DNA-dependent Protein Kinase-independent Activation of p53 in Response to DNA Damage*

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Phosphorylation at serine 15 of the human p53 tumor suppressor protein is induced by DNA damage and correlates with accumulation of p53 and its activation as a transcription factor. The DNA-dependent protein kinase (DNA-PK) can phosphorylate serine 15 of human p53 and the homologous serine 18 of murine p53 in vitro. Contradictory reports exist about the requirement for DNA-PK in vivo for p53 activation and cell cycle arrest in response to ionizing radiation. While primary SCID (severe combined immunodeficiency) cells, that have defective DNA-PK, show normal p53 activation and cell cycle arrest, a transcriptionally inert form of p53 is induced in the SCID cell line SCGR11. In order to unambiguously define the role of the DNA-PK catalytic subunit (DNA-PKcs) in p53 activation, we examined p53 phosphorylation in mouse embryonic fibroblasts (MEFs) from DNA-PKcs-null mice. We found a similar pattern of serine 18 phosphorylation and accumulation of p53 in response to irradiation in both control and DNA-PKcs-null MEFs. The induced p53 was capable of sequence-specific DNA binding even in the absence of DNA-PKcs. Transactivation of the cyclin-dependent-kinase inhibitor p21, a downstream target of p53, and the G1 cell cycle checkpoint were also found to be normal in the DNA-PKcs−/− MEFs. Our results demonstrate that DNA-PKcs, unlike the related ATM protein, is not essential for the activation of p53 and G1 cell cycle arrest in response to ionizing radiation.

When cells are exposed to DNA-damaging agents, there is accumulation of the p53 tumor suppressor protein and its activation as a transcription factor. This ultimately results in the arrest of cell cycle progression until the damage is repaired. Human p53 is phosphorylated within its transactivation domain at serine 15 in response to DNA damage (1). Serine 15 phosphorylation results in reduced interaction of p53 with its negative regulator, the oncoprotein Mdm2, which, in turn, leads to the stabilization and activation of p53 (2). Subsequent up-regulation of downstream target genes by p53, particularly the cyclin-dependent-kinase inhibitor p21, results in the arrest of cells in the G1 phase of the cell cycle (3).

The DNA-dependent protein kinase (DNA-PK)1 consists of a heterodimeric DNA-binding complex of Ku70 and Ku80 and a large catalytic subunit, DNA-PKcs. DNA-PKcs is a member of a subgroup of the phosphatidylinositol kinase superfamily, other members of which function in DNA damage responses and/or cell cycle control (4). DNA-PKcs is a serine/threonine protein kinase that is activated by DNA double-strand breaks (DSBs). DNA-PK is required for repair of DNA DSBs, and cells deficient in DNA-PKcs are hypersensitive to ionizing radiation (IR) and radiomimetic drugs.

DNA-PK has been an attractive candidate for a molecule that activates p53 in response to ionizing radiation thereby linking DNA damage to cell cycle arrest. DNA-PK phosphorylates human p53 at serine 15 and murine p53 at the homologous serine 18 residue in vitro (5, 6). Phosphorylation of p53 at serine 15 by purified DNA-PK leads to reduced interaction of p53 with Mdm2 in vitro and correlates with the stabilization of p53 and its activation as a transcription factor (2). It has recently been reported that a SCID (severe combined immunodeficiency) cell line SCGR11 accumulates a transcriptionally inactive form of p53 upon irradiation (7). As SCID cells have defective DNA-PKcs (8), it would appear that DNA-PK indeed acts upstream of p53 in response to DNA damage.

However, the role of DNA-PK in p53 activation has been called into question by reports of normal p53 induction and cell cycle arrest in response to IR in primary cells from SCID mice (9–12). Moreover, the ATM protein, encoded by the gene responsible for the human genetic disorder ataxia telangiectasia (AT), is required for the phosphorylation of p53 at serine 15 in vivo in response to IR (13, 14). AT cells have normal levels of DNA-PK (15), yet show reduced and delayed serine-15 phosphorylation in response to IR (1). These results, on the other hand, suggest that while DNA-PK may phosphorylate p53 in vitro, it may not play an essential role in the activation of p53 nor can it substitute for the ATM protein in response to IR.

In order to definitively delineate the role of DNA-PK in p53 activation we utilized cells derived from DNA-PKcs-null mice. These cells, unlike primary SCID cells (7), are completely deficient in DNA-PK kinase activity (16). Transformed cell lines like SCGR11 often have mutations in p53. Therefore, studies were performed on early passage fibroblasts derived from 13.5-

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1 The abbreviations used are: DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; DSB, double-strand break; IR, ionizing radiation; SCID, severe combined immunodeficiency; AT, ataxia telangiectasia; MEF, mouse embryonic fibroblast; Gy, gray; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; BrdUrd, bromodeoxyuridine; HO, Hoechst 33342; MI, mithramycin.

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day-old mouse embryos. Control and DNA-PKcs-null cells were irradiated and compared with respect to accumulation of p53 protein, serine 18 phosphorylation of p53, sequence-specific DNA binding by p53, up-regulation of p21 gene expression, and integrity of the G1 cell cycle checkpoint.

**EXPERIMENTAL PROCEDURES**

Establishment of DNA-PKcs +/+ and −/− Mouse Embryonic Fibroblasts (MEFs) and Irradiation of Cells—Primary fibroblasts were isolated from 13.5-day-old mouse embryos. The embryos were genotyped by PCR which distinguishes the endogenous from the targeted DNA-PKcs allele (16). Cells were maintained in a humidified atmosphere with 5% CO2 in a 5% CO2 incubator at 37 °C. The integrity of the G1 cell cycle checkpoint.

DNA binding by p53, up-regulation of p21 gene expression, and accumulation of p53 were observed after 10 days. Moreover, both accumulation and phosphorylation of p53 in response to IR—DNA-PKcs +/+ and −/− MEFs were grown to 80% confluence and irradiated with a cumulative dose of 6 Gy. Whole cell extracts for DNA-PKcs Western blotting were prepared from mock-irradiated and irradiated MEFs at 2 h post-irradiation using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA). After digestion of contaminating genomic DNA by DNase I (Ambion, Austin, TX), cDNA synthesis was carried out with the SuperScript preamplification system (Life Technologies, Inc.) according to the included protocol. PCR primers used for RT-PCR were MD-3 (5′-ATCGAAAGGTCTAAGGCCAAT-3′) and MD-5 (5′-CGTACGGTGGTGGCTACTCG-3′) for amplification between exons 1 and 4 of DNA-PKcs and GA-5 (5′-AGAAGACTGTGGATGCGCC-3′) and GA-3 (5′-AGGTTGGCTACTGC-3′) for control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification.

Whole-cell extracts for DNA-PKcs Western blotting were prepared from mock-irradiated and irradiated MEFs at 2 h post-irradiation as described previously (17). The protein concentration of extracts was determined by Bradford analysis using bovine serum albumin as a standard. Western blot analysis of DNA-PKcs was performed as described previously (17). The antibodies used for Western blotting are anti-p53 peptide (2), and anti-actin (C-11) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Western blotting revealed the presence of equal amounts of DNA-PKcs protein in control and irradiated +/+ MEFs, but no detectable protein in the −/− MEFs and its absence in the −/+ MEFs was confirmed by reverse transcription-PCR (RT-PCR). RT-PCR products between exon 1 and exon 4 of DNA-PKcs were clearly absent in the −/− MEFs (Fig. 1A). Western blotting revealed the presence of equal amounts of DNA-PKcs protein in control and irradiated +/+ MEFs, but no detectable protein in the −/− MEFs (Fig. 1B). The +/+ and −/− MEFs were also characterized by low dose rate irradiation with 133Cs γ-rays (1.4 Gy/day for 10 days). After irradiation, the cells were allowed to recover for 10 days. While we observed growing DNA-PKcs +/+ MEFs, no surviving DNA-PKcs −/− MEFs were observed after 10 days.

**Results**

Establishment and Characterization of Embryonic Fibroblasts from DNA-PKcs-null Mice—We have generated DNA-PKcs-null mice by disrupting the DNA-PKcs gene by homologous recombination (16). MEFs were isolated from DNA-PKcs +/+ and −/− sibling embryos. The genotype of the embryos was determined by PCR (16) that distinguishes endogenous from the disrupted DNA-PKcs allele (data not shown). The presence of intact DNA-PKcs transcripts in the +/+ MEFs and its absence in the −/+ MEFs was confirmed by reverse transcription-PCR (RT-PCR). RT-PCR products between exon 1 and exon 4 of DNA-PKcs were clearly absent in the −/− MEFs (Fig. 1A). Western blotting revealed the presence of equal amounts of DNA-PKcs protein in control and irradiated +/+ MEFs, but no detectable protein in the −/− MEFs (Fig. 1B). The +/+ and −/− MEFs were also characterized by low dose rate irradiation with 133Cs γ-rays (1.4 Gy/day for 10 days). After irradiation, the cells were allowed to recover for 10 days. While we observed growing DNA-PKcs +/+ MEFs, no surviving DNA-PKcs −/− MEFs were observed after 10 days.
specific DNA binding by EMSA using double-stranded oligonucleotides bearing the consensus p53 DNA-binding motif (Fig. 3). Binding reactions were carried out in the presence of a specific anti-p53 antibody PAB421 that activates sequence-specific DNA binding by p53 (22). The bands indicated by an arrow in Fig. 3 represent sequence-specific DNA binding by p53 as they are not obtained in the absence of PAB421 (Fig. 3, lane 6) or in the presence of an unrelated antibody (Fig. 3, lane 7). Enhancement of sequence-specific DNA binding by p53 was observed upon irradiation of the +/- MEFs (Fig. 3, compare lanes 2 and 3) and also upon irradiation of the -/- MEFs (Fig. 3, compare lanes 4 and 5).

Up-regulation of p21 Gene Expression upon Irradiation of Both DNA-PKcs +/- and -/- MEFs—Activated p53 can transactivate the cyclin-dependent-kinase inhibitor p21 (23). Therefore, in order to test whether the observed phosphorylation, accumulation and DNA binding of p53 corresponded to activation of transcription in vivo, we quantitated the induction of p21 messenger RNA upon irradiation of DNA-PKcs +/- and -/- MEFs by quantitative RT-PCR. We found that the +/- and -/- MEFs are indistinguishable with respect to the enhancement (4.5–5-fold) of p21 gene expression upon irradiation (Fig. 4, A and B).

The G1 Cell Cycle Checkpoint Is Intact in the DNA-PKcs -/- MEFs—To investigate the effect, if any, of the absence of DNA-PKcs on the G1 cell cycle checkpoint, DNA-PKcs +/- and -/- MEFs were mock-irradiated or irradiated at 6.0 Gy and labeled with BrdUrd immediately after irradiation. BrdUrd incorporations at 6 h post-irradiation were quantified from multiparameter flow cytometry measurements (21). The bivariate contour histograms (Fig. 5A) depict the distributions of cells with different BrdUrd (MI-HO fluorescence) and DNA (MI fluorescence) contents in the control and irradiated cultures. By 6 h post-irradiation, cells were depleted from the S phase (boxed in Fig. 5A) in both DNA-PKcs +/- and -/- cultures indicating induction of arrest at a G1 checkpoint. The ratios of percentage of total cells in G1 to percentage of total cells in S phase (percent cells in G1/percent cells in S) for control and irradiated cultures were plotted (Fig. 5B). The extent of G1 arrest was comparable for the DNA-PKcs +/- and -/- cultures (Fig. 5B).

**DISCUSSION**

In this paper we present evidence proving that DNA-PKcs is not essential for p53 activation and cell cycle arrest in response to IR. Upon irradiating DNA-PKcs-null MEFs, we observe normal p53 accumulation and serine 18 phosphorylation, sequence-specific DNA binding by p53, transactivation of p21 gene expression, and normal G1 cell cycle arrest in the absence of DNA-PKcs.

Earlier reports on p53 activation in the absence of DNA-PKcs were based upon experiments with primary cells from SCID mice (9–12). However, primary SCID cells have been reported to retain residual DNA-PK kinase activity (7). This could be because the SCID mutation resides just downstream of the conserved kinase motifs of the DNA-PKcs gene resulting in a truncated product missing only 83 amino acid residues from the extreme carboxyl-terminal end (8). In order to avoid the ambiguity associated with SCID cells, we established MEFs derived from DNA-PKcs-null mice as these cells are completely deficient in DNA-PKcs and, unlike many transformed cell lines, would not have mutations in p53.

The DNA-PKcs-null mice, generated by disrupting the DNA-PKcs gene by homologous recombination, show severe immunodeficiency and radiation hypersensitivity (16). Cells from the DNA-PKcs-null mice have no detectable DNA-PKcs protein or kinase activity (16). The DNA-PKcs +/- and -/- MEFs used for this study were extensively characterized with respect to DNA-PKcs status and phenotype by (i) PCR (for genotyping), (ii) RT-PCR (for confirming the presence of intact DNA-PKcs transcripts in the +/- but not in the -/- MEFs), (iii) Western
Phosphorylation of human p53 within its transactivation domain at serine 15 or murine p53 at serine 18 is an early and important step in the activation and accumulation of this protein in response to IR (24). This phosphorylation event could contribute to both increased p53 half-life and increased transcriptional activity by decreasing the ability of the negative regulator Mdm2 to bind to p53 (2). However, none of the earlier reports describing the involvement or noninvolvement of DNA-PK in p53 activation have focused on this phosphorylation event. Our results demonstrate that DNA-PKcs, unlike ATM, is not essential for the phosphorylation or accumulation of p53 in response to IR.

It is interesting that phosphorylation and accumulation of p53 are actually enhanced in the DNA-PKcs−/− MEFs relative to the +/+ MEFs. Our observations are consistent with those of Araki et al.2 who observed a similar enhancement in transformed mouse cell lines with defective DNA-PKcs and with those of Gurley and Kemp (11) who observed a prolonged induction of p53 protein in the intestinal crypt cells of irradiated SCID mice. The ATM protein can bind to DNA ends like DNA-PKcs, and the p53 serine 15 kinase activity of ATM is stimulated by the presence of damaged DNA.3 We speculate that in the absence of DNA-PKcs, ATM may have greater access to DNA DSBs as it no longer has to compete with DNA-PKcs for end binding. This, coupled with the persistence of DNA DSBs in the absence of DNA-PK, could result in enhanced activation of ATM, which may explain the enhanced phosphorylation and accumulation of p53 observed in the DNA-PKcs−/− MEFs.

Our observations regarding induction of sequence-specific DNA binding by p53 upon irradiation of the DNA-PKcs−/− MEFs are not in agreement with those of Woo et al. (7) who fail to detect any DNA binding using extracts from SCGR11 cells. This difference is possibly due to the fact that p53 from SCGR11 is mutated in its DNA-binding domain.2 The mutation is a “T” to “C” transversion resulting in a substitution of leucine at position 191 to arginine (DDBJ accession number AB021961). The vast majority of p53 missense mutations are clustered within the DNA-binding region and the inability of p53 from SCGR11 to bind to DNA would be due to the defect in its DNA-binding domain and not, as proposed by Woo et al. (7), due to the absence of DNA-PKcs in these cells.

Woo et al. (7) also reported that cytoplasmic extracts from γ-irradiated M059J cells (that lack DNA-PKcs) could not activate in vitro–translated p53 for DNA binding (7). However, M059J cells also have drastically reduced levels of the ATM protein (25). The ATM protein is essential for p53 activation upon irradiation and the lack of p53 activation by M059J cytoplasmic extracts may not be due to the absence of DNA-PKcs but due to the reduced levels of ATM.

p53 activation in response to IR leads to transactivation of the cyclin-dependent-kinase inhibitor p21 and G1 cell cycle arrest (24). In order to correlate the observed p53 accumulation, phosphorylation, and DNA binding with its activation as a transcription factor, we looked at the induction of p21 gene expression upon irradiation and the integrity of the G1 cell cycle checkpoint in the DNA-PKcs−/− MEFs. We found that the DNA-PKcs−/− and −/− MEFs are indistinguishable with regard to fold induction of p21 gene transcription upon irradiation. We also found that both DNA-PK−/− and −/− cultures arrested in the G1 phase of the cell cycle by 6 h post-irradiation. These results are consistent with earlier reports of normal p21 gene induction and cell cycle arrest in primary SCID cells (9–12).

Our observations involving DNA-PKcs−/− MEFs indicate unequivocally that DNA-PKcs, unlike the related ATM protein, is not essential for the activation of p53 and G1 cell cycle arrest in response to ionizing radiation. An alternate interpretation could be that DNA-PKcs is still involved in p53 activation, as suggested by in vitro experiments (2, 5, 6), but the loss of DNA-PKcs activity is concealed by the presence of other p53-dependent pathways like the one involving ATM. In this re-
gard, it should be noted that AT cells have mutated ATM protein but contain wild type DNA-PKcs. So, if DNA-PKcs were to play a major role in p53 activation, then p53 phosphorylation in A-T cells would not be significantly reduced and delayed (1). Therefore, while DNA-PKcs is involved in the repair of DNA DSBs (4, 17), our experiments clearly rule out an essential role for DNA-PKcs in the activation of a p53-mediated DNA damage signaling pathway.

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Activation of p53 in DNA-PKcs-null cells