ABSTRACT

XRCC4 is a protein associated with DNA Ligase IV, which is thought to join two DNA ends at the final step of DNA double-strand break repair through non-homologous end joining. In response to treatment with ionizing radiation or DNA damaging agents, XRCC4 undergoes DNA-PK-dependent phosphorylation. Furthermore, Ser260 and Ser320 (or Ser318 in alternatively spliced form) of XRCC4 were identified as the major phosphorylation sites by purified DNA-PK in vitro through mass spectrometry. However, it has not been clear whether these sites are phosphorylated in vivo in response to DNA damage. In the present study, we generated an antibody that reacts with XRCC4 phosphorylated at Ser320 and examined in cellulo phosphorylation status of XRCC4 Ser320. The phosphorylation of XRCC4 Ser320 was induced by γ-ray irradiation and treatment with Zeocin. The phosphorylation of XRCC4 Ser320 was detected even after 1 Gy irradiation and increased in a manner dependent on radiation dose. The phosphorylation was observed immediately after irradiation and remained mostly unchanged for up to 4 h. The phosphorylation was inhibited by DNA-PK inhibitor NU7441 and was undetectable in DNA-PKcs-deficient cells, indicating that the phosphorylation was mainly mediated by DNA-PK. These results suggested potential usefulness of the phosphorylation status of XRCC4 Ser320 as an indicator of DNA-PK functionality in living cells.

KEYWORDS: DNA double-strand break repair, non-homologous end joining (NHEJ), DNA-dependent protein kinase (DNA-PK), XRCC4, phosphorylation
XRCC4, XLF, and PAXX show remarkable similarities in 3D structure and are considered to comprise a superfamily [11–15]. XLF is thought to support LIG4 activity toward incompatible or mismatched DNA ends [19, 20]. In addition, XRCC4 and XLF may form filaments bridging two DNA ends [21]. PAXX has been shown to interact with Ku and to stabilize the NHEJ complex [13–15].

DNA-PK has been shown to phosphorylate XRCC4 in vitro [9, 22, 23]. It has also been demonstrated that XRCC4 undergoes phosphorylation in living cells in response to treatment with ionizing radiation or a DSB-inducing agent in a manner dependent on DNA-PKcs [24, 25]. Several groups have identified Ser260 and Ser320 (termed ‘Ser318’, reflecting the alternatively spliced form) as the major phosphorylation sites in XRCC4 by purification DNA-PK in vitro through mass spectrometry [26–28]. However, the XRCC4 mutants lacking these phosphorylation sites can fully restore radiosensitivity and V(D)J recombination in XRCC4-deficient XR-1 cells and also exhibit normal activity in DNA joining reaction in a cell-free system, leading to the conclusion that XRCC4 phosphorylation by DNA-PK is unnecessary for these functions [26, 27]. Nevertheless, it is presently unclear whether these sites are phosphorylated in living cells in response to treatment with ionizing radiation or a DSB-inducing agent in a manner dependent on DNA-PKcs [24, 25]. Several groups have identified Ser260 and Ser320 (termed ‘Ser318’, reflecting the alternatively spliced form) as the major phosphorylation sites in XRCC4 by purification DNA-PK in vitro through mass spectrometry [26–28]. However, the XRCC4 mutants lacking these phosphorylation sites can fully restore radiosensitivity and V(D)J recombination in XRCC4-deficient XR-1 cells and also exhibit normal activity in DNA joining reaction in a cell-free system, leading to the conclusion that XRCC4 phosphorylation by DNA-PK is unnecessary for these functions [26, 27]. Nevertheless, it is presently unclear whether these sites are phosphorylated in living cells in response to DNA damage. In the present study, we generated a phosphorylation-specific antibody against XRCC4 Ser320 and examined its phosphorylation status in living cells after irradiation.

**MATERIALS AND METHODS**

**Generation of antibody**

A rabbit polyclonal antibody α-XRCC4-pS320, which can react with Ser320-phosphorylated XRCC4, was generated essentially as described earlier [29]. Peptides XRCC4-S320-C of the sequence corresponding to XRCC4 314–326 with a cysteine appended at the C-terminus (ISAENNMSLETLRNC) and XRCC4-S320-P, with the same sequence but phosphorylated at Ser320, were synthesized by Greiner BIO ONE. Immunization and bleeding were conducted by Protein Purify (Isezaki, Gunma, Japan).

To purify the phosphorylation-specific antibodies, the sera from immunized rabbits were passed several times through a Hi-Trap NHS-activated column (GE Healthcare, Buckinghamshire, UK) that had been coupled with an XRCC4-S320-C. The flow-through fraction was then passed through a Hi-Trap NHS-activated column coupled with an XRCC4-S320-P. The bound antibody was eluted from the column with 0.2 M glycine-HCl (pH2.8) and collected into a prechilled tube with one-eighth volume of 2 M Tris-HCl (pH8.4). As a preservative, one-ninth volume of 1% sodium azide in water was added.

**Cell culture**

The human cervical carcinoma cell line HeLa was cultured in RPMI1640 medium (Nacalai Tesque; Kyoto, Japan) supplemented with 10% fetal bovine serum (HyClone; Logan, UT, USA), 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Human glioma cell lines M059 K and M059J, the latter of which lacks DNA-PKcs [7], were cultured in DMEM/Ham’s F-12 medium supplemented with 10% bovine calf serum (HyClone), 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Human XRCC4 cDNA was obtained by polymerase chain reaction (PCR) from the cDNA pool of human T cell leukemia MOLT-4 and integrated into p3XFLAG-CMV-10 vector (Sigma–Aldrich; St Louis, MO, USA) [30]. Point mutations were introduced using PrimeSTAR Mutagenesis Kit (Takara Bio; Otsu, Shiga, Japan). Sequences of PCR primers for mutagenesis are as follows (underlined nucleotides correspond to mutated amino acids): S320A-F, AAC ATG GCT TTA GAA ACT CTG AGA AAC AGC; S320A-R, TTC TAA AGC CAT GTT TTC AGC TGA GAT GTG. The entire XRCC4 open reading frame was sequenced by Fasmac (Atsugi, Kanagawa, Japan) and found to be correct.

To knockdown the endogenous XRCC4, small interfering RNA (siRNA) targeting 3’-untranslated region (UTR) was introduced [31]. The sequences of duplex were 5’- CUU UGU UUU CUA UUC AUU UdCdT -3’ and 5’- AAA UGA AUA GAA AAC AUA GdTdC -3’, where ‘d’ indicates deoxyribonucleotide. Scrambled duplex of the following sequences served as the control: 5’- UCU CUA UUA GUU CUC UdTdT -3’, 5’- AGA CUA CUA AUA GAG AdAdA -3’. Oligonucleotides were synthesized by Japan Bioservice (Saitama, Japan). The siRNA was transfected into HeLa using Lipofectamine RNAi MAX (Invitrogen; Carlsbad, CA, USA), and 24 h later, XRCC4 cDNA was transfected using Lipofectamine 2000 (Invitrogen).

**Treatment with radiation and chemicals**

Cells were irradiated using a 60Co γ-ray source at the Tokyo Institute of Technology (222 TBq in February 2010). The dose rate was measured using an ionizing chamber–type exposure dosimeter C-110 (Oyo Giken, Tokyo, Japan).

The DSB-inducing agent Zeocin [32] was purchased as an aquatic solution of 100 mg/ml from Invivogen (San Diego, CA, USA) and added to the culture medium at a final concentration of 100 µg/ml for 18 h.

The DNA-PK inhibitor, NU7441 [33], and ATM inhibitor, KU55933 [34], were purchased from Tocris Bioscience (Bristol, UK) and EMD Biochemicals (San Diego, CA, USA), respectively. NU7441 and KU55933 were dissolved in dimethylsulfoxide at a concentration of 5 mM and added to the culture medium at a final concentration of 10 µM 2 h prior to irradiation.

**Western blotting**

After irradiation or treatment with Zeocin, cells were harvested by centrifugation at 290g for 5 min and washed twice in Ca²⁺, Mg²⁺-free Dulbecco’s phosphate buffered saline. The pellet of cells was suspended in IP Lysis Buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.5% Triton X-100 supplemented with a cocktail of protease inhibitors and phosphatase inhibitors) at a concentration of 10⁶ cells/ml and, after 30 min on ice, centrifuged at 20 000g for 7 min. The supernatant was mixed with an equal volume of 2XSDS-PAGE loading buffer (125 mM Tris-HCl (pH 6.8), 4% w/v sodium lauryl sulfate, 20% v/v glycerol, 5% v/v β-mercaptoethanol, 0.02% w/v bromophenol blue, 0.01% w/v crystal violet), heated in boiling water for 5 min and then loaded onto SDS-PAGE gel.

The remaining procedures for Western blotting essentially followed our earlier publication [30]. Anti-XRCC4 rabbit polyclonal antibody, α-XRCC4, was described earlier [30]. Anti-PCNA rabbit...
polyclonal antibody (FL261) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit immunoglobulins swine polyclonal antibody conjugated with horseradish peroxidase (P0399) was purchased from DAKO (Glostrup, Denmark). Protein Ladder One Triple-color Broad Range (Nacalai Tesque) was used as the standard of molecular mass.

RESULTS

XRCC4 Ser320 undergoes phosphorylation in response to radiation

To examine whether XRCC4 Ser320 phosphorylation can be detected using the antibody α-XRCC4-pS320 generated above, the lysate of HeLa cells, either unirradiated or after 20 Gy of γ-irradiation, was subjected to Western blotting analysis. In order to verify the specificity, we also intended to knock down endogenous XRCC4 by siRNA against its 3′-UTR. Western blotting using α-XRCC4 showed that XRCC4 was successfully knocked down in HeLa cells (Fig. 1, top, compare lanes 1 and 2 with lanes 3 to 8).

In the blot probed with α-XRCC4-pS320, there was the appearance of a band of ~55 kDa (which was greatly enhanced by irradiation) in HeLa cells transfected with scrambled RNA duplex as the control (Fig. 1, middle, lanes 1 and 2). This band was diminished in cells transfected with XRCC4 3′-UTR siRNA (lanes 3 and 4). When wild-type XRCC4 was introduced, bands reappeared in the middle of 55 kDa and 71 kDa markers (lanes 5 and 6), which correspond to the position of exogenous, FLAG-tagged XRCC4. It might be noted that exogenously expressed XRCC4 was much more strongly phosphorylated than endogenous XRCC4, probably due to higher expression level. In addition, intense phosphorylation of exogenously expressed XRCC4 was observed, even without irradiation. Nevertheless, quantitative analysis using a charge-coupled device camera-based image analyzer (ImageQuant 350, GE Healthcare) showed that the phosphorylation was enhanced 1.8-fold in irradiated cells as compared with in unirradiated cells. These bands were absent in cells transfected with mutant XRCC4, in which Ser320 had been replaced with alanine (XRCC45320A) (lanes 7 and 8). In the aggregate, these results demonstrated that the band of ~55 kDa, which was enhanced by irradiation, represents the Ser320 phosphorylated form of XRCC4.

Dose response and time-course of XRCC4 Ser320 phosphorylation

To examine the detection limit of XRCC4 Ser320 phosphorylation by using α-XRCC4-pS320, HeLa cells were harvested 30 min after irradiation with 1–20 Gy of γ-rays. XRCC4 Ser320 phosphorylation increased, even after 1 Gy of γ-ray irradiation (Fig. 2A). XRCC4 Ser320 phosphorylation was detectable immediately after irradiation and it remained mostly unchanged for up to 4 h after irradiation (Fig. 2B).

XRCC4 Ser320 phosphorylation is mainly mediated by DNA-PK

To examine the role of DNA-PK in the phosphorylation of XRCC4 Ser320, we employed an inhibitor of DNA-PK (NU7441). Since ataxia-telangiectasia mutated (ATM) exhibits similarity to DNA-PKcs in structure and biochemical properties [35–37], we also tested the effects of an inhibitor of ATM (KU55933). NU7441 greatly diminished XRCC4 Ser320 phosphorylation (Fig. 3A, compare lane 2 and lane 4). On the other hand, KU55933 did not affect XRCC4 Ser320 phosphorylation (Fig. 3A, compare lane 2 and lane 6). These results showed that DNA-PK rather than ATM has the major role in XRCC4 Ser320 phosphorylation.

XRCC4 Ser320 phosphorylation was examined in M059 K and M059J cells, the latter of which lacks DNA-PKcs (Fig. 3B). These
cells were treated with Zeocin, which induces DSBs. XRCC4 Ser320 phosphorylation was increased by Zeocin treatment in M059 K, but not in M059 J.

**DISCUSSION**

Here we generated a phosphorylation-specific antibody against XRCC4 Ser320 and demonstrated that this serine undergoes phosphorylation in response to radiation and treatment with Zeocin.

In the initial study, radiation-induced XRCC4 phosphorylation was detected as the change in electrophoretic mobility and an extremely high radiation dose, such as 40 or 100 Gy, was applied [24]. As the phosphorylated form was enriched in the chromatin-bound fraction, it was detectable after 20 Gy irradiation, but barely detectable after 10 Gy irradiation [30]. In the present study, XRCC4 Ser320 phosphorylation was detected even after 1 Gy irradiation and increased with radiation dose (Fig. 2A).

Imamichi et al. showed that ionizing radiation–induced phosphorylation of XRCC4 in the chromatin-bound fraction was largely, but not completely, inhibited by DNA-PK inhibitor [38]. They also showed that, while ATM inhibitor alone did not reduce the phosphorylation significantly, the combination of DNA-PK inhibitor and ATM inhibitor diminished the phosphorylation to a greater extent than DNA-PK inhibitor alone [38]. These observations led to the conclusion that, although DNA-PK plays the major role in radiation-induced XRCC4 phosphorylation, ATM might also phosphorylate XRCC4 in response to radiation [38]. In the present study, XRCC4 Ser320 phosphorylation was strongly suppressed by DNA-PK inhibitor, but there was still some phosphorylation remaining (Fig. 3A). In addition, phosphorylation seemed to decrease somewhat in the presence of ATM inhibitor in addition to DNA-PK inhibitor (Fig. 3A). Considering this, a minor contribution by ATM cannot be excluded. Nevertheless, the present results indicate that XRCC4 Ser320 phosphorylation is mainly mediated by DNA-PK. The contribution of DNA-PK and ATM could be different for other XRCC4 phosphorylation sites.

It was shown that XRCC4 mutants lacking Ser320 (XRCC4S320A) restored normal radioresistance and V(D)J recombination in a cell-free system [26, 27]. We also created XRCC4S320A and observed that it could restore radioresistance to XRCC4-deficient M10 cells and also displayed normal activity in DNA joining reaction in a cell-free system [38]. We also created XRCC4S320A and observed that it could restore radioresistance to XRCC4-deficient M10 cells and also displayed normal activity in DNA joining reaction in a cell-free system [38]. We also created XRCC4S320A and observed that it could restore radioresistance to XRCC4-deficient M10 cells and also displayed normal activity in DNA joining reaction in a cell-free system [38].
than in rodent cells [7, 39–41], the importance of XRCC4 Ser320 phosphorylation in human cells might be different from that in rodent cells.

Although further studies are demanded in order to clarify the biological significance of XRCC4 Ser320 phosphorylation, it would serve as an indicator of DNA-PK functionality in living cells.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. Note that part of these results are involved in an application to the Japan Patent Office.

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