Human Polynucleotide Kinase Participates in Repair of DNA Double-Strand Breaks by Nonhomologous End Joining but not Homologous Recombination

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Abstract

Human polynucleotide kinase (hPNK) is a bifunctional enzyme possessing a 5′-DNA kinase activity and a 3′-phosphatase activity. Studies based on cell extracts and purified proteins have indicated that hPNK can act on single-strand breaks and double-strand breaks (DSB) to restore the termini to the chemical form required for further action by DNA repair polymerases and ligases (i.e., 5′-phosphate and 3′-hydroxyl termini). These studies have revealed that hPNK can bind to XRCC4, and as a result, hPNK has been implicated as a participant in the nonhomologous end joining (NHEJ) pathway for DSB repair. We sought to confirm the role of hPNK in NHEJ in the cellular setting using a genetic approach. hPNK was stably down-regulated by RNA interference expression in M059K glioblastoma cells, which are NHEJ positive, and M059J cells, which are NHEJ deficient due to a lack of DNA-PK catalytic subunit (DNA-PKcs). Whereas depletion of hPNK significantly sensitized M059K cells to ionizing radiation, no additional sensitization was conferred to M059J cells, clearly implying that hPNK operates in the same DNA repair pathway as DNA-PKcs. On the other hand, depletion of hPNK did not increase the level of sister chromatid exchanges, indicating that hPNK is not involved in the homologous recombination DSB repair pathway. We also provide evidence that the action of hPNK in the repair of camptothecin-induced topoisomerase 1 "dead-end" complexes is independent of DNA-PKcs and that hPNK is not involved in the nucleotide excision repair pathway. [Cancer Res 2007;67(14):6619–25]

Introduction

Strand breaks constitute a significant proportion of the lesions generated by a broad range of genotoxic agents, either directly or during the course of DNA repair. Most of the termini carry chemical modifications that require processing before further repair can proceed. All 3′-strand termini have to be restored to hydroxyl groups and 5′-termini to phosphate groups, to allow DNA polymerases and ligases to catalyze repair synthesis and strand rejoining. Human polynucleotide kinase (hPNK) is a bifunctional enzyme mediating the correction of these strand-break termini by its 5′-kinase and 3′-phosphatase activities (1, 2).

In vitro studies have provided evidence that hPNK is involved in single-strand break (SSB) and base excision repair (3–6) and in double-strand break (DSB) repair through nonhomologous end joining (NHEJ; refs. 7, 8) and a newly described XRCC1/DNA ligase III–dependent DSB repair pathway (9, 10). In their study, Chappell et al. (7) used an assay for NHEJ, which monitors end-to-end ligation of restriction enzyme-cleaved DNA by human cell extracts. Plasmid DNA substrates lacking a 5′-phosphate failed to undergo ligation when treated with extract immunodepleted for hPNK. This study also indicated that the 5′-phosphorylation was dependent on XRCC4, an observation substantiated by Koch et al. (8), who provided evidence for physical association between hPNK and XRCC4 phosphorylated at Thr^{233}. Audebert et al. (10) similarly made use of synthetic substrates, in this case, short double-stranded oligonucleotides, to monitor ligation catalyzed by cell-free extracts.

The importance of eukaryotic PNK in the cellular response to genotoxic agents was initially established in *Schizosaccharomyces pombe* (11). Deletion of PNK in fission yeast resulted in cellular hypersensitivity to ionizing radiation and camptothecin, a topoisomerase I inhibitor. Both of these agents are capable of generating DNA SSB and DSB with termini requiring either or both PNK activities (12–15). RNA interference (RNAi) silencing of hPNK in A549 human lung carcinoma cells yielded a similar response to that seen in yeast (16). The cells displayed an increased sensitivity to ionizing radiation and camptothecin, as well as a significantly elevated spontaneous mutation frequency, suggesting that hPNK plays important roles countering exogenous and endogenous DNA damage and helping to maintain genomic stability.

Because DSBs are considered to contribute significantly toward cell lethality induced by ionizing radiation and genomic instability, we sought direct cell-based evidence for involvement of hPNK in DSB repair, particularly the NHEJ pathway, by comparing the influence of hPNK down-regulation on the radiation response of NHEJ-proficient and NHEJ-deficient cells. NHEJ is a multienzymatic pathway initially involving damage recognition by binding of the Ku70/80 heterodimer followed by attachment of the DNA-PK catalytic subunit (DNA-PKcs). This complex then recruits XRCC4, XLF, and DNA ligase IV to complete strand rejoining (17, 18). The requirement of other processing enzymes, such as hPNK and DNA polymerases λ and μ, has been implicated from experiments with purified proteins or cell extracts (7, 8, 19), but not fully confirmed with intact cells. To investigate the involvement of hPNK in the NHEJ repair pathway, we stably down-regulated hPNK in the DNA-PKcs–deficient human glioma cell line M059J and the NHEJ-proficient cell line M059K isolated from the same glioma (20, 21).

Note: F. Karimi-Busheri and A. Rasouli-Nia contributed equally to this work.

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Materials and Methods

Cell lines and culture conditions. The isolation and characterization of the M059K and M059J glioma cell lines and the hPNK-depleted A549 human lung adenocarcinoma cell line have been described previously (16, 20, 21). Parental A549 cells were obtained from the American Type Culture Collection. The cells were cultured in DMEM/nutrient mixture F-12 (DMEM/F-12; 1:1 ratio) supplemented with 10% FCS, 50 units/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate and maintained at 37°C under 5% CO_2 in a humidified incubator. All culture supplies were purchased from Invitrogen.

Transfection procedure. The construction of the pSUPER plasmid (22) containing a duplex directed against a 20-base sequence in the hPNK mRNA has been described previously (16). M059J and M059K cells were plated in 60-mm dishes. When they reached 50% to 70% confluency, the cells were cotransfected with 2.4 μg pSUPER [with or without inserted small interfering RNA (siRNA) sequence] and 0.5 μg pGFPneo plasmid DNA and with 10 μL Lipofectamine 2000 (Invitrogen) diluted in 600 μL serum- and antibiotic-free DMEM. After incubation at 37°C for 24 h, cells were trypsinized and further cultured at a 1:10 dilution in complete medium. The following day, medium was replaced with selective medium containing 300 μg/mL G418 (Invitrogen) and the cells were grown in this medium for 2 weeks. G418-resistant colonies were established in six-well plates, expanded, and cloned independently.

Immunofluorescence detection of hPNK. Approximately 10^5 cells were seeded on a glass coverslip and incubated overnight at 37°C in a CO_2 incubator. On the next day, cells were fixed in 2% paraformaldehyde for 20 min at room temperature, washed with PBS, and permeabilized with 100% cold methanol at −20°C for 30 min. The cells were then blocked with 5% milk powder in PBS for 1 h at room temperature. Monoclonal antibody (mAb) to hPNK (Cytostore), diluted 250-fold in 5% milk powder, was placed on the coverslips and incubated for 1 h. After washing the slides several times with PBS and PBS containing 0.1% Tween 20, the cells were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes) diluted 1:500 in PBS for 1 h in the dark. Cells were again washed

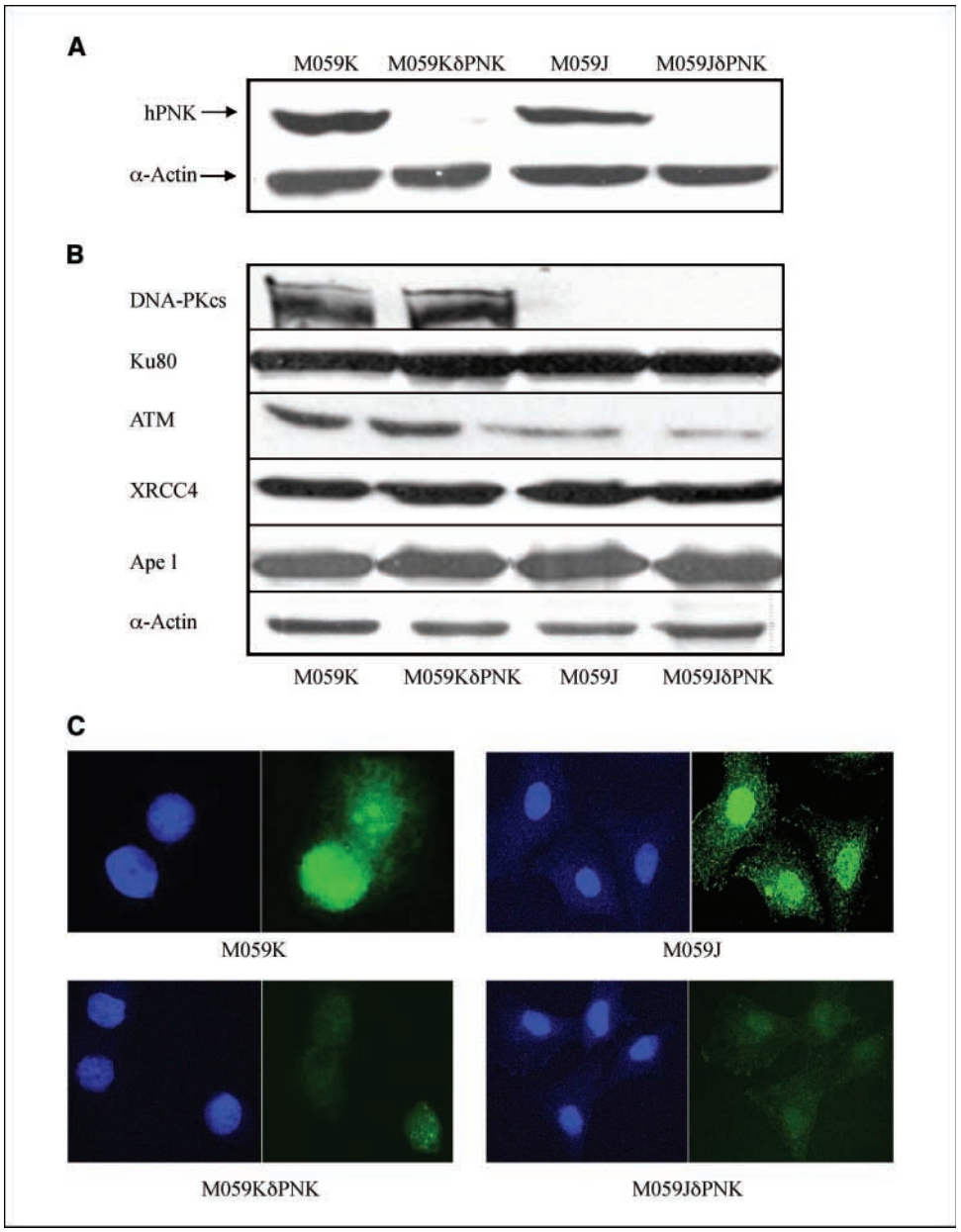

Figure 1. RNAi-mediated stable suppression of *hPNK* gene expression in M059J and M059K human glioblastoma cell lines. *A,* Western blot of hPNK and actin in M059J and M059K cells, and their respective hPNK knockdown cell lines (M059JδPNK and M059KδPNK). *B,* Western blot analysis of other DNA repair proteins to determine any possible alterations in expression resulting from hPNK down-regulation. *C,* immunofluorescence of hPNK (*right*) and nuclear staining by DAPI (*left*) in M059J and M059K controls and M059JδPNK and M059KδPNK cells.

twice with PBS and PBS-Tween 20 and finally rinsed with water. The coverslips were mounted on a microscope slide using 95% glycerol in PBS containing 3 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) and viewed with a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss International) mounted on a Zeiss Axiovert 100M microscope.

Enzyme activity. Both kinase and phosphatase activity were measured as described earlier (2, 16). Briefly, for kinase activity, 10 μL crude cell lysate was tested for the transfer of phosphate from [γ-^{32}P]ATP to the 5′-OH of a 21-mer oligonucleotide substrate. Phosphatase activity was determined by the conversion of a 21-mer oligonucleotide (p21p), bearing a ^{32}P label at the 5′-terminus as well as a 3′-phosphate, to the 3′-dephosphorylated 21-mer (p21). For both activities, reaction was carried out in 70 mmol/L Tris-HCl (pH 7.6), 10 mmol/L $MgCl_2$, and 5 mmol/L DTT at 37°C for 30 min.

Preparation of cell-free extract and Western immunoblot analysis. Cells were trypsinized and a cell pellet was obtained by centrifugation at 1,000 rpm for 10 min at 4°C. The cells were first washed with PBS followed by resuspension in 10 to 15 mL of low salt buffer [10 mmol/L HEPES, 25 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 0.1 mmol/L DTT (pH 7.2)] plus protease inhibitor cocktail (Sigma; ref. 21). The cell pellet was resuspended in 2.5 times the packed cell volume, incubated on ice for 10 min, and either directly used or frozen on dry ice for long-term storage at −80°C. Aliquots of the cell extracts were quickly thawed at 37°C and adjusted to 0.5 mol/L NaCl and 10 mmol/L $MgCl_2$ and incubated on ice for 5 min and then spun at 10,000 × *g* for 3 min at 4°C. Supernatant was removed and stored in a new tube, whereas the pellet was reextracted with extraction buffer 1:10 diluted by 50 mmol/L HEPES (pH 7.5; ref. 23).

For Western blotting, 50 μg protein was applied and run on a 10% SDS-polyacrylamide gel. Presence or absence of the hPNK was determined by a mouse monocolonal antibody against hPNK (24), probed with the secondary antibody conjugated to horseradish peroxidase, and detected with enhanced chemiluminescence reagent (GE Healthcare).

Assessment of clonogenic survival. Cells from exponentially growing subcultures were seeded in 60-mm culture dishes in DMEM/F-12 supplemented with 10% FCS at densities of 200 to 2,000 cells per dish as described earlier (16). After overnight incubation at 37°C to allow for cell attachment, the cells were subjected to γ-radiation (^{60}Co Gammacell, Atomic Energy of Canada Ltd.) or UVC radiation (G15T8 15W germicidal bulb, General Electric) or exposed to graded doses of camptothecin (Sigma) for 1 h at 37°C. (For UV exposure, the medium was removed and the cells were washed with PBS and then drained before irradiation). Drug-containing medium was then replaced with fresh medium containing 15% FCS and the plates were incubated for a period of 2 to 3 weeks at 37°C. The resulting colonies were fixed and stained with 10% methylene blue and counted. Cell survival was plotted as a function of dose and fitted using Prism version 3.03 software (GraphPad Software, Inc.).

Monitoring of H2AX phosphorylation. The induction and repair of DSBs following ionizing radiation was assessed by monitoring the formation and loss of γH2AX molecules. We followed the modified protocol of Furuta et al. (25) as described earlier (16). Briefly, for each determination, 1×10^5 cells were seeded onto a glass coverslip retained in 35-mm dishes and left overnight at 37°C. On the next day, cells were exposed to 5 Gy γ-radiation and incubated at 37°C for various times up to 24 h. Cells were probed with a mAb to phosphorylated histone H2AX (Ser^{139}; Upstate) and subsequently incubated with Alexa Fluor 488 goat anti-mouse secondary antibody. The presence of phosphorylated H2AX foci was determined by Zeiss LSM510 laser scanning confocal microscope and the average integrated intensity/nucleus was analyzed using Metamorph Offline 6.1 software (Universal Imaging) as described (16).

Sister chromatid exchange. Log-phase cells were incubated with 10 μg/mL 5-bromodeoxyuridine (BrdUrd) for 48 to 72 h at 37°C in DMEM/F-12 supplemented with 20% FCS. For the final 4 h of the incubation, cells were blocked at mitosis by 0.1 μg/mL colcemid. The cells were harvested and fixed in methanol/glacial acetic acid (3:1) and applied to prewashed slides (26). Differential staining was achieved by immunofluorescence staining. In this method, sister chromatids are differentially stained by immunostaining of mitotic chromosomes followed by treatment of cells with antibody against BrdUrd (Becton Dickson) and Alexa Fluor 488–conjugated secondary antibody. Coverslips were then mounted on slides by using mounting solution, 95% glycerol in PBS containing 5 μg/mL DAPI. Slides were stored at 4°C in the dark before scoring. At least 30 metaphases were analyzed by confocal microscopy.

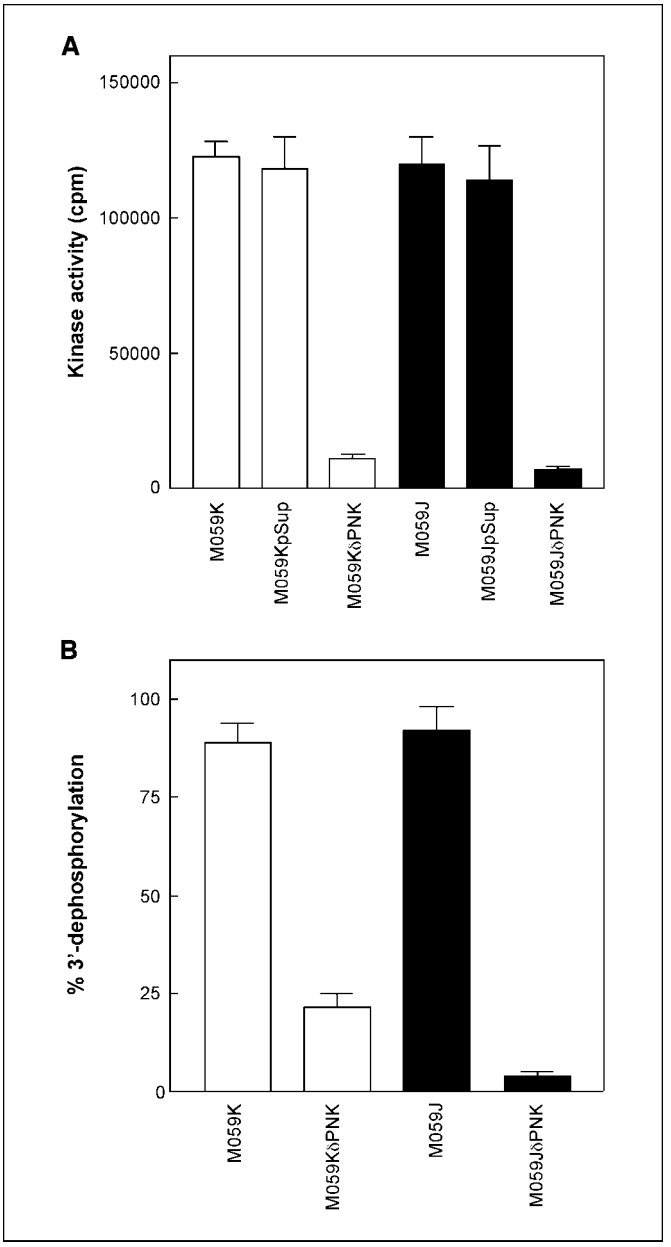

Figure 2. DNA kinase and phosphatase activity. DNA kinase activity in crude cell extracts (*A*) and DNA 3′-phosphatase activity in crude cell extracts (*B*) determined by monitoring the removal of the 3′-phosphate from a 5′ ^{32}P-labeled 21-mer oligonucleotide (p21p) as described in Materials and Methods.

Results and Discussion

siRNA-mediated knockdown of hPNK in M059K and M059J cells. Stable knockdown of hPNK in the M059 cells was achieved by expression of a siRNA sequence directed against nucleotides 1391 to 1410 (5′-AGAGATGACGGACTCCTCT-3′) of the hPNK cDNA using the pSUPER vector system (22). We did a series of experiments to validate the efficiency of our knockdown cell lines,

M059JδPNK and M059KδPNK. Western blot analysis (Fig. 1*A*), immunofluorescence (Fig. 1*C*), and functional kinase and phosphatase assays (Fig. 2*A* and *B*) showed a marked reduction in the expression of hPNK in both cell lines. Biweekly Western blotting of lysates from cells grown over a 3-month period further indicated that the knockdown of hPNK was highly stable (data not shown). Also as a control, we checked the expression of several other DNA repair proteins (Fig. 1*B*), including human apurinic/apyrimidinic endonuclease (Ape1), and did not find any changes in the expression level of these proteins as a result of hPNK down-regulation, implying that enzymes that may act on the same lesions as hPNK (e.g., the phosphatase activity of Ape1) are not up-regulated to compensate for the depletion of hPNK. The reduction in hPNK expression also had no influence on the DNA repair transducer protein ATM in either cell line. As noted previously, the level of ATM expression is lower in M059J cells than M059K cells (27).

Influence of hPNK depletion on the radiosensitivity of M059J and M059K cells and DSB repair. To assess the cellular sensitivity of the M059KδPNK and M059JδPNK cells, we measured their clonogenic survival in response to ionizing radiation, camptothecin and UV light. The radiation survival curves (Fig. 3*A*) revealed that the M059KδPNK cells were significantly more sensitive than either the parental M059K cells or the control cells transfected with the unmodified pSUPER vector (M059KpSup). The ~2-fold sensitization was very similar to our previous data obtained with hPNK-depleted A549 cells (16). In contrast, down-regulation of hPNK in M059J cells did not further sensitize them to ionizing radiation. The depletion of hPNK in M059K cells did not sensitize the cells to the same extent as the lack of DNA-PKcs (i.e., the M059J cells). Several explanations can be put forward for this observation. One straightforward explanation is that the knockdown of hPNK was incomplete and the residual enzyme was able to process a proportion of the strand-break termini. An alternative, equally plausible, explanation is that not all DSB acted on by DNA-PKcs require hPNK processing. Irradiation under aerated conditions generates termini with 3′-phosphate and 3′-phosphoglycolate end groups in approximately similar proportions and only ~15% of 5′-termini lack a 5′-phosphate group (12–14, 28). Although the 3′-phosphate termini are extremely poor substrates for Ape1 (6, 29), Ape1 can remove a significant proportion of phosphoglycolate groups, especially from 3′-recessed termini (30–32), although phosphoglycolates at 3′-overhanging termini require another processing pathway involving hPNK (33).

To determine the relationship between clonogenic survival and DNA repair, we examined DSB repair by measuring the formation and disappearance of phosphorylated histone H2AX (γH2AX) in these cell lines following exposure to 5 Gy γ-radiation. The results, shown in Fig. 3*B*, indicate that the reduction of hPNK expression in M059K cells led to elevated and more prolonged H2AX phosphorylation. Even by 24 h, the level of γH2AX had not quite returned to background in the M059KδPNK cells. Nonetheless, the level was not as high or prolonged as that seen with the M059J cells, presumably due to the lack of DNA-PKcs in the latter cells (34). Furthermore, hPNK down-regulation did not significantly alter the γH2AX profile in the M059J cells. Thus, the DSB repair profile seen with the four cell lines correlates well with the survival data.

Influence of hPNK depletion on homologous recombination. The data above confirm an important role for hPNK in NHEJ. To ascertain the effect of hPNK depletion on an alternative major

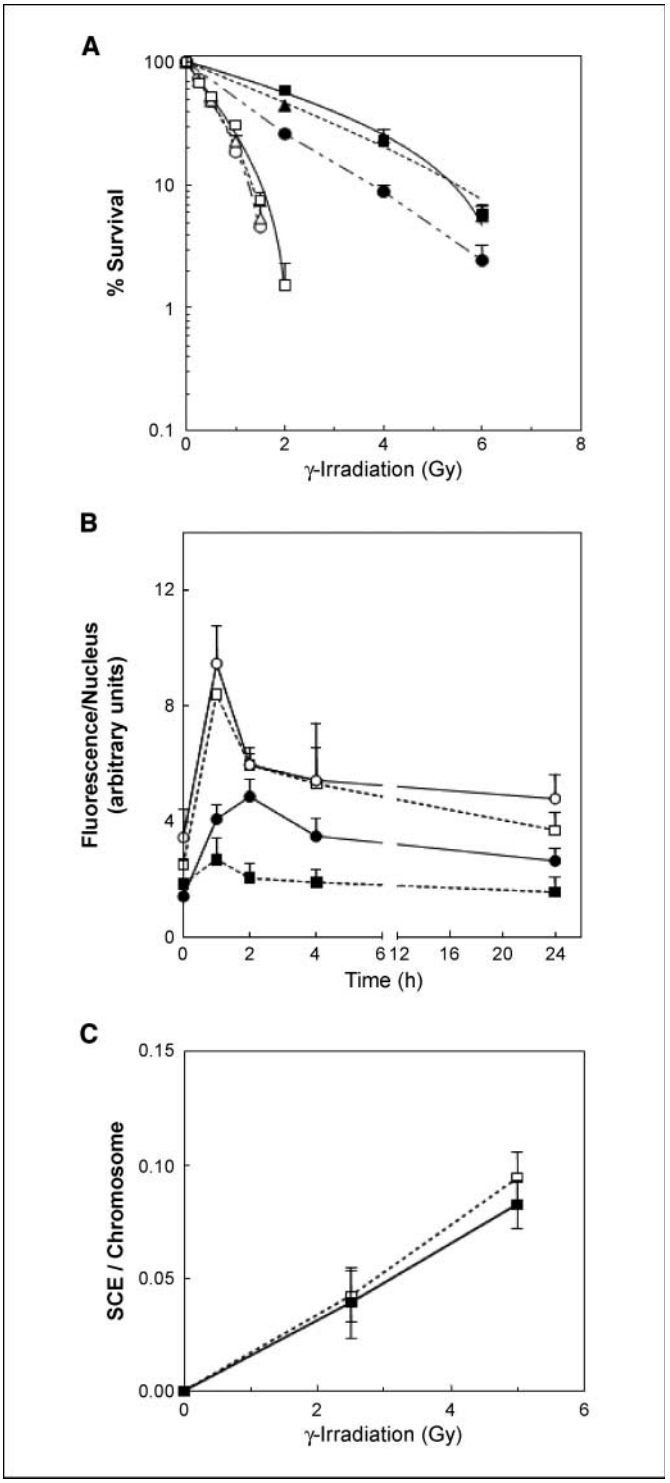

Figure 3. Influence of hPNK depletion on cellular responses to ionizing radiation. *A,* clonogenic survival of hPNK-depleted M059J and M059K cells following increasing doses of γ-radiation: M059J (□), M059JpSup (Δ), M059JδPNK (○), M059K (■), M059KpSup (▲), and M059KδPNK (●) cells. *Points,* mean surviving values of three independent experiments; *bars,* SE. *B,* DNA DSB repair determined by quantification of the average integrated fluorescence intensity/nucleus due to phosphorylation of histone H2AX as a function of time after 5 Gy irradiation: M059J (□), M059JδPNK (○), M059K (■), and M059KδPNK (●) cells. *Points,* mean surviving values of three independent experiments; *bars,* SE. *C,* induction of SCEs in A549 (■) and hPNK-depleted A549 (C-ter3; □) cell lines as a function of γ-irradiation. Cells were irradiated with 2.5 and 5 Gy and analyzed for the induction of SCEs. *Points,* the number of SCE/chromosome in at least 30 metaphases; *bars,* SE.

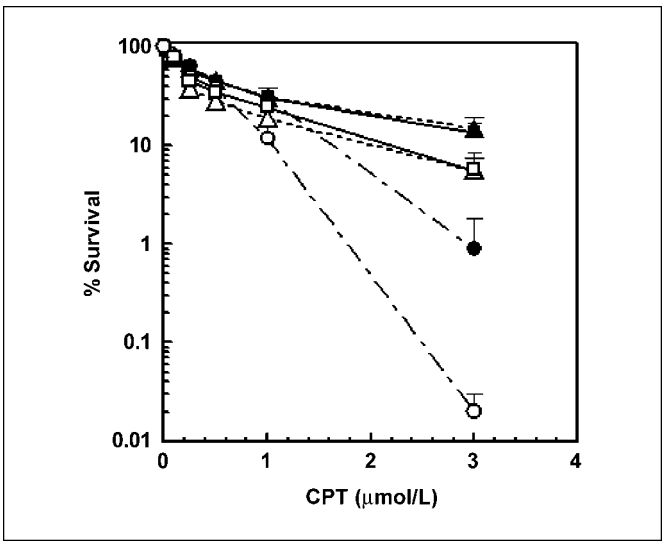

Figure 4. Response to camptothecin. Clonogenic survival of hPNK-depleted M059J and M059K cells following increasing doses of camptothecin: M059J (□), M059JpSup (Δ), M059JδPNK (○), M059K (■), M059KpSup (▲), and M059KδPNK (●) cells. *Points,* mean surviving values of three independent experiments; *bars,* SE.

pathway for DSB, homologous recombination, we analyzed the formation of sister chromatid exchanges (SCE) following irradiation (35). Because we were unable to obtain chromosome spreads from M059J and M059K cells of sufficient quality to quantify SCEs, we used A549 human lung adenocarcinoma cells and the hPNK-depleted A549 cells (C-ter3) described previously (16). Figure 3*C* indicates that the increase in the level of SCE following 2.5 and 5 Gy irradiation was almost identical in the wild-type (WT) A549 and C-ter3 cells, implying that hPNK is not required for homologous recombination and that the processing of strand-break termini in this repair pathway is handled by a different set of enzymes including exonucleases and endonucleases, such as the WRN helicase/nuclease and Mre11 exonuclease (36, 37).

Influence of hPNK and DNA-PKcs depletion on the response to camptothecin. As shown in Fig. 4, the response pattern of M059JδPNK and M059KδPNK cells and their respective parental cells toward camptothecin differed from the response pattern seen with radiation. The difference in sensitivity to this agent between the two parental cell lines was modest compared with their responses to ionizing radiation, indicating that the role of the NHEJ pathway is less critical to the survival of these cells following exposure to this topoisomerase I inhibitor. These data agree well with those of Shao et al. (38), who also examined the influence of camptothecin on M059J and M059K cells, and are qualitatively similar to those obtained with V79 hamster cells and a NHEJ-deficient variant V3-3 (39), showing a slightly elevated sensitivity to camptothecin in the V3-3 cells. On the other hand, Adachi et al. (40) found that DT40 chicken cells lacking NHEJ proteins (Ku70 or DNA-PKcs) displayed considerable resistance to camptothecin compared with WT DT40 cells. This dichotomy still needs to be resolved. The loss of hPNK markedly sensitized both M059J and M059K, especially to the higher doses of camptothecin. This reflects the fact that the repair of a significant proportion of, though not all, camptothecin-induced topoisomerase 1-DNA "dead-end" complexes is mediated by DNA-protein hydrolysis catalyzed by tyrosyl-DNA phosphodiesterase (Tdp1) followed by 3′-dephosphorylation and 5′-phosphorylation by hPNK (41, 42). The influence of the reduction of hPNK on cytotoxicity after exposure to the low doses of camptothecin (<0.5–1 μmol/L) is less clear. Together with our previous data obtained with hPNK-depleted A549 cells (16), it seems that the influence is minimal. Others have linked sensitivity to low doses of camptothecin to heightened sensitivity of cells in S phase (43) and have provided evidence that camptothecin-induced DSBs resulting from stalled replication forks are resolved by homologous recombination (39, 44).

PNK is not involved in nucleotide excision repair. As we have noted previously, reduced expression of hPNK sensitized A549 cells to UV light, most notably at high doses (Fig. 5*A*). We have proposed that the increased sensitivity to UV afforded by hPNK depletion arises from UV-induced strand breakage rather than participation of hPNK in nucleotide excision repair (NER) of UV base damage, such as cyclobutane pyrimidine dimers or 6,4-photoproducts. It has been determined that exposure of P3 teratocarcinoma cells to 10 J/m^2 introduces ~140 SSBs per cell compared with ~840 SSBs induced by 1 Gy X-rays (45), and DSBs have also been observed

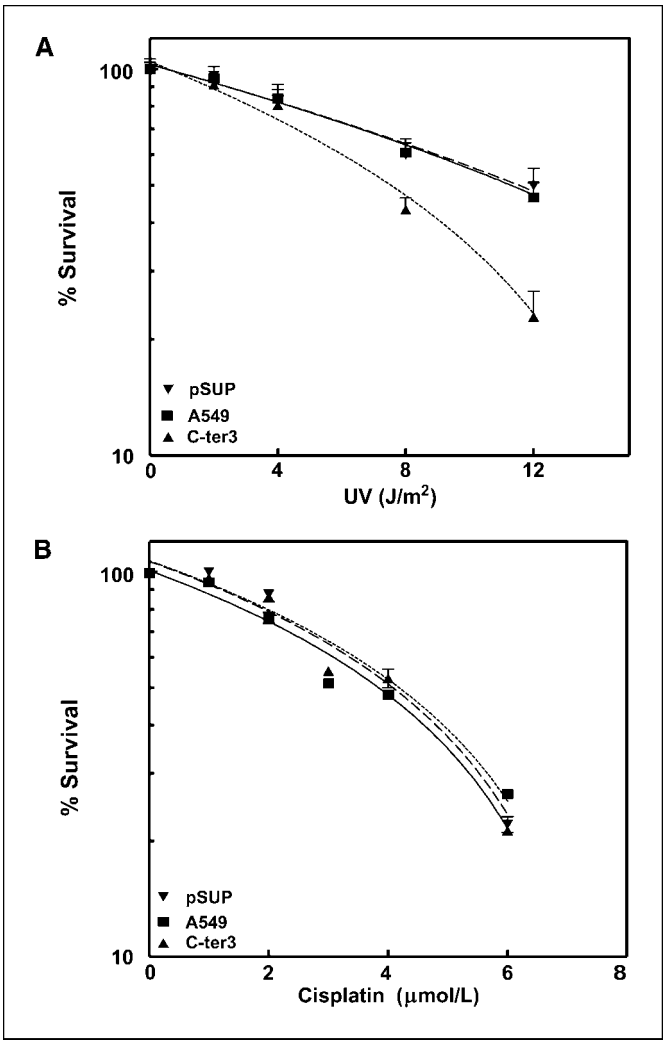

Figure 5. Clonogenic survival curves of A549, C-ter3, and vector-only control cells after exposure to UV and cisplatin. Cells were exposed to increasing doses of UV (*A*) or treated with increasing concentrations of cisplatin (*B*) and cell survival was measured as described in Materials and Methods: A549 (■), A549pSup (▼), and C-ter3 (▲) cells. *Points,* mean value of a minimum of three independent experiments; *bars,* SE.

following UV irradiation with 10 J/m^2 (46). On the other hand, Koch et al. (8) reported an interaction between the forkhead-associated domain of hPNK and DDB1, a component of the DNA damage binding complex involved in NER (47). To investigate the potential involvement of hPNK in NER, we compared the survival of A549 and C-ter3 cells after incubation with cisplatin. This agent produces cisplatin DNA adducts that are primarily repaired by NER (48) and few, if any, direct strand breaks (49, 50). The survival curves (Fig. 5*B*) of the two cell lines are almost identical, clearly ruling out any contribution of hPNK activity in NER and adding to the likelihood that UV-induced strand breaks are responsible for the sensitization resulting from the hPNK depletion.

In conclusion, the data presented here have further clarified the contribution of hPNK to the cellular response to ionizing radiation, camptothecin, UV light, and cisplatin. Together with data obtained from cell-free systems (7, 8), we have clearly established that hPNK participates in the NHEJ pathway, presumably processing the strand break termini before ligation. On the other hand, the SCE data indicate that the enzyme is not required for homologous recombination. The experiments presented here were not specifically designed to assess the contribution of hPNK to the XRCC1/DNA ligase III–dependent NHEJ DSB repair pathway (9, 10). Iliakis et al. (51, 52) have proposed that this pathway, which does not use DNA-PK, serves as a backup to the conventional NHEJ pathway. Although *in vitro* studies have indicated that M059J cells possess this repair pathway (51), which may account for a proportion of the residual DSB repair capacity of these cells, our survival and DSB repair data (Fig. 3*A* and *B*) would suggest that the requirement for hPNK in this pathway may not be significant. Further studies will be required to fully establish the role of this backup pathway in the cellular response to ionizing radiation. It should also be noted that because hPNK is also involved in the SSB and base excision repair pathways (4–6), our observation that the depletion of PNK in M059J cells did not significantly increase the radiosensitivity of these cells further strengthens the argument that DSBs are the most important lesions responsible for radiation-induced cytotoxicity (53, 54). (However, such breaks may still arise from the processing of other types of complex DNA lesions and/or replication arrest opposite SSBs). Both hPNK and DNA-PKcs play a role in the repair of camptothecin-induced DNA-topoisomerase I dead-end complexes, but not necessarily in the same pathway, because the M059J cells, like the M059K cells, were further sensitized by hPNK depletion. The involvement of hPNK in the cellular response to UV light is most likely restricted to the repair of UV-induced strand breaks with no direct participation in NER, a point confirmed by the absence of increased sensitization to cisplatin in hPNK knockdown cells. There is currently increasing interest in targeting DNA repair enzymes, such as DNA-PK, poly(ADP-ribose) polymerase, and ATM, for therapeutic advantage (55). Our findings suggest that hPNK could be a useful target for potentiating tumor cytotoxicity by ionizing radiation or topoisomerase I inhibitors, but not agents such as cisplatin.

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