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Involvement of human polynucleotide kinase in double-strand break repair by non-homologous end joining

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The efficient repair of double-strand breaks (DSBs) in DNA is critical for the maintenance of genome stability. In mammalian cells, repair can occur by homologous recombination or by non-homologous end joining (NHEJ). DNA breaks caused by reactive oxygen or ionizing radiation often contain non-conventional end groups that must be processed to restore the ligatable 3′-OH and 5′-phosphate moieties which are necessary for efficient repair by NHEJ. Here, using cell-free extracts that efficiently catalyse NHEJ *in vitro*, we show that human polynucleotide kinase (PNK) promotes phosphate replacement at damaged termini, but only within the context of the NHEJ apparatus. Phosphorylation of terminal 5′-OH groups by PNK was blocked by depletion of the NHEJ factor XRCC4, or by an inactivating mutation in DNA-PK$_{cs}$, indicating that the DNA kinase activity in the extract is coupled with active NHEJ processes. Moreover, we find that end-joining activity can be restored to PNK-depleted extracts by addition of human PNK, but not bacteriophage T4 PNK. This work provides the first demonstration of a direct, specific role for human PNK in DSB repair.

Keywords: DNA repair/double-strand break repair/genomic instability/Ku/recombination

Introduction

Breakage of the sugar-phosphate backbone of DNA is a common event in cells that are irradiated or suffer the effects of genotoxic agents. Major forms of breakage include the introduction of single- or double-strand DNA breaks (DSBs), which must be efficiently repaired to restore the integrity and functionality of the genome. In mammalian cells, DSBs are repaired by non-homologous end joining (NHEJ), a process that depends on the DNA end-binding protein Ku70/80, a protein kinase DNA-PK$_{cs}$ and the XRCC4/ligase IV heterodimer (reviewed by Chu, 1997; Critchlow and Jackson, 1998; Kanaar *et al*., 1998).

Whereas DNA ligation requires the presence of 5′-phosphate and 3′-OH termini, many chemically distinct groups are found at break points. For example, ionizing radiation, alkylating agents such as BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] and camptothecin promote the formation of non-ligatable 5′-OH and/or 3′-phosphate terminal groups (Coquerelle *et al*., 1973; Henner *et al*., 1983; Eisenbrand *et al*., 1986; Pouliot *et al*., 1999). Such groups need to be removed or modified to enable efficient repair.

Polynucleotide kinase (PNK), which catalyses the phosphorylation of 5′-OH termini and removes 3′-phosphate groups, is thought to play an important role in the modification of damage-induced DNA termini in human cells (Jilani *et al*., 1999; Karimi-Busheri *et al*., 1999). Consistent with this, inactivation of the *Schizosaccharomyces pombe* homologue of PNK, *PNK1*, results in hypersensitivity to ionizing radiation without affecting growth under normal conditions (Meijer *et al*., 2002).

A role for PNK in single-strand break repair has recently been established. First, it was shown that human PNK, DNA polymerase β and DNA ligase I can repair nicks and short gaps bounded by 3′-phosphate and 5′-OH termini (Karimi-Busheri *et al*., 1998). Secondly, direct interactions between PNK and polymerase β, DNA ligase III and XRCC1 have been demonstrated, and the four proteins appear to associate in a multiprotein complex that can promote the repair of single-strand breaks typical of those produced by ionizing radiation or reactive oxygen species (Whitehouse *et al*., 2001). Moreover, XRCC1 was shown to stimulate the DNA kinase/phosphatase activities of PNK, suggestive of a concerted role for XRCC1 and PNK in the processing of damaged termini at single-strand breaks in duplex DNA. In contrast to its role in the repair of single-strand DNA breaks, a direct involvement of PNK in DSB repair has not been demonstrated.

Recently, using human cell-free extracts, an *in vitro* system for the repair of DSBs by NHEJ was described (Baumann and West, 1998; Hanakahi *et al*., 2000). Using linearized plasmid DNA molecules, end joining was shown to be dependent upon Ku70/80, DNA-PK$_{cs}$, DNA ligase IV and XRCC4. However, in this assay, end joining was demonstrated only with simple restriction endonuclease termini that contain easily ligatable 5′-phosphate and 3′-OH termini. In the present work, we have used this *in vitro* system to analyse the repair of DNA termini containing 5′-OH groups. We find that repair occurs efficiently in human cell-free extracts, and is dependent upon the presence of PNK. Surprisingly, phosphorylation of the terminal 5′-OH group by PNK failed to occur in extracts from a DNA-PK$_{cs}$-defective cell line, or when NHEJ was blocked by addition of XRCC4 antibodies. These results indicate that the DNA kinase activity in the extract is coupled with NHEJ. Extracts that were immunodepleted for PNK exhibited a reduced end-joining efficiency, but activity could be restored by addition of purified human PNK, but not bacteriophage T4 PNK. These studies indicate that human PNK is an integral

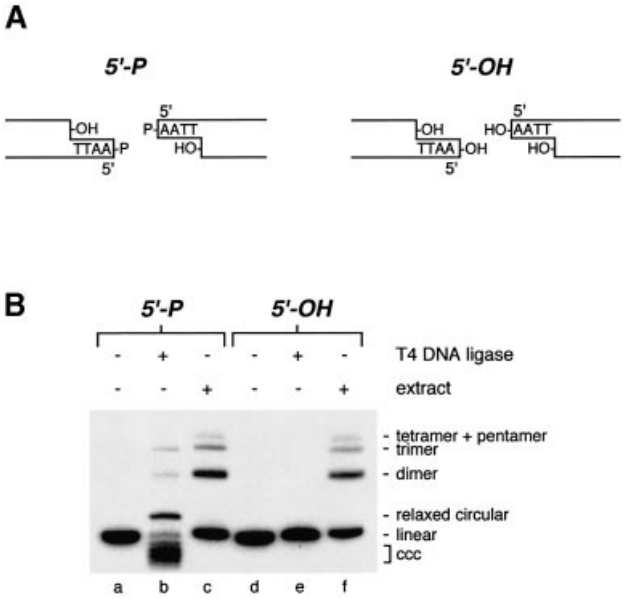

Fig. 1. End joining of 5′-OH termini by human cell-free extract. (**A**) Schematic representation of the DNA termini used in end-joining reactions. Plasmid DNA was linearized with *Eco*RI to produce protruding 5′-phosphate (5′-P) termini (left). Further treatment with CIP resulted in the formation of protruding 5′-OH termini (right). (**B**) Uniformly ^{32}P-labelled *Eco*RI-linearized plasmid DNAs (40 ng), with protruding 5′-phosphate or 5′-OH termini as indicated, were incubated with T4 DNA ligase (80 U) or GM00558 cell-free extract (80 μg). After incubation, the products were deproteinized and analysed by agarose gel electrophoresis.

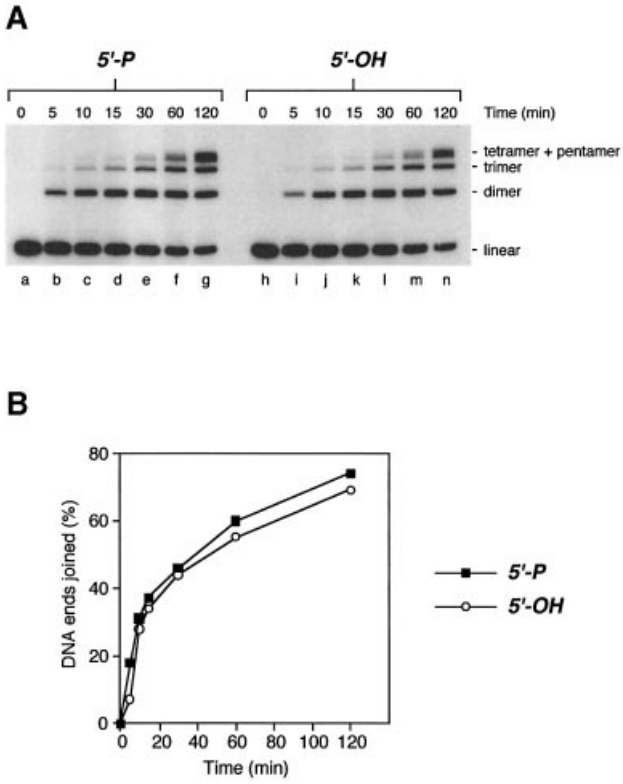

Fig. 2. Comparison of the rate of joining of 5′-phosphate and 5′-OH termini. (**A**) Large scale end-joining reactions (160 μl) contained uniformly ^{32}P-labelled *Eco*RI-linearized plasmid DNA (320 ng) with 5′-phosphate or 5′-OH termini, and GM00558 extract (640 μg). Reactions were incubated at 37°C and at the indicated times 20-μl samples were removed, deproteinized and the DNA products analysed by agarose gel electrophoresis. (**B**) Quantification of the time course shown in (A). The DNA products were quantified by phosphoimaging, and end joining is expressed as the percentage of multimeric DNA species out of total DNA. Squares, 5′-phosphate termini; circles, 5′-OH termini.

partner of the end joining machinery that is active on modified DNA termini at DSBs.

Results

Joining of DNA termini with 5′-OH groups by cell-free extracts

Plasmid DNA was linearized with *Eco*RI and then dephosphorylated with calf intestinal phosphatase (CIP) to produce 5′-OH termini. The ability of cell-free extracts, prepared from the human lymphoblastoid cell-line GM00558, to join these termini was compared with ligatable 5′-phosphate termini. The end structures are shown in Figure 1A. We found that the human cell-free extracts promoted efficient end joining of DNAs with 5′-phosphate or 5′-OH termini to form linear multimeric DNA species (Figure 1B, lanes c and f). Denaturing gel electrophoresis revealed that both strands were fully religated (data not shown). In contrast, T4 DNA ligase could only rejoin DNA molecules with 5′-phosphate termini (Figure 1B, compare lanes b and e). Time course experiments indicated that the human extracts catalysed comparable rates of rejoining with the 5′-phosphate or 5′-OH termini (Figure 2A and B), indicating that the phosphorylation of 5′-OH termini is not a rate-limiting step in this reaction.

Previously, it was shown that the rejoining of linear duplex DNA by human cell-free extracts was dependent upon the NHEJ factors, indicating the value of this *in vitro* system as a model for DSB repair (Baumann and West, 1998). To establish that the joining of 5′-OH termini was also dependent on the NHEJ proteins, we compared the end joining activity of extracts prepared from M059J (DNA-PK$_{cs}$ defective) and M059K (control) cell lines, both of which derive from human malignant glioma biopsies (Lees-Miller *et al*., 1995). In contrast to the M059K extract, which catalysed efficient end joining (Figure 3A, lane g), the DNA-PK$_{cs}$-deficient extract failed to promote multimer formation (lane h). Similarly, end joining by the GM00558 extract was inhibited by polyclonal antibodies raised against XRCC4 (Figure 3A, compare lanes e and f with lane b). All reactions were dependent upon the presence of Mg^{2+} and ATP co-factors (Figure 3A, lanes c and d). These results show that the rejoining of 5′-OH termini is dependent upon those factors previously shown to be involved in the NHEJ reaction *in vitro*.

The joining of protruding 5′-phosphate termini by human extracts is known to be accurate (Baumann and West, 1998). To determine whether 5′-OH termini were also joined accurately, the products of end joining were subjected to digestion with *Eco*RI, the same restriction enzyme that was used to initially linearize the plasmid DNA. For both 5′-phosphate and 5′-OH termini, we found that all end joining products were recut with *Eco*RI (Figure 3B, compare lanes b and c, and d and e). Thus, the

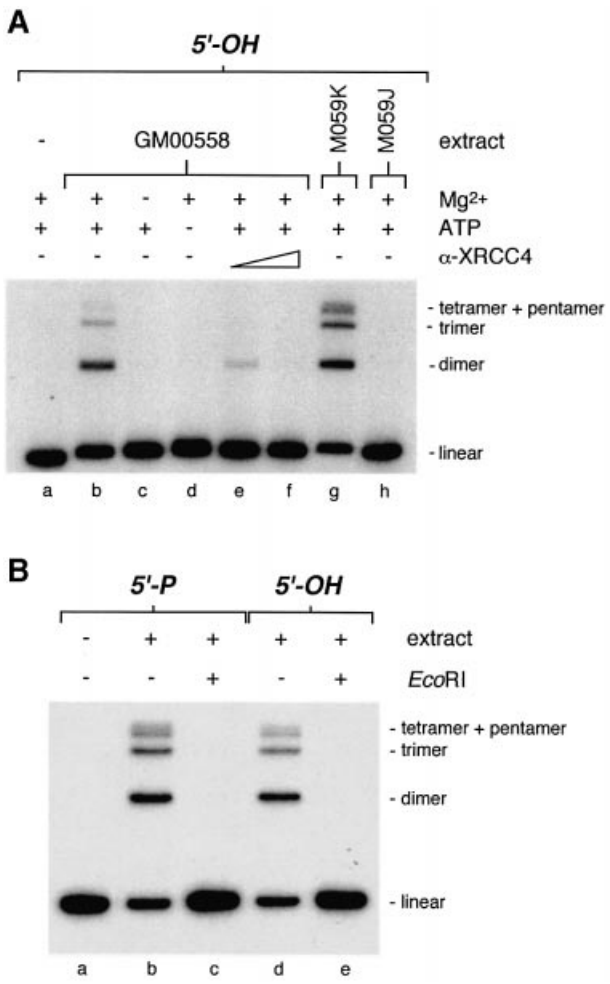

Fig. 3. Requirements and accuracy of extract-catalysed joining of 5′-OH termini. (**A**) Protein and co-factor requirements. *Eco*RI-linearized plasmid DNA with 5′-OH termini was incubated with cell-free extracts prepared from GM00558, M059K and M059J (DNA-PK_{cs} defective) cell lines, as described in the legend to Figure 1. Where indicated, Mg^{2+} or ATP was omitted from the end-joining buffer, or XRCC4 polyclonal antibodies (α-XRCC4) were added to a final dilution of 1:1250 or 1:250. (**B**) Accuracy of end joining. Large scale end-joining reactions (100 μl) containing *Eco*RI-linearized plasmid DNA (200 ng) [with 5′-phosphate (5′-P) or 5′-OH termini as indicated] and GM00558 extract (400 μg) were incubated for 2 h at 37°C. The DNA was deproteinized and aliquots (40 ng) incubated, with (+) or without (–) *Eco*RI as indicated, to recut the joint site. All products were separated by agarose gel electrophoresis and visualized by autoradiography.

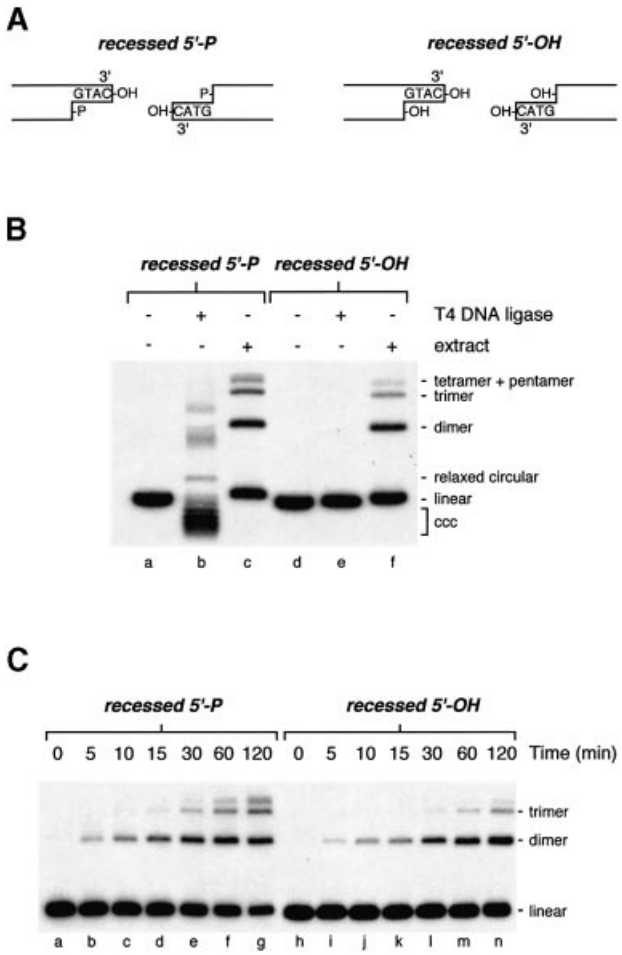

Fig. 4. End joining of recessed 5′-OH termini by human cell-free extract. (**A**) Schematic representation of termini. Plasmid DNA was linearized with *Kpn*I to produce recessed 5′-phosphate (5′-P) termini (left) and dephosphorylated with CIP giving 5′-OH termini (right). (**B**) End-joining reactions were carried out as described in the legend to Figure 1 using the recessed 5′-termini (5′-P) shown in (A). (**C**) Time course of end joining. Large scale reactions (160 μl) containing DNA with recessed 5′-phosphate (5′-P) or 5′-OH termini (320 ng) were incubated with cell-free extract and at the indicated times 20 μl samples were removed, deproteinized and analysed by agarose gel electrophoresis.

end joining reactions are accurate and there are no base losses or additions at the joint sites.

The experiments described above were carried out with protruding 5′-phosphate and 5′-OH termini produced by *Eco*RI digestion. Similar experiments were therefore carried out using *Kpn*I, which gives rise to 5′-recessed termini (Figure 4A). We found that human extracts, in contrast to the T4 DNA ligase control, were again capable of promoting the joining of both 5′-phosphate and 5′-OH termini (Figure 4B). However, the rate of end joining with the recessed 5′-OH termini was reduced slightly in comparison with the 5′-phosphate termini (Figure 4C). Moreover, the accuracy of end joining was reduced, with 18% of the joined 5′-OH termini resistant to restriction digestion with *Kpn*I compared with only 6% of the 5′-phosphate termini (data not shown). These results lead us to suggest that phosphate replacement at protruding termini occurs more efficiently than when the 5′-OH residue is recessed and the terminal base is paired with its complementary strand.

Replacement of the 5′-phosphate group is dependent upon active NHEJ

To determine whether the processing of 5′-OH termini could occur in the absence of end joining, a multistage assay was developed. *Eco*RI-linearized CIP-treated DNA with protruding 5′-OH termini was incubated with extracts, under conditions where NHEJ was inhibited, either by anti-XRCC4 antibodies or by the use of DNA-PK_{cs}-defective extracts (stage 1). The products of the reaction were then deproteinized and analysed for the presence of ligatable 5′-phosphate termini by incubation

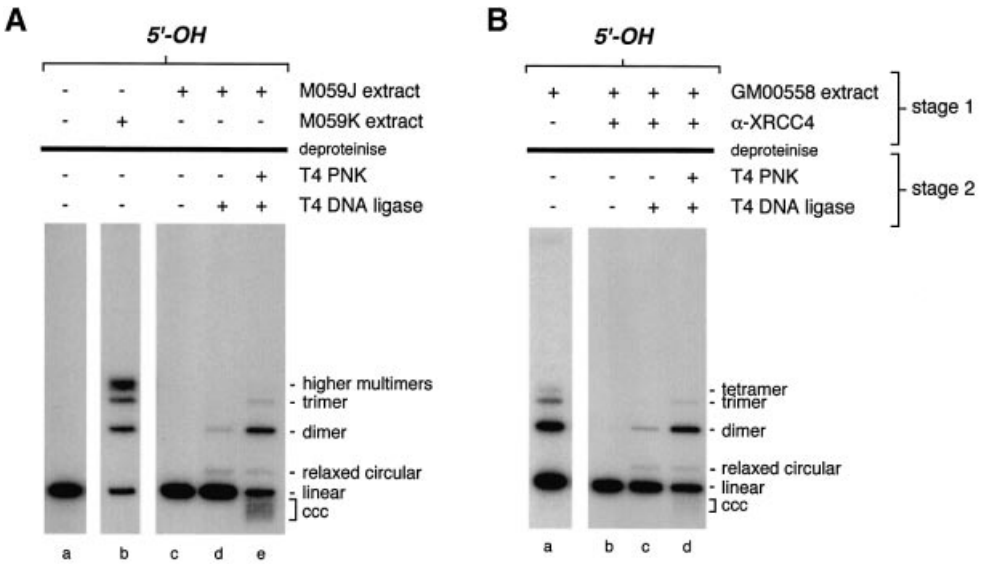

Fig. 5. Role for DNA-PK-dependent NHEJ in the processing of 5′-OH termini. (**A**) Requirement for DNA-PK_{cs} in the processing of 5′-OH termini. Uniformly ^{32}P-labelled *Eco*RI-linearized plasmid DNA with 5′-OH termini (120 ng) was incubated with M059K or M059J (DNA-PK_{cs} defective) extracts (240 μg) for 2 h at 37°C as indicated. The products were then deproteinized and aliquots of DNA (40 ng) were incubated alone, or with T4 DNA polynucleotide kinase (5 U) and/or T4 DNA ligase (80 U). (**B**) Requirement for XRCC4 in the processing of 5′-OH termini. End-joining reactions were carried out as described in (A) using GM00558 extracts, except that XRCC4 antiserum (α-XRCC4, 1:200 dilution) was added where indicated. Following deproteinization, aliquots of DNA (40 ng) were incubated with T4 PNK (5 U) and T4 DNA ligase (80 U) as shown. All DNA products were analysed by agarose gel electrophoresis and visualized by autoradiography.

with T4 DNA ligase (stage 2). The results are shown in Figure 5A and B. We found that DNA which had been incubated in extracts prepared from the DNA-PK_{cs}-defective cell line M059J served as a poor substrate for T4 DNA ligase (Figure 5A, lane d), but that ligation efficiency could be restored by inclusion of T4 polynucleotide kinase in the stage 2 reaction. These results show that conversion of the 5′-OH to a ligatable 5′-phosphate requires the NHEJ factor DNA-PK_{cs}. Similarly, we found that the addition of anti-XRCC4 antibodies to reactions catalysed by GM00558 extracts blocked phosphate addition to the 5′-OH termini, because we again found that the products of the stage 1 reaction were rejoined inefficiently by T4 DNA ligase (Figure 5B, lane c).

These results show that the inhibition of NHEJ, by inactivation of either DNA-PK_{cs} or XRCC4, affects the ability of the extract to promote the conversion of terminal 5′-OH groups to 5′-phosphates. Our interpretation of these data is that phosphate addition occurs by a mechanism that is dependent upon NHEJ, and that DNA kinase activity is coupled with DNA end joining.

Involvement of human PNK in NHEJ

Reasoning that human polynucleotide kinase is likely to be responsible for the observed kinase activity in NHEJ-competent cell-free extracts, antibodies raised against human PNK were used to immunodeplete PNK from GM00558 extracts. Western blotting confirmed that the majority of the PNK was depleted (data not shown). The PNK-depleted extracts were then assayed for their ability to promote the end joining of linear DNA molecules with 5′-OH termini. We found that the PNK-depleted extracts were severely compromised in their ability to promote multimer formation compared with a mock RAD51-depleted control (Figure 6A). Indeed, at the highest extract concentration used we observed only 7% end joining compared with >30% with the mock-depleted extract (Figure 6A, compare lanes e and j). Control experiments showed that the PNK-depleted extracts were capable of joining DNA molecules with 5′-phosphate termini, albeit with an efficiency that was less than the mock-treated control (data not shown).

To verify that the defect in end joining was caused by the absence of PNK, reactions containing the PNK-depleted extracts (50 μg) were supplemented with purified human PNK (Figure 6B, lanes a–e). We found that ~25 ng purified PNK restored normal end-joining efficiency. An analysis of the amount of PNK in an extract by quantitative western blotting showed that 50 μg of cell-free extract contained ~9 ng of PNK (data not shown), indicating that the levels of PNK required for complementation were in the physiological range. In addition, we found that the efficiency of end joining could be stimulated significantly by the presence of excess PNK (Figure 6B, lane e).

The data presented in Figure 5 led us to suggest that the kinase activity on 5′-OH termini was, in some way, coupled with the process of NHEJ. Since addition of human PNK to a PNK-depleted extract restored end-joining activity, we next tested whether bacteriophage T4 PNK could also complement the reaction. In contrast to human PNK, however, we found that T4 PNK failed to restore end-joining activity (Figure 6B, lanes f–j). These results show that the co-operation between PNK and the NHEJ machinery is species specific, and imply that a non-specific kinase such as T4 PNK is unable to access DNA termini bound by the human NHEJ factors.

Discussion

The primary conclusions of the work presented here are as follows: (i) DNA molecules with non-ligatable 5′-OH

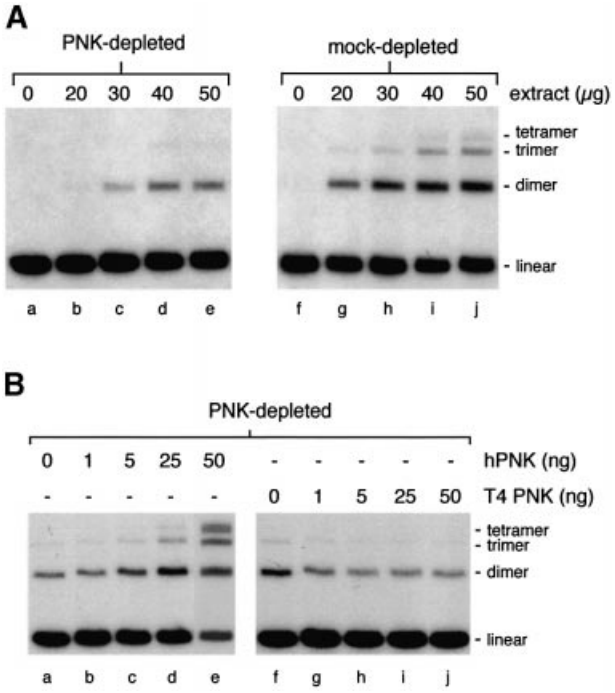

Fig. 6. End joining of 5′-OH termini exhibits a specific requirement for human polynucleotide kinase. (**A**) Immunodepletion of PNK. GM00558 extracts were immunodepleted for PNK or mock (RAD51) depleted using polyclonal antibodies. The depleted extracts were then analysed for their ability to promote end joining using *Eco*RI-linearized plasmid DNA with 5′-OH termini (20 ng). (**B**) Reconstitution of end-joining activity by complementation with human PNK. *Eco*RI-linearized plasmid DNA with 5′-OH termini (20 ng) was incubated with PNK-depleted extracts (50 μg) supplemented with the indicated amounts of purified human or T4 PNK.

termini can be efficiently processed by human cell-free extracts to render them ligatable; (ii) conversion of the 5′-OH moiety to 5′-phosphate is catalysed by polynucleotide kinase; (iii) conversion fails to occur when NHEJ is inhibited by inactivation of either XRCC4 or DNA-PK_{cs}; and (iv) T4 PNK cannot substitute for human PNK in reconstitution experiments with PNK-depleted extracts. Taken together, these data lead us to suggest that the processes of phosphate addition and DNA end joining occur by a mechanism that is inter-dependent, i.e. NHEJ cannot occur without phosphorylation, and phosphorylation cannot occur without proper assembly of the NHEJ end-binding proteins. While these studies provide the first direct evidence for a role of PNK in NHEJ, the results are not entirely unexpected in light of observations showing that *S.pombe* mutants carrying *pnk1* mutations are sensitive to ionizing radiation and yet grow normally in the absence of DNA damage (Meijer *et al*., 2002).

A role for PNK in the repair of 5′-OH groups at single-strand DNA breaks has already been established (Jilani *et al*., 1999; Whitehouse *et al*., 2001). Of particular interest are observations that human PNK interacts with XRCC1, a protein which associates with DNA ligase III and is required for single-strand break repair and genome stability (Thompson *et al*., 1990; Thompson and West, 2000). This interaction is likely to facilitate the targeting of PNK to single-strand break sites, where PNK, DNA polymerase β, XRCC1 and DNA ligase III promote gap filling and repair. In addition, XRCC1 has been shown to stimulate both the 5′-kinase and 3′-phosphatase activities of PNK (Whitehouse *et al*., 2001).

The present study indicates that PNK is also required for double-strand break repair. Moreover, the evidence provided here indicates that the NHEJ apparatus not only accommodates PNK, but that there may be specific interactions between the factors involved in this repair process. Indeed, in preliminary studies that will require further analysis, we note that PNK (mol. wt 57.1 kDa), co-elutes during phosphocellulose and gel filtration chromatography with Ku70/80 and DNA ligase IV in a high molecular weight complex of >500 kDa (data not shown). This association, which was observed during gel filtration at 100 mM KCl, may be quite weak since it is lost at high salt conditions in which Ku70/80 elutes in the position expected of a heterodimer and PNK elutes in its monomeric form (data not shown).

At the present time, little is known about the detailed mechanism of NHEJ in mammalian cells. The reaction involves DNA-PK (consisting of the protein kinase DNA-PK_{cs} and end-binding protein Ku70/80) and the DNA ligase IV/XRCC4 heterodimer (Chu, 1997). The Ku70/80 heterodimer associates with DNA termini and facilitates DNA–DNA interactions, leading to end-bridging (Bliss and Lane, 1997; Ramsden and Gellert, 1998). At this stage, Ku70/80 is thought to cradle the DNA and confine its movement to a helical path that leads to alignment of the two DNA ends (Walker *et al*., 2001). The fit to major and minor groove contours is steric, and few contacts are made either to the DNA bases or to the sugar phosphate backbone. Thus, the shape of the DNA binding site on Ku appears well designed to structurally support but not obscure DNA ends. Upon DNA-PK_{cs} binding, Ku shifts inwards ~10 bp and DNA-PK_{cs} itself adopts a position at the DNA end (Yoo and Dynan, 1999). We therefore suggest that the end-binding complex containing Ku70/80 and DNA-PK_{cs} can recruit and accommodate both DNA ligase IV/XRCC4 complex (Chen *et al*., 2000; McElhinny *et al*., 2000) and end-modifying activities such as PNK. Access to the DNA may be facilitated by the relatively small size of PNK and/or associations with the other factors of the NHEJ apparatus.

Materials and methods

Cell lines

The lymphoblastoid cell line GM00558 was obtained from the NIGMS human genetic mutant cell repository, and the glioblastoma lines M059J and M059K were generous gifts from Dr Susan Lees-Miller (University of Calgary, Canada).

Cell-free extracts

Whole-cell extracts were prepared essentially as described previously (Baumann and West, 1998). Typically, 6–10 l of cells were harvested, washed twice in iced phosphate-buffered saline and once in hypotonic lysis buffer [HLB; 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 5 mM dithiothreitol (DTT)]. The pellet was resuspended in 1 vol. HLB and incubated on ice for 20 min. The cells were lysed by homogenization using a B dounce pestle in the presence of a cocktail of protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, pepstatin, chymostatin and leupeptin). After 20 min, 0.5 vol. hypertonic lysis buffer (50 mM Tris–HCl pH 7.5, 1 M KCl, 2 mM EDTA, 2 mM DTT) was added and the suspension was centrifuged for 3 h at 37 000 r.p.m. in a Beckman SW41-Ti rotor (at 4°C). The supernatant was removed carefully to avoid the upper lipid and lower nucleic acid containing regions, and dialysed for 2 h

against buffer E (20 mM Tris–HCl pH 8.0, 100 mM KOAc, 20% glycerol, 0.5 mM EDTA, 1 mM DTT). Protein concentrations were determined by Bradford assays, and the extract fast-frozen and stored at –70°C.

Proteins and DNA

T4 polynucleotide kinase and T4 DNA ligase were purchased from NEB. Human PNK was purified as described previously (Mani *et al*., 2001). The 5.5 kbp plasmid $pACSG_2$ (PharMingen) was amplified in DH5α in the presence of [^{32}P]orthophosphate and the DNA prepared using Qiagen Maxi plasmid purification kits. Uniformly ^{32}P-labelled form I DNA was linearized with *Eco*RI (to produce protruding 5′-termini) or *Kpn*I (to produce recessed 5′-termini). Dephosphorylation was carried out by treatment with CIP.

End-joining assay

DNA end-joining reactions (20 µl) typically contained 40 ng of uniformly ^{32}P-labelled plasmid DNA and 80 µg of cell-free extract in 50 mM HEPES pH 8.0, 40 mM KOAc, 1 mM $Mg(OAC)_2$, 1 mM ATP, 1 mM DTT, 100 µg/ml bovine serum albumin. Reactions were incubated for 2 h at 37°C, except where indicated. ^{32}P-labelled products were deproteinized and analysed by electrophoresis through 0.6% agarose gels followed by autoradiography or phosphoimaging.

Antibodies and immunodepletions

Polyclonal antibodies against human PNK (Karimi-Busheri *et al*., 1999), XRCC4 (Critchlow *et al*., 1997) and RAD51 (Davies *et al*., 2001) have been described elsewhere. For immunodepletions, cell-free extracts (150–200 µl) were incubated with polyclonal antibodies raised against PNK or RAD51 at a concentration of 1 µl antibody/10 µl extract for 2 h at 4°C on a rotary wheel. Protein A beads (Pharmacia) were pre-washed and resuspended in buffer E and aliquots (100–120 µl) added to the extract. After 1.5 h at 4°C, the beads were pelleted by low-speed centrifugation, and the supernatant was carefully removed, stored in aliquots at –70°C and used in end-joining assays.

Acknowledgements

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