (11, 12), reintroduction of human DNA-PKcs largely rescued the resistance of SJ7 cells to ionizing radiation (Fig. 3C). Approximately 25% of the SJ7-PKWt cells survived exposure to 4 Gy, compared with a 60% survival rate of C.B-17 cells and 2% for SJ7 cells. Similar results were obtained with three additional clones expressing human DNA-PKcs at various levels (data not shown). Unexpectedly, despite fully reconstituting DNA-PK activity in the SJ7 cells, DNA-PKcsT2638/2647 (SJ7-PK38/47 cells) was completely unable to rescue radioresistance. This result did not reflect the peculiarities of a single clone or the relative level of expression of DNA-PKcsT2638/2647A. Within the SJ7-PK38/47 cells, because we have obtained exactly the same results with two other SJ7 clones expressing DNA-PKcsT2638/2647A at many levels (data not shown). Furthermore, we obtained the same results with six clones expressing various levels of DNA-PKcsT2609A/S2612A/T2638A/T2647A.

To directly investigate the importance of T2638/2647 for DNA repair, we monitored the rejoining of double-stranded DNA breaks in response to ionizing radiation using pulse field electrophoresis in a fraction of activity released assay (Ref. 13; Fig. 3D). Four h after exposure to 40 Gy of ionizing radiation, >30% of the initial DNA damage remained in DNA prepared from DNA-PKcs-deficient SJ7 cells. By contrast, repair in WT C.B-17 and SJ7 cells expressing WT human DNA-PKcs was largely complete, with only ~5–10% of DNA breaks remaining. Unexpectedly, despite the compromised survival, the SJ7-PK38/47 cells expressing DNA-PKcsT2638/2647A exhibited the same DNA repair efficiency as the SJ7-PKWt cells expressing WT DNA-PKcs and an efficiency comparable with the C.B-17 cells. The repair efficiency of SJ7-PK38/47 cells also closely paralleled the efficiency of C.B-17 and SJ7-PKWt cells at earlier times after irradiation (data not shown).

Our results identify T2638/2647 in DNA-PKcs as being essential for radioresistance conferred by DNA-PK. However, consistent with previous reports that the kinase activity of DNA-PK is required for NHEJ, substitutions at T2638/2647 that alleviate autophosphorylation of DNA-PKcs and leave what appears to be a fully active kinase remained competent for NHEJ. However, DNA-PKcs activity was not sufficient to allow for cell proliferation after DNA repair, because cells expressing DNA-PKcs containing these substitutions failed to recover from irradiation. The precise role of DNA-PK autophosphorylation that allows for cell survival after NHEJ awaits further investigation. However, as the consequences DNA-PKcs autophosphorylation in vitro include inactivation of kinase activity and dissociation of DNA-PKcs from Ku antigen and DNA (4), it is possible that autophosphorylation of DNA-PKcs is required for dissociation of DNA-PKcs from the DNA after rejoicing. This may remove a physical constraint to regenerate predamage chromatin structure directly or could act indirectly by allowing reversal of phosphorylation of factors modified by DNA-PK at the repair site.

T2638 and T2647 appear to be the key sites for autophosphorylation that are required for cell survival after exposure to ionizing radiation. Alanine substitution at these two sites reduced DNA-PKcs autophosphorylation by 85% and failed completely to rescue SJ7 cells exposed to ionizing radiation. Interestingly, after initial submission of this work, a report appeared describing T2609 as a DNA-PKcs autophosphorylation site that was important for NHEJ (14). T2609 phosphorylation was shown to occur specifically at sites of DNA damage. However, alanine substitution at this site allowed partial rescue of radiation resistance while severely down-regulating NHEJ. The reason for these differences in phenotype remain to be determined. However, we note that our data indicated that T26269/S2612 phosphorylation is largely dependent on previous phosphorylation of T2638/T2647. Thus, it may be that phosphorylation at T2638/2647 is the predominant determinant for cell survival subsequent to repair, whereas additional phosphorylation at T2609 is directly required for repair.

Lastly, the potential to disrupt NHEJ and DNA-PKcs function through the prevention of DNA-PK phosphorylation at T2638/2647 offers a novel and potentially attractive therapeutic target to enhance the action of therapies that target tumor cells by introducing double-stranded DNA breaks that are repaired through NHEJ.

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References

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