Cell, Vol. 104, 107–117, January 12, 2001, Copyright ©2001 by Cell Press

XRCC1 Stimulates Human Polynucleotide Kinase Activity at Damaged DNA Termini and Accelerates DNA Single-Strand Break Repair

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Summary

XRCC1 protein is required for DNA single-strand break repair and genetic stability but its biochemical role is unknown. Here, we report that XRCC1 interacts with human polynucleotide kinase in addition to its established interactions with DNA polymerase-β and DNA ligase III. Moreover, these four proteins are coassociated in multiprotein complexes in human cell extract and together they repair single-strand breaks typical of those induced by reactive oxygen species and ionizing radiation. Strikingly, XRCC1 stimulates the DNA kinase and DNA phosphatase activities of polynucleotide kinase at damaged DNA termini and thereby accelerates the overall repair reaction. These data identify a novel pathway for mammalian single-strand break repair and demonstrate a concerted role for XRCC1 and PNK in the initial step of processing damaged DNA ends.

Introduction

DNA damage is a major threat to genetic stability as indicated by the cancer-prone phenotype of human diseases in which DNA repair is defective. Contributors to this threat are the thousands of cellular DNA single-strand breaks (SSBs) that arise each day from DNA base damage or DNA sugar damage. If not repaired, such breaks can be converted into double-strand breaks during DNA replication and can result in chromosome instability and cell death. SSBs can arise from DNA base damage indirectly, via the enzymatic excision of base damage during DNA base excision repair. In contrast, SSBs resulting from DNA sugar damage arise directly, from attack by endogenous reactive oxygen species (ROS) or those generated by hydrogen peroxide or ionizing radiation (Ward, 1998). The termini of SSBs are frequently damaged, or "blocked", possessing nonconventional end groups that must be processed to restore 3′-hydroxyl and 5′-phosphate moieties before single-strand break repair (SSBR) can be completed by gap filling and DNA ligation.

One polypeptide implicated in the repair of a broad range of SSBs is XRCC1. *XRCC1* was the first human gene involved in SSBR to be cloned and cells lacking this protein are hypersensitive to ionizing radiation (1.7-fold), hydrogen peroxide (2-fold), camptothecin (3-fold), and alkylating agents (10-fold) (Thompson et al., 1990; Thompson and West, 2000). The importance of XRCC1 to genetic stability is indicated by an elevated frequency of spontaneous chromosome aberrations and deletions in *XRCC1* mutant cells and by embryonic lethality in XRCC1$^{-/-}$ mice (Tebbs et al., 1999; Thompson and West, 2000). In addition, a genetic polymorphism has been identified in human XRCC1 that is correlated with elevated somatic mutation and cancer risk (Shen et al., 1998; Lunn et al., 1999; Sturgis et al., 1999; Divine et al., 2001). At the molecular level, XRCC1 interacts with poly (ADP-ribose) polymerase (Caldecott et al., 1996; Masson et al., 1998), DNA polymerase-β (Caldecott et al., 1996; Kubota et al., 1996), and DNA ligase IIIα (Caldecott et al., 1994, 1995). Despite these observations, however, the biochemical role of XRCC1 remains unclear. Here, we have employed human XRCC1 as bait in a yeast 2-hybrid screen in an attempt to identify additional proteins with which XRCC1 interacts and to determine the role of this protein in SSBR.

Results

XRCC1 Protein Interacts with Human Polynucleotide Kinase

Yeast Y190 cells harboring the 2-hybrid construct pAS-XRCC1 were employed to screen a human cDNA library for genes that encode XRCC1-interacting proteins. From 1.5×10^6 transformants, 186 clones were obtained that displayed histidine prototrophy and β-galactosidase activity. Of thirty-nine clones so far examined, two encoded unknown proteins, thirty-one encoded DNA polymerase-β (Polβ), and four encoded DNA ligase IIIα (Lig3). Intriguingly, the remaining two clones encoded human polynucleotide kinase (PNK), a protein possessing 3′-DNA phosphatase and 5′-DNA kinase activity and which can thus restore normal termini to DNA strand breaks possessing 3′-phosphate and 5′-hydroxyl end groups (Jilani et al., 1999; Karimi-Busheri et al., 1999). 3′-phosphate termini are present at 70% of direct SSBs induced by endogenous ROS or ionizing radiation, and also at any SSBs arising at abasic sites via enzymatic or thermal β,δ elimination (Bailly et al., 1989; Sugiyama et al., 1994; Ward, 1998). 5′-hydroxyl termini have been

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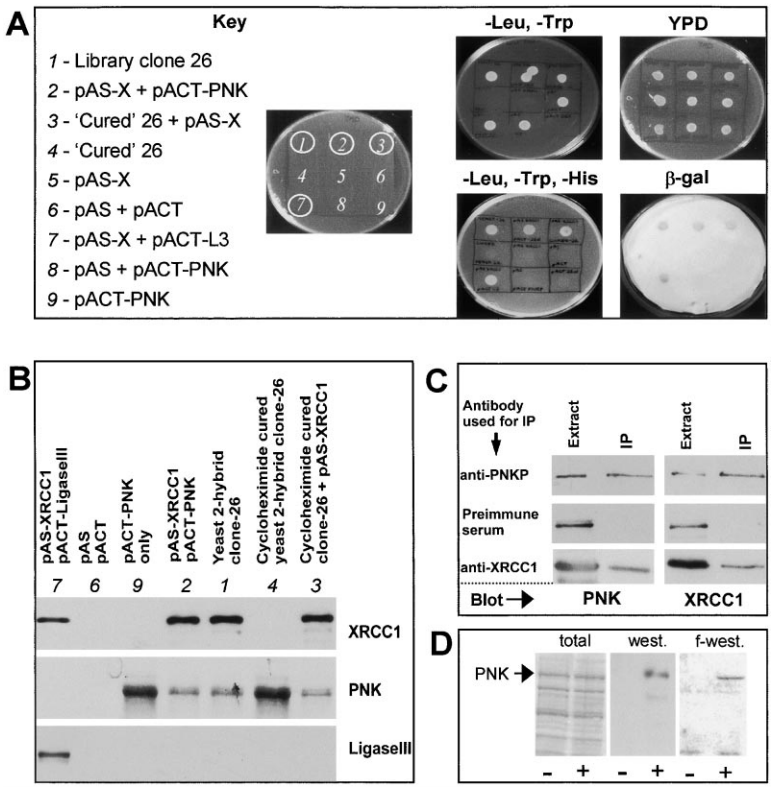


Figure 1. Human XRCC1 and PNK Physically Interact

(A) Pair-wise yeast 2-hybrid analysis was conducted on yeast Y190 cells harboring the constructs indicated in the key. Cells were plated on complete medium ("YPD"), minimal medium lacking leucine and tryptophan to select for both pAS and pACT constructs ("-leu, -trp"), and minimal medium additionally lacking histidine and containing 50 mM 3-aminotriazole (3-AT) to select for 2-hybrid protein–protein interactions ("-leu, -trp, -his"). Colonies from YPD plates were examined for β-galactosidase activity by a colony filter assay ("β-gal"). Strains exhibiting both histidine-prototrophy and β-gal activity are circled in the key. "Cured 26" refers to a derivative of clone 26 generated by selecting for loss of pAS-X plasmid by growth on media containing cycloheximide.

(B) Cell extract from yeast Y190 cells harboring the indicated 2-hybrid constructs was fractionated by SDS-PAGE and immunoblotted with anti-XRCC1 33-2-5 mAb or anti-PNK or anti-Lig3 TL25 polyclonal antisera. Italicized numbers refer to the Y190 strains in the key in (A).

(C) Cell extract from Y190 cells harboring pAS-X and pACT-PNK (library clone 26) was immunoprecipitated with anti-PNK antisera (top panels), preimmune sera (middle panels), or anti-XRCC1 mAb (bottom panels). Aliquots of the crude extract ("extract") and immunoprecipitates ("IP") were fractionated by SDS-PAGE and immunoblotted with anti-PNK antisera (left panels) or anti-XRCC1 mAb (right panels).

(D) Cell extract (30 μg total protein) from *E. coli* cells lacking human PNK ("−") or weakly expressing this protein ("+") were fractionated by SDS-PAGE and transferred to nitrocellulose. Nitrocellulose strips were either stained for total protein with amido black (left two lanes), immunoblotted with anti-PNK polyclonal antisera (middle two lanes), or incubated with ^{32}P-labeled XRCC1 by far-Western analysis (right two lanes) as previously described (Wei et al., 1995).

detected in DNA recovered from irradiated thymocytes (Coquerelle et al., 1973; Lennartz et al., 1975; Ward, 1998), and both 5′-hydroxyl and 3′-phosphate termini are present at SSBs created by abortive topoisomerase I activity (Nitiss, 1998). Despite its potential utility at SSBs, however, direct evidence for a role for PNK in mammalian DNA repair has been lacking.

The His$^+$/β-gal$^+$ phenotype of a Y190: pAS-XRCC1 library clone harboring pACT-PNK (Clone "26") is presented (Figure 1A, "1"), along with a number of positive and negative controls. Loss of pAS-XRCC1 from Clone 26, achieved by selecting for growth in the presence of cycloheximide, resulted in a corresponding loss of the His$^+$/β-gal$^+$ phenotype (Figure 1A, "4"), and reintroduction of pAS-XRCC1 by transformation restored this phenotype (Figure 1A, "3"). Cotransformation of empty Y190 cells with purified pAS-XRCC1 and pACT-PNK plasmids similarly conferred a His$^+$/β-gal$^+$ phenotype (Figure 1A, "2"). Immunoblotting confirmed that the strains employed expressed the expected proteins (Figure 1B). Biochemical evidence for an interaction between XRCC1 and PNK emerged from the observed coimmunoprecipitation of these proteins from Y190:pAS-XRCC1/pACT-PNK extract by either anti-XRCC1 or anti-PNK antibodies (Figure 1C), and from Far-western analysis (Figure 1D). For the latter approach, extracts from *E. coli* lacking human PNK or expressing low levels of this protein were fractionated by SDS-PAGE and either stained for total protein with amido black (Figure 1D, left panel) or electroblotted to nitrocellulose. The nitrocellulose filters were subsequently either immunoblotted with anti-PNK antibodies (Figure 1D, middle panel) or incubated with a [^{32}P]-XRCC1 probe (Figure 1D, right panel). Although PNK was expressed at levels too low to be detected with amido black, both anti-PNK antibodies and [^{32}P]-XRCC1 detected the recombinant polypeptide. Taken together, these data demonstrate that PNK protein interacts with XRCC1, providing the first direct evidence for a DNA kinase/DNA phosphatase activity in mammalian SSBR.

The Coassociation of XRCC1, PNK, Polβ, and Lig3 in Multiprotein Complexes

To determine whether XRCC1 and PNK are associated in mammalian cells, we examined whether they could be coimmunoprecipitated from human cell extract. Indeed, both XRCC1 and PNK were coprecipitated from A549 cell extract by either anti-XRCC1 or anti-PNK antibodies (Figure 2A, top panel). XRCC1 also interacts and is associated in cells with Lig3 and Polβ (Caldecott et al., 1994, 1995, 1996; Kubota et al., 1996). In fact, almost all cellular XRCC1 and Lig3 are tightly bound together, and the intracellular stability of Lig3 is dependent on this interaction (Caldecott et al., 1995; Taylor et al., 1998, 2000a). We therefore determined whether PNK also coprecipitates with Polβ and Lig3, to examine whether all four proteins might be coassociated in a multiprotein complex. In-

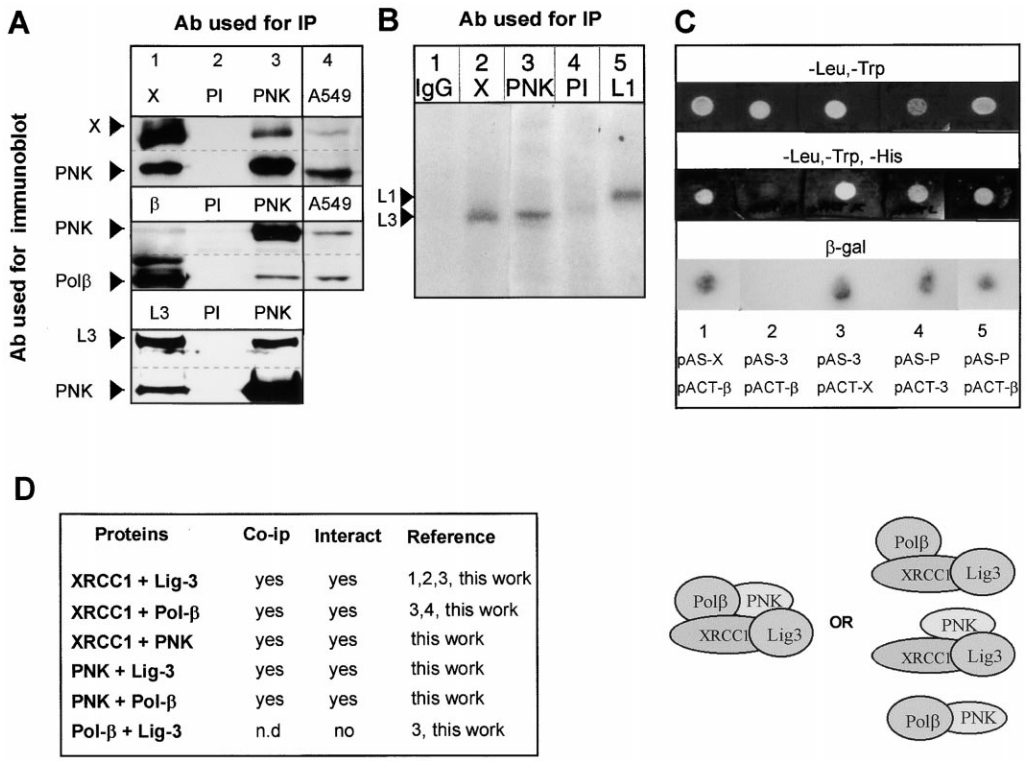


Figure 2. PNK Interacts and Coassociates with XRCC1, Polβ, and Lig3

(A) Cell extract from human A549 cells was subjected to immunoprecipitation with anti-XRCC1 polyclonal antisera (GeneTex inc. San Antonio) (lane 1, top panel), anti-Polβ polyclonal antisera (lane 1, middle panel), anti-Lig3 polyclonal antisera (lane 1, bottom panel), preimmune rabbit serum (lane 2, all panels), or anti-PNK polyclonal antisera (lane 3, all panels). Aliquots of the immunoprecipitates (lanes 1–3, all panels) and A549 total cell extract (lanes 4, top and middle panels) were fractionated by SDS-PAGE and immunoblotted for XRCC1 and PNK (top panel), Polβ and PNK (middle panel), or Lig3 and PNK (bottom panel).

(B) HeLa cell extract was subjected to immunoprecipitation with murine IgG (lane 1), anti-XRCC1 mAb (lane 2), anti-PNK antiserum (ACIV, lane 3), rabbit preimmune serum (lane 4), or rabbit 1186 anti-Lig-1 antiserum (lane 5). The immunoprecipitates were incubated with [α-^{32}P]ATP to adenylate DNA ligase polypeptides prior to their fractionation by SDS-PAGE. Labeled proteins were detected by autoradiography. "L1" and "L3" denote Lig-1 and Lig-3.

(C) XRCC1, PNK, Polβ, and Lig3 were examined for interaction with each other by 2-hybrid analysis. Yeast Y190 cells harboring the 2-hybrid constructs indicated at the bottom ("1–5") were plated onto minimal medium lacking leucine and tryptophan to select for pAS and pACT constructs ("-leu, -trp"), or minimal medium additionally lacking histidine and containing 50 mM 3-AT to select for 2-hybrid protein–protein interaction ("-leu, -trp, -his"). Colonies from complete media were examined for β-gal activity ("β-gal") by a colony filter assay.

(D) Left, summary of immunoprecipitation and yeast 2-hybrid experiments. [1](Caldecott et al., 1994); [2](Caldecott et al., 1995); [3](Caldecott et al., 1996); [4](Kubota et al., 1996). Right, alternative models to explain the summarized data, depicting the association of XRCC1, PNK, Polβ, and Lig3 in a single protein complex or multiple independent complexes. The two possibilities are not mutually exclusive. Note that XRCC1 and Lig3 are always bound together.

deed, both anti-Polβ and anti-Lig3 antibodies immunoprecipitated PNK, and anti-PNK antibodies immunoprecipitated both Polβ and Lig3 (Figure 2A, middle and bottom panels). The coimmunoprecipitation of Lig3 with PNK was also evident if anti-PNK immunoprecipitates were incubated with [α-^{32}P]ATP to detect DNA ligases via their ability to form a covalent complex with AMP (Figure 2B). A comparison of the ratio of signal intensities in the immunoprecipitated material and total cell extract, in conjunction with gel filtration and immunofluorescence experiments, suggest that the association of PNK and Polβ with XRCC1-Lig3 complex is normally limited to approximately 5%–15% of the total cellular complement of each protein (data not shown). Nevertheless, these data indicate that a proportion of these four proteins interact with each other and are coassociated in human cell extract at any one time, either as a single multiprotein complex or as multiple smaller complexes.

We next conducted further 2-hybrid experiments to determine whether additional interactions occur between these four proteins, other than those identified for XRCC1, as might be expected if they comprise one or more multiprotein complexes. Indeed, in addition to interacting with XRCC1 (Figure 2C, lanes 1 and 3), Polβ and Lig3 proteins both interacted with PNK (Figure 2C, lanes 4 and 5). In contrast, Polβ and Lig3 did not appear to interact with each other (Figure 2C, lane 2). A summary of these and previously published data, and a schematic of the possible multiprotein complexes minimally required to explain these data, is presented (Figure 2D).

XRCC1 Accelerates SSBR Reconstituted with Recombinant Human Proteins

The observation that XRCC1 binds a DNA kinase/phosphatase (PNK), a DNA polymerase (Polβ), and a DNA ligase (Lig3) is intriguing, since the latter three proteins

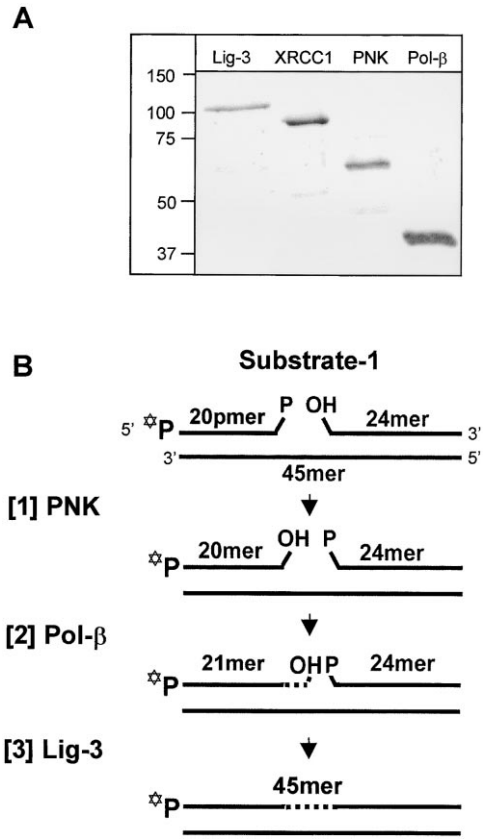


Figure 3. In Vitro SSBR Reactions

(A) Recombinant human proteins. 1 μg of each of the indicated proteins purified from *E. coli* were fractionated by SDS-PAGE and stained with Coomassie blue. The position of molecular weight markers (Biorad) is indicated.

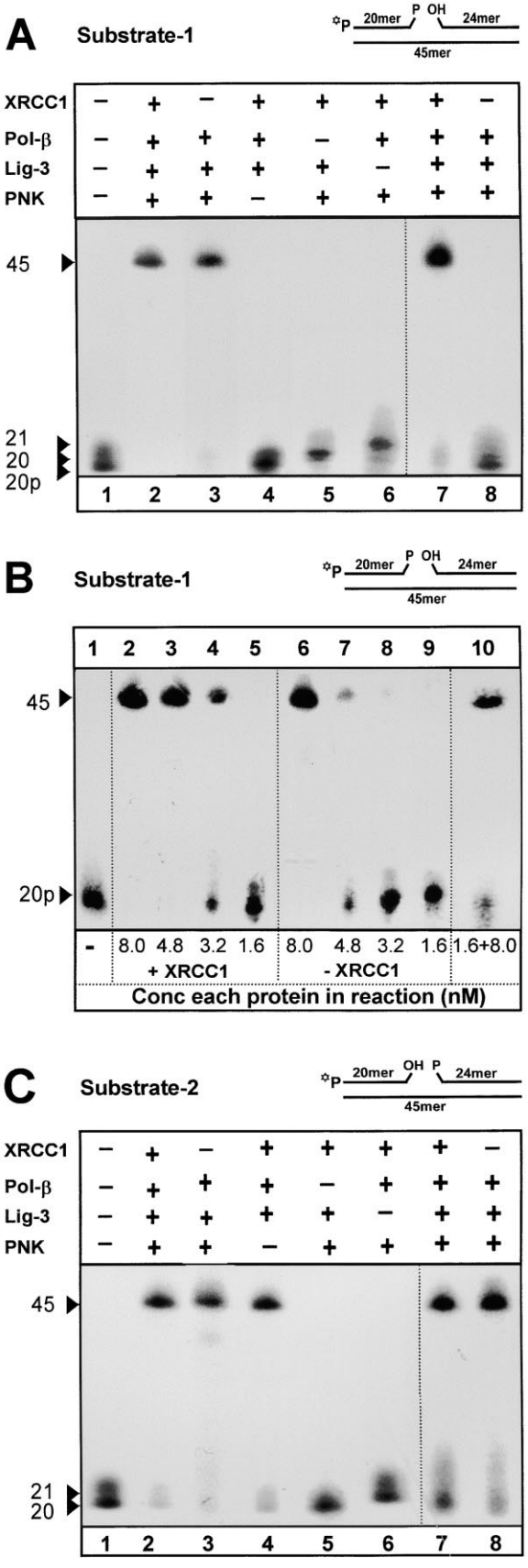
(B) Structure of the oligonucleotide duplex substrate-1 and the predicted intermediates of its repair. The initial duplex substrate (top) harbors a 1 bp gap with 5′-hydroxyl and 3′-phosphate termini. The asterisk denotes the position of a ^{32}P label. [1] The phosphatase activity of PNK restores a conventional 3′-hydroxyl terminus to the ^{32}P-labeled 20-pmer creating a ^{32}P-labeled 20-mer. The kinase activity of PNK also restores a conventional 5′-phosphate terminus but this event is not detected unless [γ-^{32}P]ATP is present. [2] Polβ fills the 1 bp gap (dotted line), creating a ^{32}P-labeled 21-mer. [3] Lig3 ligates the single-strand nick, creating a ^{32}P-labeled 45-mer.

together possess the core enzymatic activities theoretically sufficient for the repair of cellular SSBs possessing 3′-phosphate and/or 5′-hydroxyl termini. To examine this possibility, we attempted to reconstitute SSBR using recombinant human proteins and a defined oligonucleotide duplex substrate. The recombinant proteins and oligonucleotide duplex employed for these experiments, along with the expected DNA intermediates of SSBR, are presented (Figures 3A and 3B). The repair of substrate 1 was completed within 1 hr when incubated with a mixture of the four proteins, as indicated by the complete conversion of labeled 20pmer to 45-mer (Figure 4A, compare lanes 1 and 2). Whereas omission of XRCC1 did not appear to influence the repair reaction under the conditions employed (Figure 4A, lane 3), omission of PNK prevented removal of the 3′-phosphate from the 20pmer and consequently any further processing of the SSB (lane 4). Omission of Polβ or Lig3 from the reaction also prevented repair, resulting in accumulation of the expected 20-mer or 21-mer intermediates, respectively (lanes 5 and 6). Because the radiolabel in the above experiments was present on the 20pmer, only the phosphatase activity of PNK could be directly measured. However, the observed ability of Lig3 to complete repair confirmed that DNA kinase activity did occur during the SSBR reaction because the presence of a 5′-terminal phosphate is a prerequisite for DNA ligation.

If PNK, Polβ, and Lig3 comprise the core enzymatic activities sufficient for SSBR, what role does XRCC1 play in this process? Although XRCC1 was dispensable in the reactions described above, which contained each recombinant protein at 25 nM, it was considered possible that XRCC1 may serve to stimulate SSBR if one or more of the core enzymatic activities are limiting. Indeed, a requirement for XRCC1 for SSBR was observed if the concentration of each protein was reduced to 4 nM (Figure 4A, lanes 7 and 8). Additional protein titration experiments confirmed that XRCC1 stimulated SSBR over a range of concentrations of PNK, Polβ, and Lig3 (Figure 4B, lanes 1–9). Moreover, although XRCC1 was unable to stimulate SSBR at the lowest concentration of proteins employed (each protein at 1.6 nM), it did so if the amount of XRCC1 in this reaction was increased 5-fold (Figure 4B, lanes 5, 9, and 10). These data demonstrate that XRCC1 accelerates SSBR in vitro.

XRCC1 Stimulates the DNA Phosphatase and DNA Kinase Activities of PNK

In contrast to substrate-1, the repair of a derivative of this substrate that lacks damaged termini (substrate-2) did not require PNK (Figure 4C, lane 4). This confirms that PNK was required during the repair of substrate-1 for processing damaged termini. More interestingly, XRCC1 was similarly dispensable for repair of substrate-2, indicating that the requirement for this protein during the repair of substrate-1 was also for processing damaged DNA ends (Figure 4C, lanes 7 and 8). What role might XRCC1 play in this process? The simplest explanation for these results is that XRCC1 stimulates the activity of PNK if the concentration of the latter is limiting during SSBR. To test this notion, we allowed the products of DNA kinase and DNA phosphatase activity to accumulate during the SSBR reaction by omitting dNTPs and thereby preventing subsequent gap filling and DNA ligation. DNA phosphatase activity was greater than 8-fold more efficient in reactions containing PNK, XRCC1, Polβ, and Lig3 than in reactions containing PNK alone (Figure 5A, compare panels 1 and 2). Omission of PNK from the reaction abolished phosphatase activity, confirming that the extra activity conferred by one or more of XRCC1, Polβ, and Lig3 reflected stimulation of PNK rather than another source of phosphatase (Figure 5A, panel 4). Although the 3′-phosphatase activity of PNK was greatest in the presence of all three proteins (Figure 5A, panel 2), XRCC1 was both required (Figure 5A, panel 3) and sufficient (Figure 5A, panel 5) for most of this stimulation. In contrast, Polβ, Lig3, and BSA were unable to stimulate PNK phosphatase activity by themselves (Figure 5A, compare panels 1 and 6–8). These experiments demonstrate that XRCC1 stimulates the 3′-phosphatase activity of PNK.



To examine whether XRCC1 also stimulates the 5′-kinase activity of PNK, a derivative of substrate-1 was employed that was unlabeled and in which the only damaged terminus was a 5′-hydroxyl (Figure 5B, top right). In addition, the DNA kinase reactions were conducted in the presence of [γ-^{32}P]ATP. The 5′-kinase activity of PNK was more than 6-fold greater in the presence of XRCC1, Polβ, and Lig3 than when present alone (Figure 5B, compare panels 1 and 2). Once again, XRCC1 was required and sufficient for much of this stimulation (Figure 5B, compare panel 1 with 2, 3, and 5), though it was noted that Lig3 also stimulated PNK to some extent (Figure 5B, compare panels 1 and 7). Taken together, these data demonstrate that XRCC1 stimulates both the 3′-DNA phosphatase and 5′-DNA kinase activities of PNK and thereby accelerates SSBR, in vitro.

XRCC1 Is Required for End Processing and SSBR Conducted by Whole-Cell Extract

We wished to examine whether XRCC1 is required to stimulate PNK activity in cells. We therefore compared whole-cell extract prepared from *XRCC1* mutant EM9 cells lacking XRCC1 with that from EM9 cells corrected with human XRCC1 cDNA for their ability to support SSBR in vitro. We have demonstrated previously that SSBR proficiency and cellular resistance to DNA damage is restored in EM9 cells that express human XRCC1 (denoted EM9-X cells) (Caldecott et al., 1992; Taylor et al., 2000a). Strikingly, EM9 cell extracts were less able to conduct repair of substrate-1 than were extracts from EM9-X (Figure 6A, top panel, compare lanes 2–4 and 8–10). Furthermore, the addition of recombinant human XRCC1 to EM9 cell extract stimulated SSBR to a level similar or greater than that observed with EM9-X cell extract (Figure 6A, lanes 11–13). Recombinant XRCC1 stimulated SSBR supported by EM9-X cell extract to a much lesser extent, suggesting that its ability to stimulate EM9 cell extract reflected true complementation of the XRCC1 defect (Figure 6A, lanes 5–7). Strikingly,

Figure 4. XRCC1 Accelerates SSBR

(A) 45-mer duplex substrate-1 (80 nM) harboring a 1 bp gap with 3′-phosphate and 5′-hydroxyl termini was incubated in the absence of protein (lane-1) or in the presence of 25 nM (lanes 2–6) or 4 nM (lanes 7and 8) of each of the indicated recombinant human proteins for 1 hr at 37°C. Reaction products were separated by denaturing PAGE (15% gels) and visualized by phosphorimager.

(B) 45-mer duplex substrate-1 (80 nM) was incubated in the absence of recombinant proteins (lane 1) or in the presence of 1.6–8.0 nM each of human PNK, Polβ, and Lig3 (lanes 2–9), with (lanes 2–5) or without (lanes 6–9) the same amount of XRCC1. One reaction contained 1.6 nM each of PNK, Polβ, and Lig3 and 8 nM XRCC1 (lane 10). Reaction products were analyzed as above.

(C) 45-mer duplex substrate-2 (80 nM), which differs from substrate-1 by the absence of damaged termini, was incubated in the absence of protein (lane 1) or in the presence of 25 nM (lanes 2–6) or 4 nM (lanes 7and 8) of each of the indicated recombinant human proteins for 1 hr at 37°C. Reaction products were analyzed as above.

The structure of the substrates employed in these experiments is shown at the top of each panel, with the position of the ^{32}P-labeled phosphate indicated by an asterisk. The position migrated by the 20-pmer, 20-mer, 21-mer, and the 45-mer are also indicated. The slight smearing of 20-mer, 20-pmer, and 21-mer in some lanes (e.g., [A], lane 1) is an artifact of the electrophoresis conditions employed.

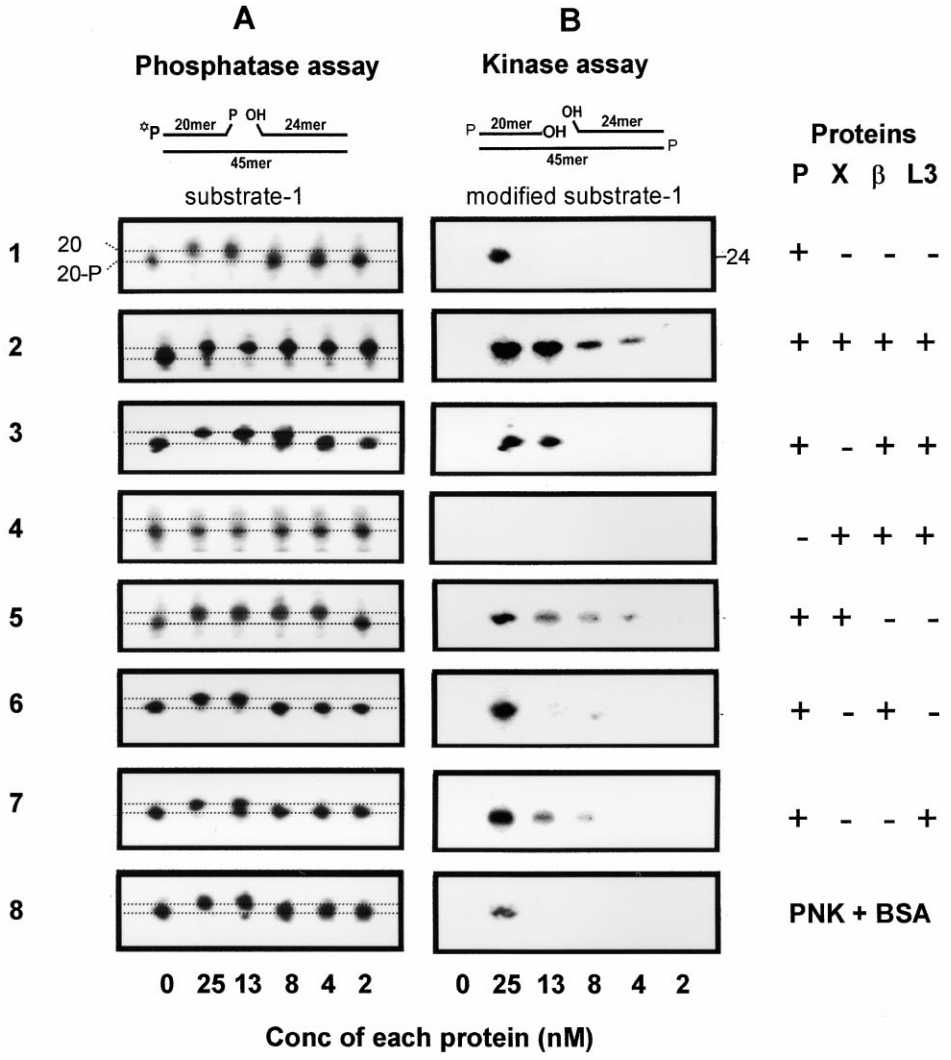


Figure 5. XRCC1 Stimulates the DNA Kinase and DNA Phosphatase Activities of PNK

(A) 3′-phosphatase assay: substrate-1 (80 nM) was incubated in the presence or absence, as indicated on the far right, of 0–25 nM of each of PNK ("P"), XRCC1 ("X"), Polβ ("B"), and Lig3 ("L3"), for 1 hr at 37°C. One set of reactions (panel 8) included 0–25 nM PNK and twice this amount of BSA (0–50 nM, respectively). dNTPs were omitted from the reactions to allow the products of end processing to accumulate. Dotted lines denote the positions of 20-pmer substrate and the 20-mer product of 3′-phosphatase activity.

(B) 5′-kinase assay: A modified version of substrate-1 (80 nM) was incubated as described above but included the additional presence of [γ-^{32}P]ATP. Reaction products were resolved by denaturing PAGE. The position of the 24-mer product of 5′-kinase activity is indicated.

however, XRCC1 was largely redundant in SSBR reactions in which end processing was not required since little difference was observed between the ability of EM9 and EM9-X extracts to conduct SSBR of substrate-2, and recombinant human XRCC1 did not stimulate this reaction (Figure 6A, lower panel). These data demonstrate that XRCC1 stimulates the repair of SSBs with damaged termini in reactions reconstituted with whole cell extract. To examine whether this requirement for XRCC1 for end processing reflects the ability of this protein to stimulate PNK activity, we examined whether addition of recombinant human PNK to EM9 cell extract would circumvent the need for XRCC1. Indeed, the addition of PNK to EM9 extract stimulated SSBR to a level comparable to that stimulated by recombinant XRCC1 (Figure 6B, lanes 6–13) and the additional presence of human XRCC1 did not stimulate the reaction further (Figure 6B, lanes 14–17).

To examine whether recombinant PNK might also be able to circumvent the requirement for XRCC1 in vivo, we attempted to introduce this protein into *XRCC1* mutant EM9 cells by electroporation. We have shown previously that recombinant human XRCC1 restores EMS resistance in electroporated EM9 cells, whereas unrelated proteins such as BSA or maltose binding protein do not (Caldecott et al., 1995). Intriguingly, PNK did increase EMS resistance in electroporated EM9 cells, though to a lesser extent than did XRCC1 (Figure 7A, left panel). The partial nature of this correction is not surprising, since it is likely that other XRCC1 functions are also important for SSBR in vivo (e.g., the interactions with Lig3, Polβ, and PARP). Further support for a role

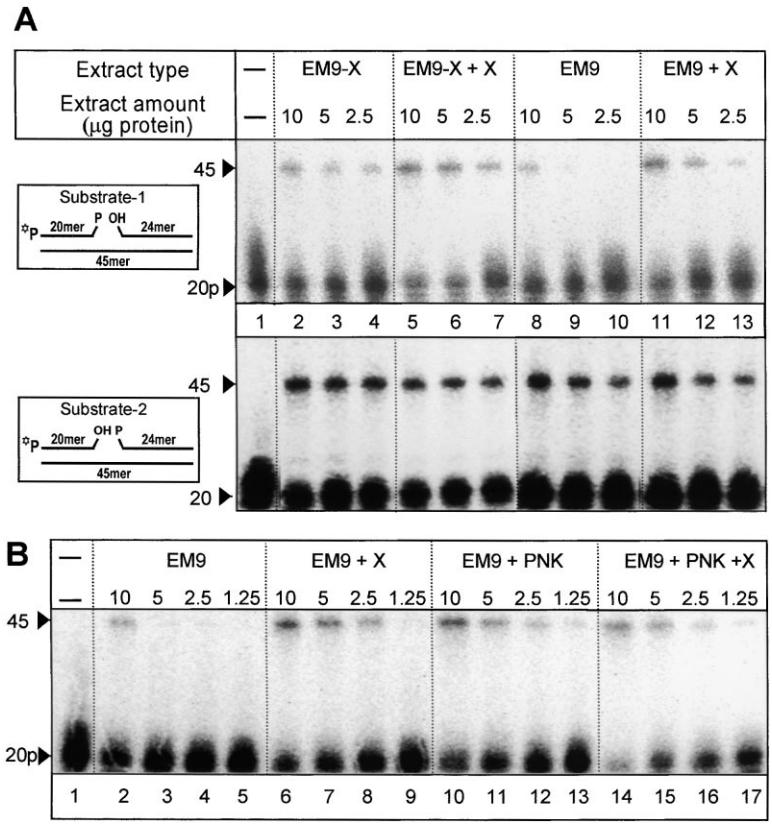


Figure 6. XRCC1 Stimulates SSBR Mediated by Whole-Cell Extract

(A) 80 nM substrate-1 (top panel) or substrate-2 (bottom panel) was incubated in the absence of protein (lane 1) or with 2.5–10 μg of EM9-X (lanes 2–7) or EM9 (lanes 8–13) cell extract protein at 37°C for 1 hr (10 μl reaction volume). Where indicated, cell extracts were supplemented with 0.5 μg recombinant human XRCC1 ("+X") per 20 μg total protein.

(B) Substrate-1 (80 nM) was incubated in the absence of protein (lane-1) or with 1.25–10 μg EM9 cell extract protein at 37°C for 1 hr (10 μl reaction volume). Where indicated, extracts were supplemented with 0.5 μg recombinant human XRCC1 ("+X") or PNK ("+PNK"), or both, per 20 μg total protein. Reaction products were separated by denaturing PAGE and visualized by phosphorimager.

for PNK in DNA repair in vivo emerged from the observation that HeLa cells transfected with human PNK cDNA exhibited elevated resistance to EMS, compared to HeLa cells transfected with empty vector or with PNK cDNA cloned in anti-sense orientation (Figure 7A, right panel). The expression of human PNK in *E. coli* has similarly been observed to increase cellular resistance to alkylating agents (Jilani et al., 1999). The most likely role for PNK following EMS is at SSBs arising from abasic sites via enzymatic or thermal β,δ-elimination, since such breaks possess 3′-phosphate damaged termini (Bailly et al., 1989; Wilson, et al., 1998).

Taken together, these data describe a concerted role for XRCC1 and PNK in processing damaged DNA termini during mammalian SSBR.

DNA Polymerase-β and DNA Ligase III Are Required for SSBR Conducted by Whole-Cell Extract

Following end processing by XRCC1 and PNK, SSBR can be completed by gap filling and DNA ligation. XRCC1 and PNK interact with Lig3 and Polβ in cells, and the latter two proteins were able to conduct these roles during the repair of direct SSBs by recombinant human proteins (see Figure 4). To examine whether Polβ and/or Lig3 might mediate these roles in cells, we examined the effect of anti-Lig3 and neutralizing anti-Polβ polyclonal antibodies on SSBR conducted by whole-cell extract. Strikingly, the immunodepletion of Polβ from wild-type EM9-X extract strongly inhibited the SSBR reaction (Figure 7B, top panel, compare lanes 2–5 with 6–9). The specificity of this inhibition was demonstrated by the observation that pre-blocking the anti-Polβ antibody with purified recombinant human Polβ ablated its ability to inhibit the reaction (Figure 7B, top panel, lanes 10–13). This pre-blocking step had much less effect on the SSBR reaction in the absence of anti-Polβ antibodies (Figure 7B, top panel, lanes 14–17). The anti-Polβ antibodies also inhibited SSBR if simply added to the reaction, consistent with their ability (Singhal et al., 1995) to neutralize Polβ activity (Figure 7B, bottom panel, compare lanes 2–5 with 6–9). Addition of anti-Lig3 polyclonal antibodies to the reaction also inhibited SSBR (Figure 7B, bottom panel lanes 10–13), whereas anti-Lig1 monoclonal antibodies did not (Figure 7B, bottom panel lanes 14–17). These data suggest that both Polβ and Lig3 were required for SSBR conducted by whole-cell extract, under the conditions employed.

Discussion

Processing Ends to Begin Repair

The single-strand break repair protein XRCC1 is required for genetic stability and for embryonic viability in mice. However, the biochemical role of this polypeptide has remained elusive. Here, we demonstrate that XRCC1 interacts with human polynucleotide kinase (PNK), providing the first direct evidence for the activity of this dual DNA kinase/DNA phosphatase during mammalian SSBR (Jilani et al., 1999). Moreover, we have reconstituted SSBR reactions using recombinant human proteins and show that XRCC1 stimulates both the DNA kinase and DNA phosphatase activities of PNK at dam-

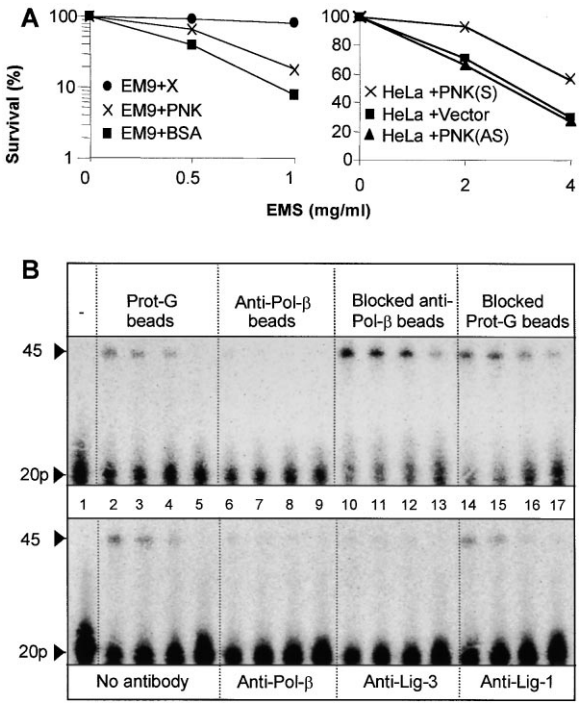


Figure 7. Involvement of Polβ and Lig3 in SSBR Mediated by Cell Extract

(A) Left, EM9 cells were electroporated in the presence of either recombinant human PNK, recombinant XRCC1 ("X"), or BSA as a negative control. The cells were then plated, exposed to 0–1 mg/ml EMS for one hour, and surviving colonies counted after 10 days. Survival was calculated by dividing the number of colonies present on treated plates by the number present on untreated plates. Right, pooled populations of stable HeLa cell transfectants harboring pcD2E empty vector ("EV") or pcD2E containing a PNK ORF cloned in either sense ("S") or anti-sense ("AS") orientation were exposed to 0–4 mg/ml EMS for 1 hr and survival calculated as described above. Experiments are the mean of five independent experiments, with similar results observed in each.

(B) Top, reactions (10 μl) contained substrate-1 (80 nM) and either lacked cell extract (lane 1) or contained 10 μg (lanes 2, 6, 10, and 14), 5 μg (lanes 3, 7, 11, and 15), 2.5 μg (lanes 4, 8, 12, and 16), or 1.25 μg (lanes 5, 9, 13, and 17) of total protein from EM9-X cell extract that was immunodepleted with the following: protein G beads (lanes 2–5), protein G/anti-Polβ antibody beads (lanes 6–9), protein G/anti-Polβ antibody beads preblocked with Polβ (lanes 10–13), or protein G beads preblocked with Polβ (lanes 14–17). Bottom, reactions (10 μl) contained substrate-1 (80 nM) and either lacked cell extract (lane 1) or contained 10 μg (lanes 2, 6, 10, and 14), 5 μg (lanes 3, 7, 11, and 15), 2.5 μg (lanes 4, 8, 12, and 16), or 1.25 μg (lanes 5, 9, 13, and 17) of total protein from EM9-X cell extract additionally containing the indicated antibodies. Reaction products were separated by denaturing PAGE and analyzed by phosphorimager.

aged termini and thereby accelerates the overall repair reaction. These data demonstrate a concerted role for XRCC1 and PNK in processing damaged DNA termini in vitro. Several observations also strongly implicate XRCC1 and PNK in this role in cells. First, protein extracts from *XRCC1* mutant EM9 cells exhibit a reduced ability to repair SSBs possessing the 5′-hydroxyl or 3′-phosphate termini that are substrates for PNK in vitro, but a largely normal ability to repair breaks possessing undamaged termini. Moreover, the role normally fulfilled by XRCC1 at 5′-hydroxyl/3′-phosphate damaged termini can be replaced by increasing the concentration of PNK, suggesting that this role is to stimulate PNK. Second, *XRCC1* mutant cells are hypersensitive to agents that can induce SSBs possessing 5′-hydroxyl or 3′-phosphate damaged termini, such as ionizing radiation, hydrogen peroxide, and camptothecin (Thompson and West, 2000).

Completing Repair after Processing Ends

Once XRCC1 and PNK have restored the termini of direct SSBs to their conventional chemistry, SSBR can be completed by gap filling and DNA ligation. The observation that XRCC1 and PNK both interact and coassociate with Polβ and Lig3 strongly implicates the latter two polypeptides in these processes. Consistent with this notion, both anti-Lig3 and neutralizing anti-Polβ antibodies inhibited SSBR mediated by wild-type extract, indicating that these polypeptides were required for this process in vitro. Several other observations support a role for Polβ and Lig3 in the repair of direct SSBs. First, Polβ inhibitors reduce gap filling in permeabilized mammalian cells treated with bleomycin, a radiomimetic agent that induces primarily direct SSBs, and a concerted role for Polβ and Lig3 has been reported during the repair of direct SSBs by human cell extract (Miller and Chinault, 1982a, 1982b; Winters et al., 1992, 1999). It is also worth noting that we observe a weak defect in SSBR in reactions employing low concentrations of EM9 whole-cell extract that is unrelated to the type of termini present at the SSBs (unpublished data). This suggests that in addition to its requirement for end processing, XRCC1 may also increase the efficiency of gap filling and/or ligation by Polβ and Lig3 at direct SSBs. A similar requirement for XRCC1 has been observed during the repair of indirect SSBs during base excision repair, due primarily to the stabilizing effect of XRCC1 on the cellular level of Lig3, but perhaps also to a weak effect on Polβ levels or activity (Cappelli et al., 1997).

Although our data implicate the participation of Polβ in gap filling at direct SSBs, they do not exclude the involvement of other DNA polymerases, such as Polδ and Polε. This is because the linear oligonucleotide substrates employed in our experiments are poor substrates for the latter enzymes. Indeed, it has been reported that one or both of Polδ and Polε can complete the repair of direct SSBs mediated by cell extract (Winters et al., 1999). This ability of different DNA polymerases to conduct gap filling in vitro suggests that some redundancy may exist in the polymerase employed for this process in cells. Indeed, such redundancy has been demonstrated for Polβ during gap filling at indirect SSBs during base excision repair (Frosina et al., 1996; Stucki et al., 1998; Sobol et al., 2000), and a similar redundancy at direct SSBs could explain the lack of sensitivity of Polβ$^{-/-}$ cells to ionizing radiation (Miura et al., 2000).

The Architecture and Coordination of Mammalian SSBR

An early step in the cellular repair of both direct and indirect SSBs appears to be rapid binding by poly (ADP-ribose) polymerase-1 (PARP-1), a molecular "nick sensor" that is activated at SSBs and synthesizes negatively charged polymers of ADP-ribose (de Murcia and de Mur-

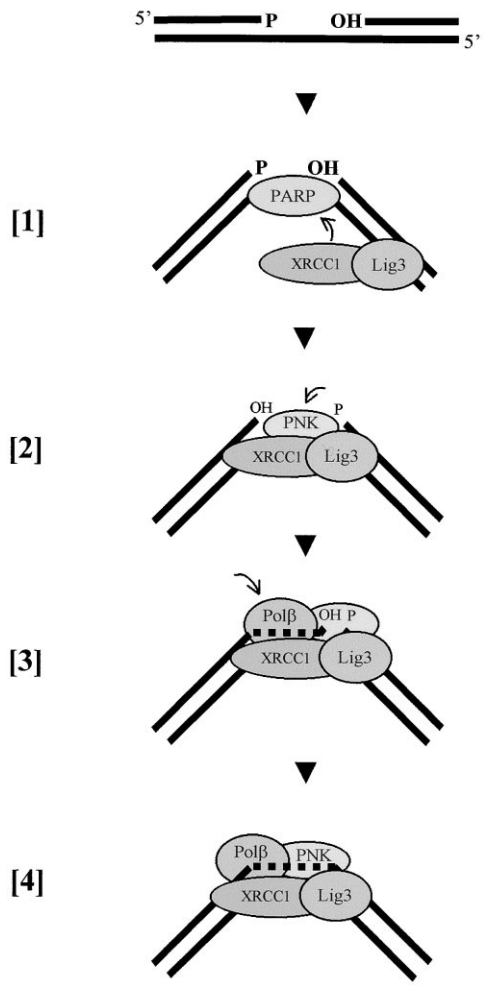


Figure 8. A Model for Mammalian SSBR

Top, a direct SSB comprised of a 1 bp gap with 3′-phosphate and 5′-hydroxyl damaged termini.

[1] PARP binds to the inside bend of an SSB and is activated, synthesising large negatively charged ADP-ribose polymers. Activated PARP sequesters XRCC1–Lig3 complex, which then replaces PARP at the inside bend of the SSB. [2] PNK is sequestered for end processing by simultaneous interaction with the SSB and with XRCC1–Lig3 complex, both subunits of which bind and stimulate PNK. [3] Polβ is sequestered for gap filling by simultaneous interaction with the SSB, XRCC1, and PNK. [4] XRCC1-Lig3 completes repair. Notice the bent configuration of DNA at the site of the SSB. Structural studies support the notion that PARP and XRCC1 bind the inside of this bend and Polβ binds the outside, on which the SSB is located (de Murcia and Menissier de Murcia, 1994; Sawaya et al., 1997; Marintchev et al., 1999; Rice, 1999).

cia Menissier, 1994; Trucco et al., 1998). The role of PARP-1 is unclear, but may include sequestering other repair proteins to sites of SSBs (Figure 8, step 1). This is supported by the observed ability of activated PARP-1 to interact with XRCC1 (Caldecott et al., 1996; Masson et al., 1998; Dantzer et al., 1999), which we propose then serves as a molecular scaffold to coalesce PNK, Polβ, and Lig3 at the SSB (Figure 8, steps 2 and 3). We also propose that XRCC1 binds the inside bend of DNA at an SSB and thereby can serve as a "docking" platform for PNK and Polβ while still allowing these enzymes access to the SSB termini on the outside bend (steps 2 and 3). This notion is prompted by elegant structural studies that suggest that XRCC1 and Polβ do indeed encompass SSBs in this way (Sawaya et al., 1997; Marintchev et al., 1999; Rice, 1999).

The model presented above is in accordance with one recently proposed for base excision repair, in which protein–DNA complexes assembled for one step of the pathway are subsequently "recognized" by a protein that mediates the next step (Rice, 1999; Mol et al., 2000a, 2000b; Wilson and Kunkel, 2000). One advantage of organizing SSBR in this way, in which the product of one enzymatic step is "handed" to the enzyme responsible for the next step, might be to prevent the DNA intermediates of the repair process from erroneous access by exonucleases or recombination proteins. A second advantage may be an increased activity of the individual polypeptides that comprise the repair process, as suggested by the stimulatory effect of XRCC1 on PNK. This could reflect an effect on DNA structure caused by one protein that subsequently increases the activity of another protein. For example, perhaps XRCC1 helps maintain the bent conformation of DNA during steps 2–4 and thereby increases access of the SSB termini to the other repair proteins. Alternatively, increased activity of individual components of the repair process may result from an effect of specific protein–protein interactions on either the catalytic mechanism of an enzyme (e.g., by affecting protein folding) or its affinity for DNA substrate (thereby increasing substrate binding). With respect to the latter, the affinity of PNK for the SSB in step 2 may be increased by the ability of this protein to "dock" with XRCC1 and Lig3. Similarly, the affinity of Polβ for the SSB in step 3 may be increased by an ability to "dock" with XRCC1 and PNK.

In summary, our data suggest a model in which an XRCC1 protein scaffold orchestrates the architectural choreography of mammalian DNA single-strand break repair. In particular, we demonstrate that XRCC1 stimulates the activity of polynucleotide kinase at the onset of SSBR and thereby accelerates the overall repair reaction.

Experimental Procedures

2-Hybrid Analysis

2-hybrid pAS1CYH2 or pACT2 constructs encoding human XRCC1 or Lig3α have been described previously (Caldecott et al., 1996) or were generated during this study (pAS-3). pACT1 constructs encoding Polβ (pACT-β) or PNK (pACT-P) were obtained from a human cDNA library present in pACT by the XRCC1 interaction screen described below. pAS-P was generated by subcloning the PNK ORF from pACT-P into pAS1CYH2. To clone human cDNA encoding proteins that interact with XRCC1, Y190 cells harboring pAS-X were transformed with a pACT human cDNA library and plated onto 25 mM 3-AT plates lacking hisitidine. DNA recovered from individual $His^+/\beta\text{-}gal^+$ clones was transformed into XL2Blue electrocompetent cells (Stratagene) and the recovered pACT plasmids sequenced (LARK Technologies) using 5′ and/or 3′ primers specific for pACT1.

Yeast, Human, and CHO Protein Extracts

Pelleted yeast Y190 cells (1×10^9) were resuspended in 1 ml of 100 mM KCl, 10% glycerol, 50 mM sodium phosphate (pH 8), 1mM DTT, and 1mM PMSF and lysed by vortexing with glass beads. Clarified extracts (2 mg/ml total protein) were stored at −80°C. Whole-cell extracts were prepared from confluent monolayers of HeLa or

AsPC-1 human cells (0.5–1.0 × 10^8cells; 0.5 ml packed cell volume), or EM9-V or EM9-X CHO cells (3 × 10^7cells), by sonication in 1.75 ml (human cells) or 0.5 ml (CHO cells) of 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1mM EDTA, 10% glycerol, 5 mM DTT, and protease inhibitors (Sigma P8340, 1/250 dilution). Clarified supernatants (15 mg/ml total protein) were stored at −80°C.

Immunoprecipitations (IP)

Y190 cell extract (500 μg total protein) was incubated with 4 μl preimmune or anti-PNK polyclonal antisera or 1 μg 33-2-5 anti-XRCC1 mAb in 1 ml of 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100 plus protease inhibitors. After 2 hr on ice, protein G beads were added (for 3 hr at 4°C) to recover antibody-bound proteins and aliquots of the immunoprecipitates were immunoblotted with the antibodies indicated in the text. For immunoprecipitations from human A549 cell extract, cells were lysed by resuspension in 0.5% CHAPS, 137 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA plus protease inhibitors. The clarified cell extract was precleared with protein A beads prior to incubation with the indicated primary antibody for 2–12 hr and protein A beads for a further 2 hr, at 4°C. For DNA ligase-AMP assays, precleared HeLa extract was incubated for 1 hr on ice with primary antibody and 2 hr at 4°C with 10% vol/vol of protein G or A. Washed beads were incubated for 15 min at room temperature in the presence of 3 μCi [α-^{32}P]-ATP and AMP-bound polypeptides detected by SDS-PAGE and autoradiography.

SSBR Reactions, 3′-Phosphatase Assays, 5′-Kinase Assays

^{32}P-labeled 45-mer substrate at a concentration of 80 nM (20 ng or 0.8 pmol in 10 μl total volume) was incubated at 37°C for 1 hr with the indicated proteins/cell extract in 50 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1mM DTT, 0.25–0.5 mM ATP, and 0.5 μM of each dNTP. Where appropriate, proteins/extracts were preincubated together at 4°C for 10 min prior to use. Where indicated, an equal volume of the indicated antibodies was included in the preincubation. Alternatively, extracts were immunodepleted with protein G beads or protein G beads containing anti-Polβ antibody (preblocked on ice for 30 min with recombinant human Polβ or mock blocked with buffer). To measure 3′-phosphatase activity, reactions were conducted as above but in the absence of dNTPs. To measure 5′-kinase activity, dNTPs were omitted, 2 μCi [γ-^{32}P]-ATP was included in the reaction, and the concentration of cold ATP was reduced to 100 μM. Reaction products were analyzed by denaturing PAGE and phosphorimager.

Recombinant Proteins, Electroporation, and HeLa Transfection

Recombinant human XRCC1, Polβ, and Lig3α were expressed in *E. coli* and purified as described (Caldecott et al., 1995; Taylor et al., 2000b). Recombinant His-tagged PNK was expressed in *E. coli* (BL Gold) for 2 hr at 30°C, following addition of 0.2 mM IPTG to logarithmic cultures. PNK was purified by cation exchange (1.6 ml Poros 20 HS column) using a 0–1 M KCl gradient and a Biocad Sprint Perfusion Chromatography System, followed by IMAC. BSA or recombinant XRCC1 or PNK (100 μg) was introduced into EM9 cells (1 × 10^6) by electroporation essentially as described (Caldecott et al., 1995). HeLa cells were transfected by $CaPO_4$ coprecipitation with 20 μg of either empty pcD2E expression vector or with pcD2E harboring the human PNK ORF (from pACT-P) in either sense or antisense orientation. For each construct, pooled populations of >100 independent transfectants were selected for 14 days in 1.5 mg/ml G418.

Acknowledgments

We thank Annie Beaudry for technical assistance, Dr. Arshad Jilani for pThioHisB-PNK construct, and Sharon Barker, Sam Wilson, Rajendra Prassad, Steve Jackson, and Susan Critchlow for antibodies. C. J. W. and R. M. T. are funded by Medical Research Council Project Grants to K. W. C. (G9603130, G9809326, and G9821041). D. D. L. is supported by the National Cancer Institute of Canada and funds from the Canadian Cancer Society, and M. W. is funded by the Medical Research Council of Canada.

Received September 5, 2000; revised December 13, 2000.

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