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Advances in Brief

Enhanced Phosphorylation of p53 Serine 18 following DNA Damage in DNA-dependent Protein Kinase Catalytic Subunit-deficient Cells

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Abstract

DNA-dependent protein kinase (DNA-PK) controls signal transduction following DNA damage. However, the molecular mechanism of the signal transduction has been elusive. A number of candidates for substrates of DNA-PK have been reported on the basis of the *in vitro* assay system. In particular, the Ser-15 amino acid residue in p53 was one of the first such *in vitro* substrates to be described, and it has drawn considerable attention due to its biological significance. Moreover, p53 Ser-15 is a site that has been shown to be phosphorylated in response to DNA damage. In addition, crucial evidence indicating that DNA-PK controls the transactivation of p53 following DNA damage was reported quite recently.

To clarify these important issues, we conducted the experiments with *dna-pkcs* null mutant cells, including gene knockout cells. As a result, we detected enhanced phosphorylation of p53 Ser-18, which corresponds to Ser-15 of human p53, and significant expression of p21 and mdm2 following ionizing radiation. Furthermore, we identified a missense point mutation in the p53 DNA-binding motif region in SCGR11 cells, which were established from severe combined immunodeficient (SCID) mice and used for previous study on the role of DNA-PK in p53 transactivation.

Our observation clearly indicates that DNA-PK catalytic subunit does not phosphorylate p53 Ser-18 *in vivo* or control the transactivation of p53 in response to DNA damage, and these results further emphasize the different pathways in which ataxia telangiectasia-mutated (ATM) and DNA-PK operate following radiation damage.

Introduction

A number of substrates for DNA-PK[2] have been detected using the *in vitro* assay system. Ser-15 in p53 was one of the first such substrates to be described (1). DNA-PK has long been an appealing candidate as a monitoring molecule of DNA damage because it has an ability to bind DNA double-strand breaks and requires DNA double-strand breaks for its enzymatic activity. The next step to be taken was to clarify whether each *in vitro* substrate was truly an *in vivo* substrate. The SCID mouse has been used to achieve this objective, and it was demonstrated that the cell cycle regulation following radiation damage and p53 stabilization and the activation of downstream pathways are normal in SCID cells (2–5). Recently, however, several observations suggesting that *scid* is a partially active mutant of *dna-pkcs* have been reported (6–9). These recent findings necessitate a reexamination of DNA-PKcs function using true *dna-pkcs* null mutants. In addition, antibodies specific for phosphorylated p53 Ser-15 were developed recently allowing researchers to analyze the phosphorylation of Ser-15 of p53 product in the *in vivo* situation (10, 11).

Surprisingly, recent data have suggested that DNA-PK activity is required for optimal p53 activation (12). Using the *dna-pkcs* null mutant SX9 cells and the *dna-pkcs* knockout cell line PK33N, therefore, we examined the *in vivo* phosphorylation of Ser-18 residue, which corresponds to Ser-15 of human p53, in the p53 product in response to ionizing radiation as well as the expression of p21 and mdm2, which are controlled by p53 product in normal cells.

Materials and Methods

Cell Culture. SX9 and SR-1 cells were derived from mouse mammary carcinoma FM3A cells. SR-1 cells were used as a wild-type control (13–15). SX9 has a ^{9572}C→T transition, which causes a Leu-3191→Pro residue substitution in the DNA-PKcs molecule (7). PK33N and PK34N cells were derived from lung fibroblasts of *dna-pkcs* knockout mice and the parent strain 129sv, respectively (16). SR-1, SX9, PK34N, and PK33N cells were cultured in α-MEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS, 1% L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown at 37°C in 5% CO_2.

Induction of DNA Damage. Cells were irradiated with 7 Gy using the X-ray apparatus Shin-ai 250 (Shimadzu, Kyoto, Japan) and harvested at indicated times.

Western Blot Analysis. Western blot analysis was carried out by standard methods. In brief, proteins from total cell lysates were separated by 10–20% gradient SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. Immunoreactive bands were visualized with ECL (Amersham-Pharmacia, Uppsala, Sweden) chemiluminescent substrate and recorded on X-ray film, and quantitation was performed with a Chemi-Imager (Alpha Innotech, San Leandro, CA).

The primary antibodies used in this study were rabbit polyclonal antiphosphorylated Ser-15-specific antibody (10, 11), mouse monoclonal anti-p53 antibody pAb421 (Calbiochem, Cambridge, MA), mouse monoclonal anti-ATM antibody 2C6 (GeneTex, San Antonio, TX), mouse monoclonal anti-α-tubulin antibody Ab-1 (Calbiochem), and mouse monoclonal anti-waf1 antibody Ab-4 (Calbiochem). Horseradish peroxidase-conjugated secondary antibodies (DAKO, Copenhagen, Denmark) were used to detect primary antibody signals. The rabbit polyclonal antiphosphorylated Ser-15-specific antibody does not react with the nonphosphorylated residue *in vivo* (10, 11).

Semiquantitative RT-PCR. Total RNAs were prepared from 5×10^6 cells with RNeasy total RNA kit (Qiagen, Hilden, Germany), and 1.3 μg of total RNA fraction was used for the first strand synthesis. The synthesis was performed using Superscript II (Life Technologies, Inc.) with oligo(dT) primer, according to the method recommended by the supplier. The primer sets of the PCR were as follows: p21, 5′-GACCATGTCCAATCCTGGTGATGTCCGACC-3′and 5′-CTCCCGTGGGCACTTCAGGGTTTTCTCTTG-3′; mdm2, 5′-ATGTGCAATACCAACATGTCTGTGTCTACC-3′ and 5′-CAGGTAGCTCATCTGTGTTCTCTTCTGTCT-3′; and β-actin, GAACCCTAAGGCCAACCGTGAAAAGATGAC-3′ and 5′-TGATCTTCATGGTGCTAGGAGCCAGAGCAG. PCR was performed with an LA PCR Kit (TaKaRa, Kyoto, Japan) under the following conditions: 1 cycle of 94°C for 1 min and

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[2] The abbreviations used are: DNA-PK, DNA-dependent protein kinase; SCID, severe combined immunodeficiency; DNA-PKcs, DNA-PK catalytic subunit; ATM, ataxia telangiectasia-mutated; RT-PCR, reverse transcriptase-PCR.

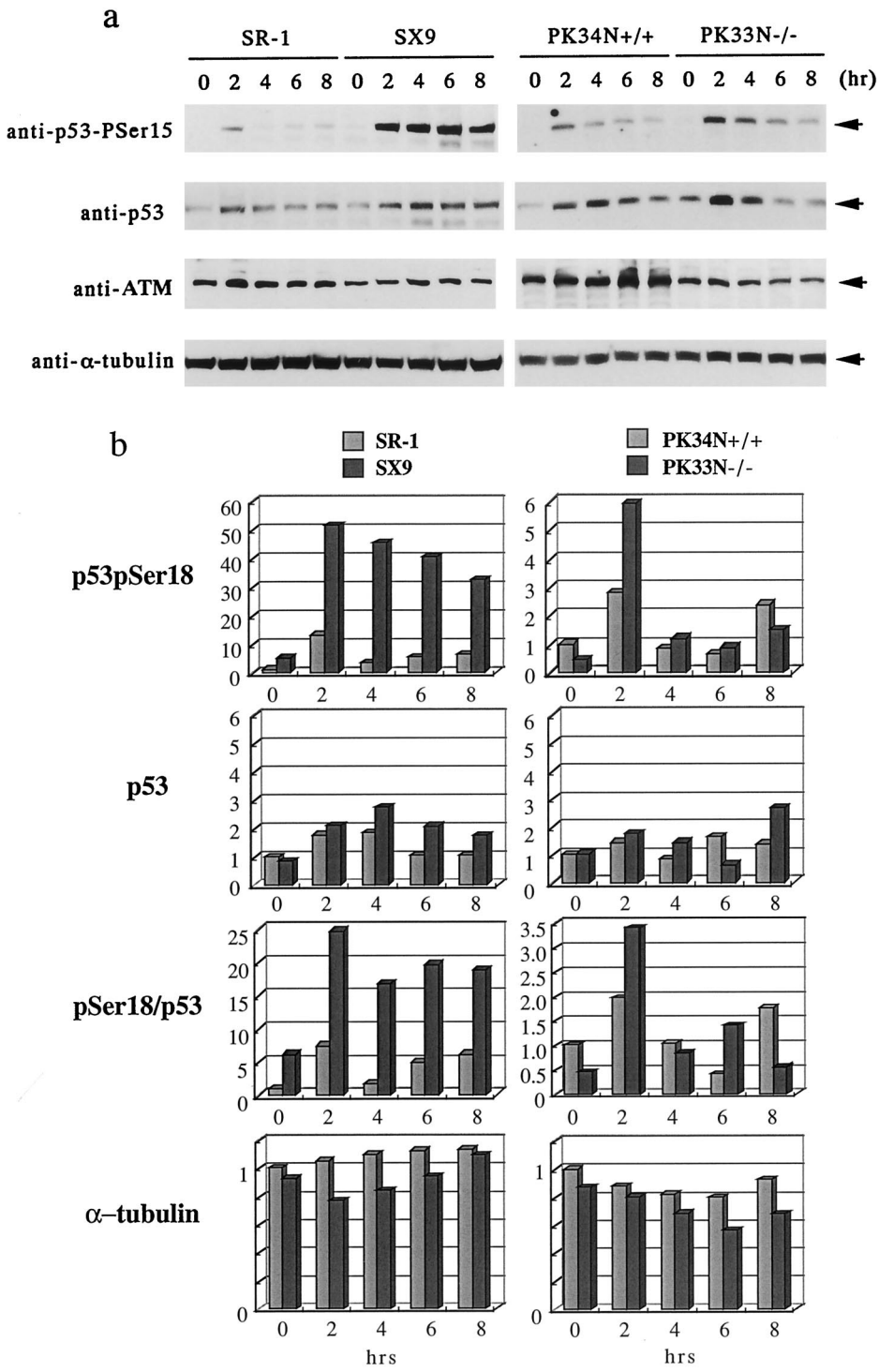

Fig. 1. *a*, Western blots of whole cell lysate from SR-1, SX9, PK34N, and PK33N cells with antiphosphorylated Ser-15-specific antibody, anti-p53 antibody, anti-ATM antibody, and anti-α-tubulin antibody. *Lanes 0, 2, 4, 6,* and *8*, extracts prepared 0, 2, 4, 6, and 8 h, respectively, after 7 Gy irradiation. *b*, induction of phosphorylation on p53 Ser-18 and amounts of p53. Western blot patterns were quantified. Intensity of p53 phosphorylated Ser-18 (*p53pSer18*), p53, and phosphorylated Ser-18 p53 (*pSer18/p53*) was normalized with the density of α-tubulin on the Western blots. *Y axis*, relative values when the intensity at 0 h of each parent cell was defined as 1; *X axis*, preparation time of each extract after irradiation. The Ser-18 protein intensity was normalized by the amount of p53.

20–30 cycles of 98°C for 20 s and 68°C for 2 min. One-twenty-fifth of the PCR products were subjected to electrophoresis.

Sequence of the Whole Region of *p53* cDNA. *p53* cDNAs were amplified by the RT-PCR technique with Pfu DNA polymerase (Stratagene, La Jolla, CA), and the amplified products were sequenced by BigDye Terminator Cycle Sequencing Kit (Applied Biotechnology, Inc. Foster City, CA; Ref. 17).

Results and Discussion

We found that *in vivo* phosphorylation of Ser-18 of p53 in response to ionizing radiation is not impaired in either *dna-pkcs*-deficient cell lines and that p53 stabilization occurs, resulting in its accumulation. Interestingly, Ser-18 phosphorylation and p53 accumulation in both cell lines were rather enhanced, as compared to those in the parental SR-1 cells and PK34N cells (Fig. 1). Sequencing of cDNA revealed no *p53* mutations in SX9 or PK33N cells (DNA Data Bank of Japan accession nos. AB017816, and AB020317, respectively). A similar pattern of phosphorylation and accumulation of p53 was observed in MEF cells from *dna-pkcs*−/− mice (data not shown).

Because ATM products have the ability to phosphorylate Ser-15 on p53 in human cells (18, 19), we measured the amount of ATM

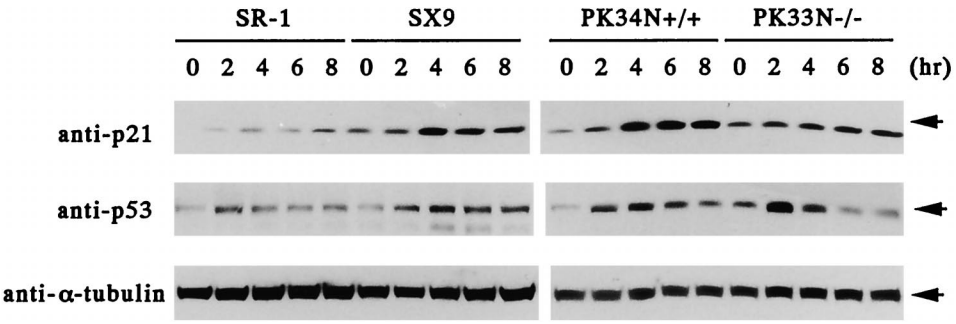

Fig. 2. Western blots of whole cell lysate from SR-1, SX9, PK34N, and PK33N cells with anti-p21 antibody. See Fig. 1 legend for lane assignments.

products in the deficient cell lines but did not observe any accumulation of ATM products in the cells. This observation indicates that the enhancement of phosphorylation of Ser-18 in the cell lines cannot be explained by the quantity of ATM products.

Extremely significant evidence indicating that DNA-PK controls p53 transactivation was obtained recently in a study using SCID mouse-derived cells and human glioma cells, both of which are known as *dna-pkcs*-deficient cells (12). To verify these findings, we tried to determine whether the proteins controlled by p53 are induced in the *dna-pkcs* null cells in response to ionizing radiation. Surprisingly enough, we found, as a result, that p21 products and mRNA for p21 and mdm2 are clearly induced in the deficient cells in response to ionizing radiation (Figs. 2 and 3). Therefore, our results indicate that DNA-PKcs is not required for p53 transactivation. One of the possible explanations for the contradictory results of our and previously reported observations is the p53 molecule itself. Therefore, we isolated full-length cDNA of *p53* from the *dna-pkcs*-deficient cells, SX9, PK33N, SCGR11, and SC3T3 (20), and determined their DNA sequence.

As a result, a point mutation was identified in the open reading frame of *p53* gene only in the SCID cell line SCGR11, which was used for previous studies (12), and its parent cell line, SC3T3 (DNA Data Bank of Japan accession no. AB021961). The mutation is a T→G transversion, which results in a ^{191}Leu→Arg residue substitution (Fig. 4). The mutation exists in the DNA-binding motif in which a significant number of mutations in p53 has been detected in cancer cases. Almost all of the mutations in the DNA-binding motif seriously

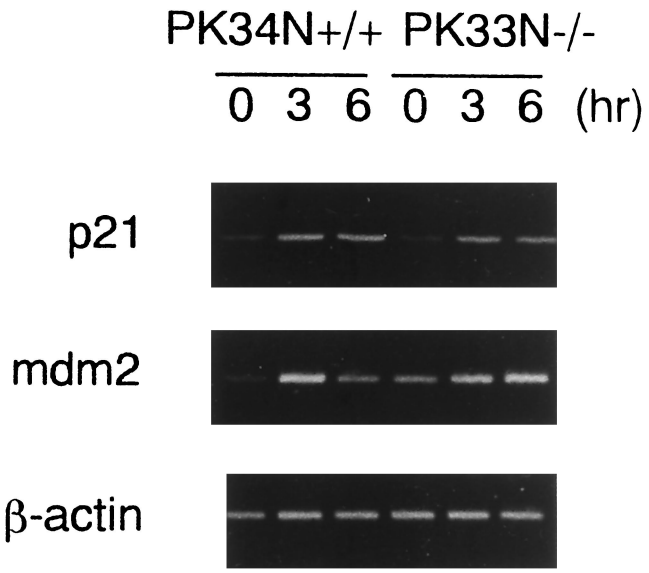

Fig. 3. RT-PCR of transcripts from PK34N and PK33N cells. *Lanes 0, 3,* and *6*, RNAs 0, 3, and 6 h, respectively, after 7 Gy irradiation.

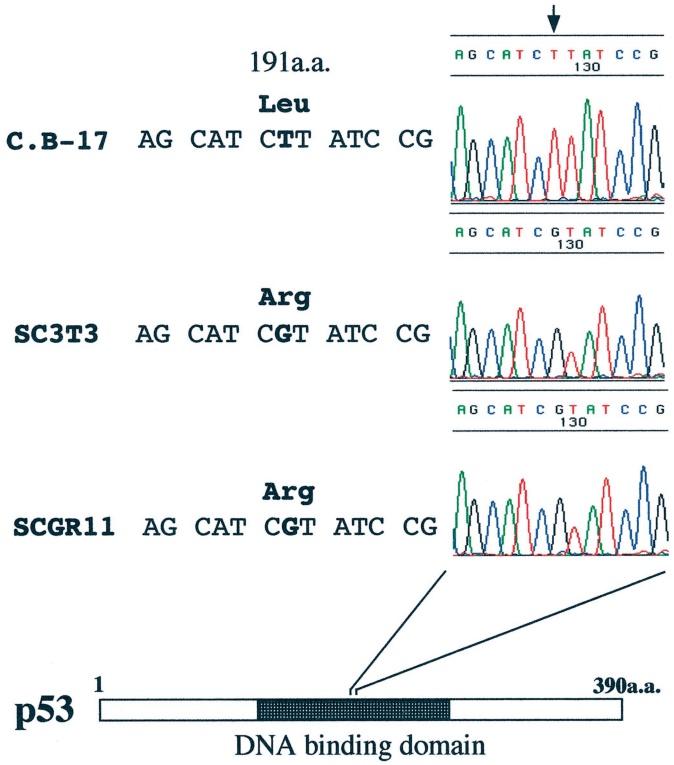

Fig. 4. Mutation in p53 of SC3T3 and SCGR11. *Arrow*, mutated nucleotide. *C.B-17* is a parent mouse strain of the SCID mouse.

affect DNA binding activity and other functions of the p53 product. The mutation can, therefore, be expected to affect DNA binding activity of p53 and p53 transactivation in SCGR11 cells. Our observation of the mutation in p53 from not only the SCGR11 cells but also the SC3T3 cell line, which is a parent cell line of the SCGR11 cells, clearly indicates that the identified mutation was caused before the establishment of parent cells of SCGR11.

In addition, it should be noted that the M059J human glioma cell line used in the reported experiment exhibits a drastic reduction in ATM products as well as DNA-PKcs products (21). These facts strongly suggest that the defect of transactivation of p53 in the *dna-pkcs* mutated cells is due to the p53 or ATM products and not to the DNA-PKcs molecules, and they clearly indicate that DNA-PKcs does not control p53 transactivation following DNA damage.

We were able to confirm the previous observation (11) of delayed Ser-15 phosphorylation in ataxia telangiectasia cells, which are ATM protein deficient. Thus our results indicate that Ser-15 phosphorylation *in vivo* is ATM dependent but DNA-PK independent and that, in fact, the absence of DNA-PK enhances the process. This process was not due to the amount of ATM molecule (Fig. 1). Furthermore,

DNA-PK does not control p53 transactivation. Our observations further emphasize the fact that ATM and DNA-PK operate in different pathways following radiation damage.

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