Cleavage and phosphorylation of XRCC4 protein induced by X-irradiation

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Abstract We report the p35 and p60 forms of XRCC4 protein, appearing in human leukemia MOLT-4 or U937 cells following X-irradiation or hyperthermia. p35 appeared in conjunction with the cleavage of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the fragmentation of internucleosomal DNA, and was suppressed by Ac-DEVD-CHO. p35 was also produced in vitro by treating MOLT-4 cell lysate with recombinant caspases, suggesting that p35 was a caspase-cleaved fragment of XRCC4 in apoptotic cell death. p60 was sensitive to treatment with phosphatase or wortmannin and was undetectable in M059J cells deficient in DNA-PKcs. However, p60 was found in ataxia-telangiectasia cells after irradiation. These results indicated p60 as a phosphorylated form of XRCC4, requiring DNA-PKcs but not ataxia-telangiectasia mutated (ATM).

Key words: XRCC4; DNA-dependent protein kinase; Ataxia-telangiectasia mutated; DNA double-strand break repair; Caspase; Apoptosis

1. Introduction

Ionizing radiation (IR) causes various damage to DNA molecules such as base alterations and single- or double-strand breaks (DSBs), resulting in cell death, mutagenesis and transformation. DSBs are repaired by various mechanisms, including non-homologous end-joining and homologous recombination. IR-sensitive rodent cells are categorized into several complementation groups, termed IR groups [1]. IR groups 4, 5, 6 and 7 are defective in the rejoining of radiation-induced DSBs and also defective in V(D)J recombination [1–3]. The genes defective in IR groups 4 to 7 are termed XRCC4 to 7 and their products are considered to be critical factors in the non-homologous end-joining of DSBs.

XRCC5, 6 and 7 encode the subunits of DNA-dependent protein kinase (DNA-PK), i.e. Ku86, Ku70 and DNA-PKcs (DNA-PK catalytic subunit), respectively [1,2]. DNA-PK is activated by binding to the ends of double-stranded DNA and can phosphorylate in vitro a number of proteins, including p53 and replication protein A [1,2]. DNA-PKcs is structurally related to ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad-3-related), which were recently shown to phosphorylate p53 in vitro and, most likely, in vivo following IR or UV irradiation [4–7]. The activities of DNA-PK, ATM and, to a lesser extent, ATR are sensitive to phosphatidylinositol 3-kinase (PI-3K) inhibitor wortmannin, probably because the catalytic domains of these kinases have sequence similarity to PI-3K [4,5,8–10].

XRCC4 is considered a DNA ligase IV-associated protein, as was demonstrated by immunoprecipitation studies [11–14]. Recombinant XRCC4 protein in vitro stimulates DNA binding and the ligation activity of DNA ligase IV [12,15]. XRCC4 may possibly stabilize DNA ligase IV protein in vivo, since DNA ligase IV protein is decreased in XRCC4-deficient cells [16]. Additionally, XRCC4 protein in vitro exhibits DNA binding activity, preferring nicked or linear DNA [15]. XRCC4 probably forms homodimers or oligomers and might promote ligation by holding DSBs together [15,17,18].

Leber et al. reported that XRCC4 in vitro enhanced the association of Ku proteins to DNA ends and to DNA-PKcs [18]. Several groups reported that recombinant XRCC4 protein was phosphorylated by DNA-PK in vitro [11,15,18]. Modesti et al. also showed that phosphorylation of XRCC4 by DNA-PK did not affect its association with DNA ligase IV but decreased the DNA binding activity [15]. However, Mizuta et al. reported that the predicted phosphorylation site, i.e. Ser53, in XRCC4 protein was not essential in V(D)J recombination [17]. Additionally, Leber et al. and Modesti et al. reported that truncated XRCC4 proteins, which were not phosphorylated by DNA-PK in vitro, could still restore normal radiosensitivity or V(D)J recombination of XRCC4-deficient cells [15,18]. Thus, the role of the XRCC4 phosphorylation in DSB repair and in V(D)J recombination is presently unclear. Moreover, there is presently no reported evidence that DNA-PK phosphorylates XRCC4 in vivo.

In the present study, we found two radiation-inducible forms, i.e. p35 and p60, derived from XRCC4 using Western blotting analysis of human leukemia MOLT-4 cells. We showed that p35 was a caspase-cleaved fragment of XRCC4, while p60 was produced through phosphorylation, which required DNA-PKcs but not ATM.

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2. Materials and methods

2.1. Cells

A human T-cell leukemia cell line, MOLT-4, and a human monocytic leukemia cell line, U937, were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS). Human glioma cell lines M059K and M059J [19] were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Minimal Essential Medium supplemented with 10% bovine calf serum. Simian virus 40-transformed human fibroblast cell lines from normal individual (LM217) and ataxia-telangiectasia patients (TAT5 and AT13LA, obtained from Dr. A.M.R. Taylor and Dr. R. Gatti, respectively) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% FCS [10,20]. MOLT-4 and U937 cells were recovered by centrifugation at $200\times g$ for 5 min, whereas other cells were recovered by treatment with 0.25% trypsin solution with 0.03% w/v EDTA disodium salt followed by centrifugation. Cell pellets were rinsed with phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 per l) and stored at −70°C until use.

2.2. Chemicals

Caspase inhibitors, Ac-YVAD-CHO and Ac-DEVD-CHO (Peptide Institute, Minoh, Osaka, Japan), were dissolved at 10 mM in DMSO (dimethylsulfoxide) and were stored at −40°C. PI-3K inhibitor wortmannin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved at 20 mM in DMSO and stored at −40°C [21].

2.3. Irradiation and heat treatment

Cells were X-ray irradiated using HF-350 (Shimadzu, Kyoto, Japan) at 2.8 Gy/min (operated at 200 kV, 20 mA with filters of 0.5 mm Cu and 1.0 mm Al) at room temperature unless otherwise indicated. Caspase inhibitors were added to the medium immediately following irradiation. Cells were heated by submerging the culture flasks in a thermostatic water bath T-105 (Thomas, Tokyo, Japan) set at 44.0(±0.05)°C [21].

2.4. Antibodies

Rabbit polyclonal antiserum against XRCC4 protein (AHP387) was purchased from Serotec (Oxford, UK). Rabbit polyclonal antibody against DNA-PKcs (YM-CS.1) was newly raised in our laboratory. For antigen preparation, MOLT-4 cells were partly supplied as pellets by Dr. Makoto Takeuchi and thr Fujisaki Institute of Hayashibara Biochemical Laboratories (Okayama, Japan). DNA-PKcs was purified from the nuclear extract of MOLT-4 cells through successive column chromatography followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [22]. The gel pieces containing DNA-PKcs were homogenized with Freund's adjuvant (Difco, Detroit, MI, USA) and injected subcutaneously into a female rabbit. For affinity purification, serum from the immunized rabbit was applied onto polyvinylidenedifluoride (PVDF) membrane (Millipore, Bedford, MA, USA) with DNA-PKcs transblotted. The membrane was rinsed with Tris-buffered saline (20 mM Tris–HCl, pH 7.6, 0.9% NaCl) and the bound antibody was eluted with 0.2 M glycine–HCl (pH 2.8). The eluate was neutralized by 2 M Tris–HCl (pH 8.4), supplemented with 0.1% bovine serum albumin and 0.1% sodium azide as preservatives and stored at 4°C until use.

In Western blotting analysis, a single main band of 470 kDa was detected either in the purified DNA-PK fraction or in the whole cell lysate from MOLT-4 cells (data not shown). This band was also detected in M059K cells, but not in M059J cells, which are deficient in DNA-PKcs expression (data not shown). These results indicate a high specificity of YM-CS.1 to DNA-PKcs.

2.5. SDS–PAGE and Western blotting analysis

Two million cells were suspended in 200 μl of standard SDS–PAGE sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% sodium laurylsulfate, 10% glycerol, 2.5% β-mercaptoethanol, 0.01% bromophenolblue, 0.005% crystal violet) and lysed by heating at 95°C for 5 min. After centrifugation at $15\,000\times g$ for 5 min, 5–40 μl of supernatant was electrophoresed through 5 or 10% polyacrylamide gel, respectively. For analysis of XRCC4, proteins separated through a 10% gel were transblotted onto PVDF membrane at 2 mA/cm^2 for 1 h in transfer buffer (10 mM Tris, 192 mM glycine, 10% v/v methanol), whereas for analysis of DNA-PKcs, proteins separated through a 5% gel were transblotted at 2 mA/cm^2 for 1.5 h in transfer buffer containing 0.1% w/v SDS. The membrane was probed with YM-CS.1 (at 100-fold dilution) or anti-XRCC4 antiserum (at 10 000-fold dilution) as the primary antibody and peroxidase-conjugated antibody against rabbit IgG (Dako, Glostrup, Denmark) as the secondary antibody and was developed using an ECL-plus kit (Amersham-Pharmacia, Buckinghamshire, UK) or Konica Immunostaining HRP-1000 kit (Konica, Hachioji, Tokyo, Japan).

2.6. DNA analysis

The method for DNA preparation and DNA ladder formation analysis has been previously described [23], with the exception that the agarose gel concentration was 1.5% instead of 1.0%.

2.7. In vitro caspase treatment and analysis of XRCC4 and DNA-PKcs

Recombinant human caspase-3 and caspase-7 were purchased from Medical and Biological Laboratories Co. (Nagoya, Japan). MOLT-4 cells were lysed in caspase buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2) [24] with three freeze–thaw cycles, i.e. repeated freezing in liquid nitrogen bath followed by thawing in water bath at 30°C, and the lysate was clarified by centrifugation at $18\,000\times g$ for 30 min at 4°C. The reaction mixture (16 μl of final volume) contained 12 μl MOLT-4 cell lysate (protein concentration of 5 mg/ml) and either recombinant caspase-3 (1.48 μg, 13 U) or caspase-7 (1.26 μg, 83 U). After incubation at 37°C for 60 min, XRCC4 and DNA-PKcs were analyzed by Western blotting, as described above.

2.8. In vitro phosphorylation

Crude cell lysate was prepared from MOLT-4 cells and was passed through a DEAE Bio-Gel A column (BioRad, Hercules, CA, USA) as described previously [25]. The flow through was dialyzed overnight at 4°C against 1.25× kinase buffer (25 mM HEPES–NaOH (pH 7.2), 187.5 mM KCl, 6.25 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol) and centrifuged at $18\,000\times g$ for 5 min. The phosphorylation reaction mixture (10 μl total volume) contained 8 μl of the cell lysate (protein concentration 5 mg/ml), 50 μM ATP and 10 ng/μl of pBluescript SKII(+) (Stratagene, La Jolla, CA, USA) with or without 100 Gy X-irradiation and was incubated at 37°C for 10 min. The reaction was stopped by the addition of 10 μl of 2×SDS–PAGE sample buffer and heating at 95°C for 5 min and XRCC4 protein was analyzed by Western blotting, as described above. Where indicated, cell lysate was preincubated with 10 μM wortmannin or 1% v/v DMSO, as a control, for 1.5 h on ice. For phosphatase treatment, 10 U of calf intestinal phosphatase (Sigma-Aldrich) was added to the reaction mixture and incubated at 37°C for 20 min.

3. Results and discussion

3.1. p35: caspase-cleaved fragment of XRCC4 protein

The human T-cell leukemia cell line, MOLT-4, is highly sensitive to X-irradiation and exhibits caspase-dependent rapid cell death with DNA fragmentation, indicating apoptosis [26–28]. We recently reported that ceramide generation, which in turn caused the phosphorylation of SAPK/JNK, was important for the radiation-induced apoptotic cell death of MOLT-4 [29]. In SDS–PAGE and Western blotting analysis, XRCC4 protein was mainly found as a 55 kDa band (p55) in unirradiated MOLT-4 cells (Fig. 1, upper panels), as reported previously [11–18]. However, we found an additional band of 35 kDa (p35), 4 h after 40 Gy (Fig. 4A) and 8 h after 10 Gy X-irradiation (Fig. 1, upper left). The amount of p35 was X-ray dose-dependent (Fig. 1, upper right). DNA-PKcs was found mostly as a 470 kDa molecule in unirradiated MOLT-4 cells, but smaller forms of 240 and 160 kDa, which have been reported as caspase-cleaved products [30–32], were found 4 h after 40 Gy (data not shown) and 8 h after 10 Gy (Fig. 1, lower left). Thus, the timing and dose-dependency of p35 appearance after irradiation was similar to the cleavage of DNA-PKcs (present results), poly(ADP-ribose) polymerase

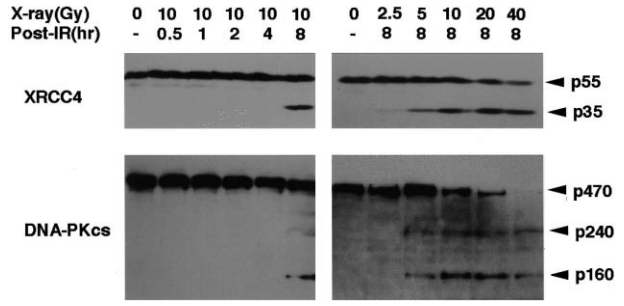

Fig. 1. XRCC4 and DNA-PKcs in X-ray irradiated MOLT-4 cells. MOLT-4 cells were irradiated with 2.5–40 Gy of X-ray and were recovered at various times after irradiation, as indicated. XRCC4 (upper panels) and DNA-PKcs (lower panels) were analyzed by Western blotting.

(PARP) [28,29] and p42/SETβ by caspase(s) ([26] and unpublished data).

Additionally, p35 was also detectable concomitantly with the smaller forms of DNA-PKcs in U937 cells 4 h after 40 Gy X-irradiation or 0.5 h after heating at 44°C for 40 min (Fig. 2, upper and middle panels). The internucleosomal DNA fragmentation appeared 4 h after 40 Gy X-irradiation and 1 or 2 h after a 44°C, 40 min treatment (Fig. 2, lower panels). Thus, like the cleavage of DNA-PKcs by caspases, the timing of p35 appearance was similar to or somewhat earlier than DNA fragmentation, which is a hallmark of apoptosis.

We examined possible effects of caspase inhibitors on p35 appearance. Ac-DEVD-CHO, an inhibitor of caspase-3/7-like proteases, but not Ac-YVAD-CHO, an inhibitor of caspase-1/4-like proteases, suppressed the appearance of p35 (Fig. 3A, upper panel) and cleaved forms of DNA-PKcs (lower panel). Additionally, p35 was produced in conjunction with 240 and 160 kDa forms of DNA-PKcs by treating MOLT-4 cell lysate with recombinant caspase-3 or caspase-7 (Fig. 3B). These results suggest that p35 was derived from p55 by cleavage by apoptosis-related proteases, like caspase-3/7.

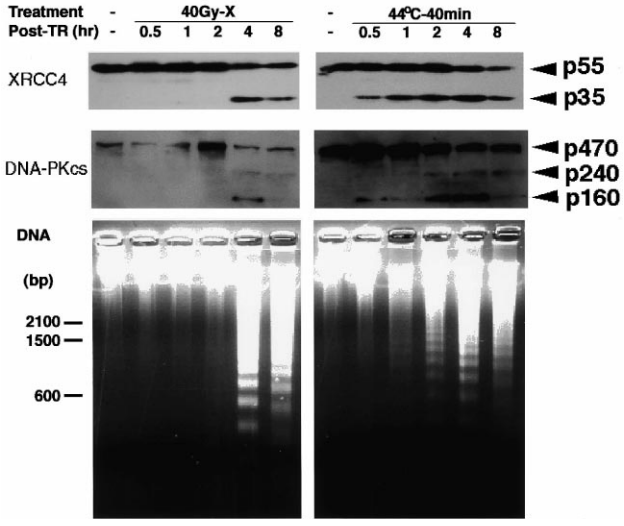

Fig. 2. XRCC4, DNA-PKcs and DNA in X-irradiated or heated U937 cells. U937 cells were either irradiated with 40 Gy of X-ray (left panels) or heated at 44°C for 40 min (right panels) and were recovered at various times after treatment. XRCC4 (upper panels) or DNA-PKcs (middle panels) was analyzed by Western blotting. In lower panels, DNA was extracted from cells and analyzed by agarose gel electrophoresis.

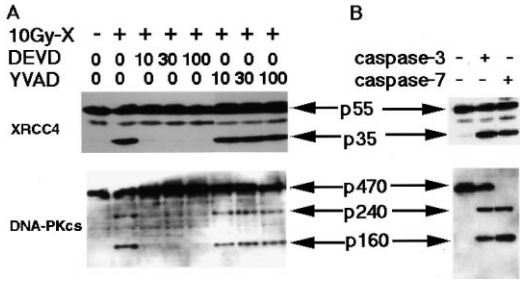

Fig. 3. A: Effect of caspase inhibitors on XRCC4 or DNA-PKcs cleavage. MOLT-4 cells were irradiated with 10 Gy of X-ray and treated with Ac-DEVD-CHO or Ac-YVAD-CHO at 10, 30 or 100 μM as a final concentration in the medium. Cells were recovered 10 h after irradiation and XRCC4 (upper panel) and DNA-PKcs (lower panel) were analyzed by Western blotting. B: In vitro caspase treatment. The lysate from unirradiated MOLT-4 cells was incubated with either recombinant human caspase-3 or caspase-7, as indicated. After incubation at 37°C for 60 min, XRCC4 or DNA-PKcs was analyzed by Western blotting.

Presently more than 10 caspase genes are found in the human genome and categorized into several subgroups with regard to structure, function and substrate specificity [33,34]. Caspase-1/4-like proteases are thought to play important roles, such as processing interleukin-1β, in the inflammatory response. Caspase-3/7-like proteases are converted from a latent form into an active form through cleavage by caspase-8/9-like proteases, disassembling the cytoskeleton, nuclear matrix or chromosomal DNA, initiating the detachment of the cell from its surrounding tissue structures and marking the dying cells for engulfment by other cells such as macrophages. These caspases have been reported to cleave a number of DNA repair proteins, including DNA-PKcs ([30–32] and the present study), PARP [35,36], the 140 kDa subunit of replication factor C [37] and Rad51 [38,39]. The present study suggested that XRCC4 was also cleaved by a caspase-3/7-like enzyme, producing a p35 fragment.

3.2. p60: phosphorylated form of XRCC4 requiring DNA-PK but not ATM

In addition to p35, a band of 60 kDa (p60) was also detected in 40 Gy X-irradiated MOLT-4 cells using anti-XRCC4 antibody (Fig. 4A). Although p60 was hardly detectable after 10 or 20 Gy irradiation, it increased with radiation dose (data not shown). The appearance of p60 was much earlier than that of p35: while p35 was detectable from 4 h after 40 Gy X-irradiation, p60 was detected even 0.5 h after irradiation (Fig. 4A,B). p60 was detectable until 4 h after irradiation but was undetectable 8 h after irradiation (Fig. 4A). p60 was also found after the incubation of crude lysate from unirradiated MOLT-4 cells with ATP and X-irradiated plasmid DNA, but not unirradiated plasmid DNA (Fig. 4C, lanes 1 and 2). p60 disappeared after phosphatase treatment, indicating that p60 is a phosphorylated form of XRCC4 (Fig. 4C, lanes 2 and 4).

Twenty μM wortmannin, an inhibitor of DNA-PK, ATM and ATR as well as PI-3K [4,5,8–10], suppressed the appearance of p60 in 40 Gy irradiated MOLT-4 cells (Fig. 4B). Additionally, 10 μM wortmannin suppressed the appearance of p60 in vitro, i.e. after incubation of MOLT-4 cell lysate with ATP and irradiated plasmid DNA (Fig. 4D). These results suggest the possibility that p60 in X-irradiated MOLT-4 cells might be derived from p55 through phosphorylation by

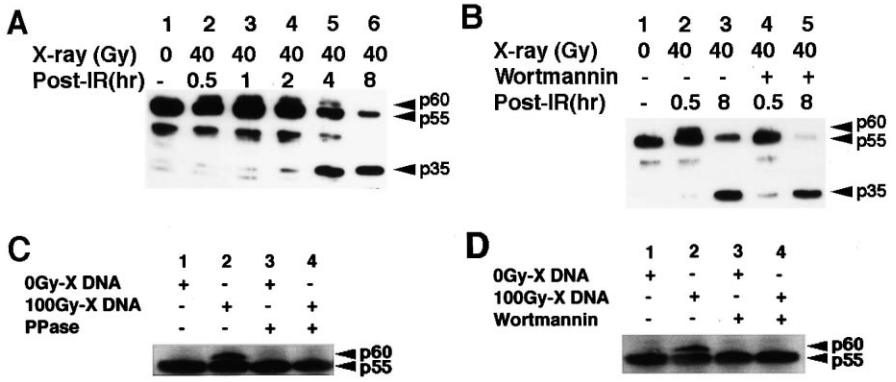

Fig. 4. A: XRCC4 protein in 40 Gy X-irradiated MOLT-4 cells. At indicated times after irradiation, cells were recovered for Western blotting analysis. B: Suppression by wortmannin of p60, but not p35, in X-irradiated MOLT-4 cells. Wortmannin (lanes 1 to 3, 20 μM final concentration) or DMSO (lanes 4 and 5), as control, was added to culture medium 1 h before 40 Gy X-irradiation. Cells were recovered 0.5 or 8 h after irradiation for Western blotting analysis. C: DNA damage-dependency and phosphatase sensitivity of p60 in vitro. The crude lysate of MOLT-4 cells, passed through a DEAE column in order to minimize contaminated cellular DNA, was incubated with either unirradiated (lane 1 and 3) or 100 Gy X-irradiated (lane 2 and 4) pBluescript II SK(+) at 37°C for 10 min. In lanes 3 and 4, the reaction mixture was further incubated at 37°C for 20 min with 10 U of calf intestinal phosphatase. D: Wortmannin sensitivity of p60 in vitro. MOLT-4 cell lysate was pretreated either with DMSO, as control (lanes 1 and 2), or with 10 μM wortmannin (lane 3 and 4) on ice for 90 min before incubation with either unirradiated (lanes 1 and 3) or 100 Gy X-irradiated (lanes 2 and 4) pBluescript II SK(+) at 37°C for 10 min.

DNA damage-responsive, wortmannin-sensitive kinases, like DNA-PK, ATM or ATR [4,5,8–10]. On the other hand, 20 μM wortmannin did not suppress the appearance of p35 in 40 Gy X-irradiated MOLT-4 cells (Fig. 4B), suggesting that p60 production was not likely a prerequisite for cleavage producing p35.

p60 was not found in DNA-PKcs-deficient M059J cells but was found in control M059K cells 0.5 h after 100 Gy X-irradiation (Fig. 5A). However, p60 was detectable 0.5 h after 100 Gy X-irradiation in two fibroblast cell lines from an ataxia-telangiectasia patient, i.e. TAT5 and AT13LA, as well as in normal human fibroblasts, LM217 (Fig. 5B). These results suggest that DNA-PKcs, but not ATM, is necessary for the radiation-induced phosphorylation of XRCC4.

In in vitro studies, DNA-PK has been reported to phosphorylate recombinant XRCC4 and to change the association of XRCC4 with DNA [11,15,18]. The present study clearly demonstrated for the first time that radiation could induce phosphorylation of XRCC4 in vivo, requiring DNA-PKcs but not ATM, although these results could not entirely exclude a possibility of indirect phosphorylation through other kinase(s).

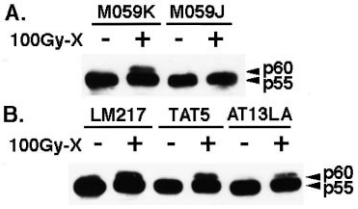

Fig. 5. Radiation-induced XRCC4 phosphorylation in DNA-PKcs- or ATM-defective cell lines. A: DNA-PKcs-positive and -negative human glioma cell lines (M059K and M059J, respectively). B: Simian virus 40-transformed fibroblast cell lines from normal individual (LM217) and ataxia-telangiectasia patient (TAT5 and AT13LA). Two million cells were pelleted by trypsinization and centrifugation and were either unirradiated or irradiated with 100 Gy (2 Gy/min) of X-ray on ice. The cell pellets were resuspended in culture medium, incubated at 37°C for 30 min and, then, recovered for Western blotting analysis.

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