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XRCC1–DNA polymerase β interaction is required for efficient base excision repair

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ABSTRACT

X-ray repair cross-complementing protein-1 (XRCC1)-deficient cells are sensitive to DNA damaging agents and have delayed processing of DNA base lesions. In support of its role in base excision repair, it was found that XRCC1 forms a tight complex with DNA ligase III α and also interacts with DNA polymerase β (Pol β) and other base excision repair (BER) proteins. We have isolated wild-type XRCC1–DNA ligase III α heterodimer and mutated XRCC1–DNA ligase III α complex that does not interact with Pol β and tested their activities in BER reconstituted with human purified proteins. We find that a point mutation in the XRCC1 protein which disrupts functional interaction with Pol β , affected the ligation efficiency of the mutant XRCC1–DNA ligase III α heterodimer in reconstituted BER reactions. We also compared sensitivity to hydrogen peroxide between wild-type CHO-9 cells, XRCC1-deficient EM-C11 cells and EM-C11 cells transfected with empty plasmid vector or with plasmid vector carrying wild-type or mutant XRCC1 gene and find that the plasmid encoding XRCC1 protein, that does not interact with Pol β has reduced ability to rescue the hydrogen peroxide sensitivity of XRCC1-deficient cells. These data suggest an important role for the XRCC1–Pol β interaction for coordinating the efficiency of the BER process.

INTRODUCTION

Base excision repair (BER) is the major pathway involved in removal of endogenous and mutagen induced DNA damage. This repair process involves a number of sequential reactions initiated by a damage-specific DNA glycosylase that recognizes and removes the damaged base by cleavage of the *N*-glycosylic bond connecting the base to the sugar phosphate backbone. The arising apurinic/apyrimidinic site (AP site) is recognized by an apurinic/apyrimidinic endonuclease 1

(APE1) that incises the phosphodiester bond 5' next to the AP site, followed by addition of one nucleotide to the 3'-OH end of the incised AP site and excision of the base-free sugar phosphate residue by DNA polymerase β (Pol β). Finally, DNA ligase completes repair by sealing the DNA ends (reviewed in 1).

Three major human DNA ligases (DNA ligase I, III α and IV) employ the same basic mechanism for DNA ligation, but participate in distinct biological processes namely DNA replication, BER and nonhomologous end joining, respectively (2). The mechanism linking DNA ligases to a particular biological process is unclear. It was proposed that other proteins might support functional diversity of the DNA ligases (3). Indeed, all of the DNA ligases mentioned above were found in tight complexes with other proteins. DNA ligase I forms a complex with PCNA (4), and DNA ligases III α and IV with X-ray cross complementing protein 1 (XRCC1) and XRCC4, respectively (5–7). Mutations in a DNA ligase partner protein frequently leads to genetic instability and sensitivity to DNA damaging agents (8–10) and all attempted knockouts of these genes were embryonic lethal (11).

Changes in the XRCC1 gene in characterized mutant cell lines (EM7, EM9, EM-C11 and EM-C12) resulted in undetectable levels of the XRCC1 protein and reduced (3–5 fold) levels of its counterpart protein, DNA ligase III α (12,13). These changes affect the mutant cells' sensitivity to a number of DNA damaging agents including alkylating agents and X-rays, pointing to a deficiency in the BER pathway. Indeed, delayed ligation during BER was demonstrated for XRCC1-deficient cells (14). Since XRCC1 has no established biological activity, it was suggested that it might serve as a scaffold protein recruiting and positioning other components during BER (15,16). This idea was further supported by the multiple interactions of human XRCC1 with other BER proteins including Pol β (15,17), polynucleotide kinase (18), poly(ADP-ribose) polymerase 1 (PARP-1) (17,19), and APE1 (20). Although the physiological role of XRCC1–PARP-1 (21,22) and XRCC1–DNA ligase III α (23) interactions have been recently demonstrated, there are no such data for other XRCC1 interactions. In this report, we address the role of the XRCC1–Pol β interaction.

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MATERIALS AND METHODS

Materials

Synthetic oligodeoxyribonucleotides purified by high-performance liquid chromatography were obtained from Midland (8-oxoguanine). [γ - 32 P]ATP (3000 Ci/mmol) was purchased from Perkin-Elmer Life Sciences.

Two-hybrid analysis

Performed as previously described (18).

Proteins

Recombinant human uracil-DNA glycosylase (UDG) was purified as described (24). Histidine-tagged human Pol β , APE1 and DNA ligase III α were purified on Ni $^{2+}$ -charged His-Bind Resin (Novagen, Cambridge, MA) as recommended by the manufacturer.

Transfection and survival experiments

The mammalian expression construct pcD2EXH, encoding human XRCC1 protein with a C-terminal decahistidine affinity tag, was described previously (5). A mutant derivative of pcD2EXH containing the V86R mutation was constructed using the 'QuikChange' protocol (Stratagene) and confirmed by sequencing. EM-C11 cells were cultured as monolayers in DMEM medium and a plasmid containing wild-type or mutant XRCC1 was introduced into EM-C11 cells using 'Fugene 6' transfection reagent (Roche) according to the manufacturer's protocol. Stable clones were isolated under G418 (1.5 mg/ml) selection. Treatment with hydrogen peroxide was performed in DMEM medium with the indicated concentrations of the mutagen for 15 min. Colonies that appeared after 8–10 days were fixed with methanol, stained with Methylene Blue and counted. Relative colony formation (%) was expressed as colonies per treatment level/colonies that appeared in the control.

Purification of XRCC1–DNA ligase III α heterodimer

To purify the XRCC1–DNA ligase III α heterodimer, cell extracts prepared from EM-C11 cells by the method of Tanaka *et al.* (25) as modified by Vodenicharov *et al.* (26) were brought to 5 mM imidazole and 200 μ l of 50% Ni-NTA agarose slurry (Qiagen) was added. After incubation at 4°C for 1 h, beads were washed five times with 0.5 ml of buffer containing 50 mM NaH $_2$ PO $_4$ pH 8.0, 0.2 M KCl, 40 mM imidazole and the XRCC1–DNA ligase III α heterodimer was eluted with the same buffer containing 250 mM imidazole (3 \times 100 μ l), dialysed overnight against buffer containing 25 mM HEPES–KOH pH 7.9, 2 mM DTT, 12 mM MgCl $_2$, 0.1 mM EDTA, 17% glycerol and 0.1 M KCl and stored at –80°C.

DNA ligase activity

DNA ligase activity of the purified DNA ligase III α and XRCC1–DNA ligase III α heterodimer was monitored using a 40mer oligonucleotide duplex containing a 3'-OH and 5'-phosphate nick at position 20. Reactions were carried out in a reaction mixture (10 μ l) that contained 45 mM HEPES pH 7.8, 70 mM KCl, 7.5 mM MgCl $_2$, 0.5 mM EDTA, 1 mM DTT, 2 mM ATP, 20 μ M each of dATP, dGTP, dCTP and TTP, 0.5 mg/ml BSA, a FAM (6-carboxyfluorescein)-labelled oligonucleotide duplex (~5 ng, 0.5 pmol) and the indicated

amount of DNA ligase III α or XRCC1–DNA ligase III α heterodimer at 37°C for 20 min. The reaction was stopped by addition of 10 μ l of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% Bromophenol Blue and 0.02% xylene cyanole). Following incubation at 90°C for 3 min, the reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel. One unit of DNA ligase activity was defined as the amount of enzyme converting 0.1 pmol of the substrate to a full-length product (40mer) at 37°C within 20 min.

Reconstituted repair assays

The reactions were reconstituted in a reaction mixture (10 μ l) that contained 45 mM HEPES pH 7.8, 70 mM KCl, 7.5 mM MgCl $_2$, 0.5 mM EDTA, 1 mM DTT, 2 mM ATP, 2 mg/ml BSA, 20 μ M each of dATP, dGTP, dCTP and TTP, and a FAM (6-carboxyfluorescein)-labelled 30mer oligonucleotide duplex containing a uracil residue at position 19 (~5 ng, 0.5 pmol). The reactions were initiated by incubation of the substrate with UDG (~645 fmol), APE1 (~300 fmol), Pol β (~50 fmol) and the indicated amount of DNA ligase III α or XRCC1–DNA ligase III α heterodimer at 37°C for 20 min. The reaction was stopped by addition of 10 μ l of gel loading buffer. Following incubation at 90°C for 3 min, the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel.

RESULTS

Purification and characterization of XRCC1–DNA ligase III α heterodimer

Recombinant XRCC1 and DNA ligase III α were shown to form a very tight complex resistant to dissociation at a high salt concentration (12) and it was also demonstrated that the two proteins co-purify over several chromatographic steps (6) and can be co-immunoprecipitated from whole cell extracts (18). To purify XRCC1–DNA ligase III α heterodimer, we used EM-C11 cells that contain a G \rightarrow A substitution at nucleotide 1169 in the XRCC1 gene which leads to a C390 \rightarrow Y amino acid change in the protein and is located in the putative functional BRCT1 domain (13). This mutation results in an undetectable level of XRCC1 protein in these cells (probably due to instability of the mutated protein) and reduced amounts of DNA ligase III α (12,13). We stably transfected these cells with wild-type His-tagged XRCC1 and pulled down the XRCC1–DNA ligase III α heterodimer using Ni-NTA agarose. Western blot analysis revealed that the pulled down fraction contained both XRCC1 and DNA ligase III α components (Fig. 1A). The complex was also tested and shown to be active in a DNA ligase activity assay with a nicked oligonucleotide (Fig. 1C and D). However, since a Coomassie-stained gel of the heterodimer in addition to XRCC1 and DNA ligase III α also contained several background protein bands characteristic for non-specific binding to Ni-NTA agarose, the complex was tested for enzymatic activities that may interfere with BER. We did not find any substantial activity of DNA polymerase, UDG, AP endonuclease or any other nucleases in a reconstituted repair assay (data not shown). In control experiments, no ligase activity could be pulled-down from XRCC1-deficient cells (EM-C11)

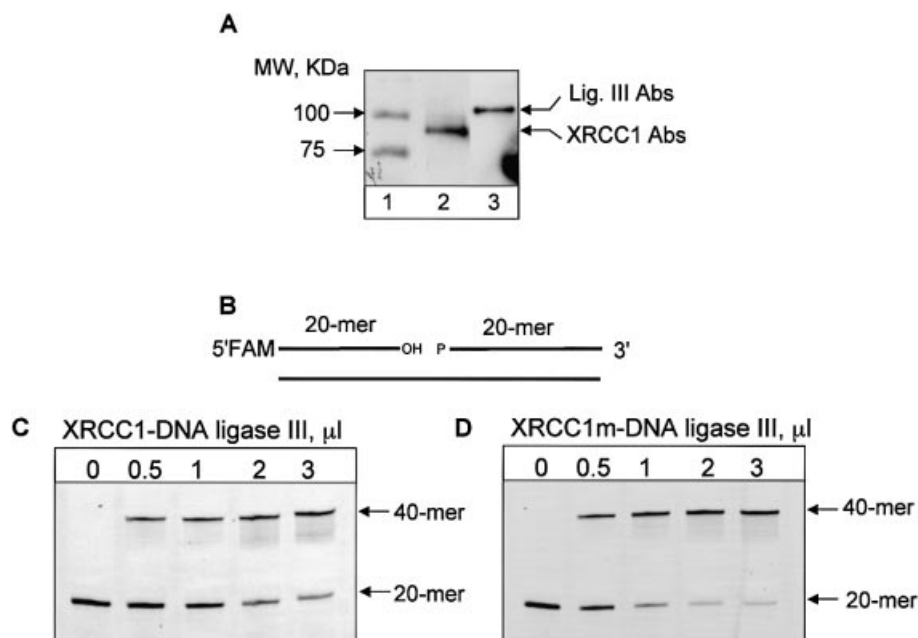


Figure 1. Isolation and characterization of XRCC1–DNA ligase III α heterodimer. (A) XRCC1–DNA ligase III α heterodimer pulled down by Ni-NTA agarose from EM-C11 cells transfected with His-tagged human XRCC1 was subjected to electrophoresis on a 10% polyacrylamide gel, transferred onto PVDF membrane, and immunoblotted against XRCC1 (lane 2) or DNA ligase III α (lane 3) antibodies. Molecular weight markers are shown on lane 1. (B) Schematic representation of nick-containing oligonucleotide substrate 5'-labelled with FAM. (C and D) DNA ligase activity of XRCC1–DNA ligase III α heterodimer. The indicated amount of wild-type (C) or mutated (D) XRCC1–DNA ligase III α complex was incubated with nick-containing substrate. Following incubation for 20 min at 37°C, reactions were stopped by addition of an equal volume of gel loading buffer and after incubation at 90°C for 3 min, the reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel.

or HeLa cells containing untagged XRCC1 (data not shown). Marintchev *et al.* (27) have recently characterized a number of XRCC1 point mutations specifically disrupting the interaction of Pol β with the N-terminal domain of XRCC1. To test whether this interaction is important for the activity of the XRCC1–DNA ligase III α heterodimer in the BER reaction, we used site-directed mutagenesis to generate a V86R mutation in a cloned human XRCC1 cDNA (Fig. 2A). Yeast 2-hybrid analysis confirmed that the V86R mutation greatly reduced or ablated the interaction with Pol β *in vivo* (Fig. 2B). This was indicated by the ability of the wild-type protein, but not mutated protein, to activate the histidine and β -galactosidase reporter genes in yeast expressing Pol β . The mutation in XRCC1 did not affect the interaction of XRCC1 with DNA ligase III α , however (data not shown).

Then we stably transfected EM-C11 cells with mutated His-tagged XRCC1 (XRCC1m) pulled down the XRCC1m–DNA ligase III α heterodimer using Ni-NTA agarose and tested its DNA ligase activity. The V86R mutation did not affect DNA ligase activity and the complex proved to be highly active (Fig. 1D).

Interaction of XRCC1–DNA ligase III α with Pol β is required for efficient ligation in reconstituted BER

The potential function of XRCC1 as a scaffold protein has been discussed in a number of papers and the interaction of XRCC1 with Pol β was suggested as a possible molecular basis for such a role (reviewed in 16,18,28), however this model has not been verified experimentally. To demonstrate that the V86R mutation in XRCC1 protein affects the ability of

the mutated XRCC1m–DNA ligase III α heterodimer to function in BER, we tested its DNA ligase activity and its ability to support BER in a reconstituted repair assay. We reconstituted short-patch repair of uracil in DNA with purified human recombinant proteins including UDG, APE1, Pol β and XRCC1–DNA ligase III α or mutated XRCC1m–DNA ligase III α heterodimer. The DNA end-joining activity of the XRCC1–DNA ligase III α heterodimer and XRCC1m–DNA ligase III α was initially measured using a single nick-containing oligonucleotide (Fig. 1C and D, correspondingly) and an equal amount of activity units was subsequently used in the reconstituted reaction (one unit of DNA ligase activity was defined as the amount of enzyme converting 0.1 pmol of the nicked substrate to full-length product at 37°C within 20 min).

For repair reactions, a 30mer oligonucleotide duplex containing a single uracil at position 19 was used as a substrate (Fig. 3A). Incubation of the 30mer substrate (Fig. 3B, lane 1) with UDG and APE1 leads to generation of the 18mer labelled product resulting from removal of uracil and incision of the AP site (Fig. 3B, lane 2). Pol β , when added to the reaction mixture, continues repair by adding one nucleotide to the 3'-end of the incised AP site and simultaneously removing the 5'-sugar phosphate, thus generating a substrate for DNA ligase (Fig. 3B, lane 3). The XRCC1–DNA ligase III α heterodimer stimulated ligation (observed as the 30mer full-length product) and stimulation depended on the amount of the heterodimer added (Fig. 3B and D). In contrast, the equivalent amount (in units of DNA ligase activity) of XRCC1m–DNA ligase III α was about 2–3 fold less efficient in reconstituted

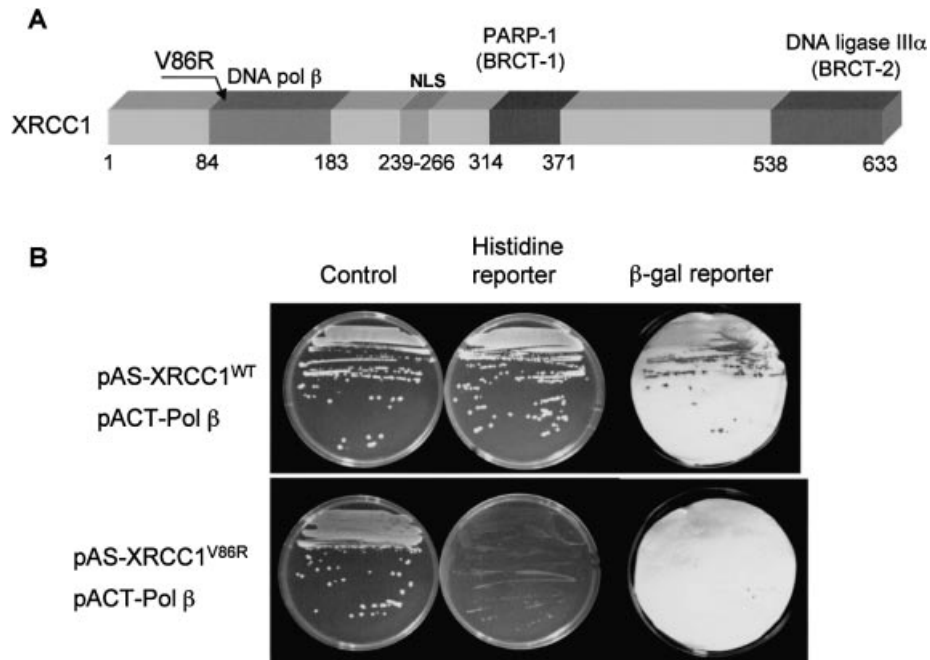


Figure 2. Mutated XRCC1 (V86R) does not interact with Pol β in a yeast two-hybrid system. (A) Schematic representation of human XRCC1. Sites of interaction with other BER proteins, the nuclear localization signal (NLS) and site-directed point mutation (V86R) affecting XRCC1-Pol β interaction are indicated. Adapted from Thompson and West (28). (B) Wild-type and mutant human XRCC1 proteins were examined for interaction with human Pol β by yeast two-hybrid analysis. Yeast Y190 cells harbouring pACT-Pol β and either pAS-XRCC1^{WT} or pAS-XRCC1^{V86R} were examined for activation of the histidine and β-galactosidase reporter genes that are indicative of a protein-protein interaction. For this purpose, Y190 cells harbouring these constructs were plated onto minimal media lacking tryptophan and leucine, to select for the pAS and pACT constructs ('Control'), and also on the above plates additionally lacking histidine and containing 50 mM 3-aminotriazole to test for protein-protein interaction ('Histidine reporter'). Colonies from the control plates above were also examined for β-galactosidase activity by colony filter assays ('β-gal reporter').

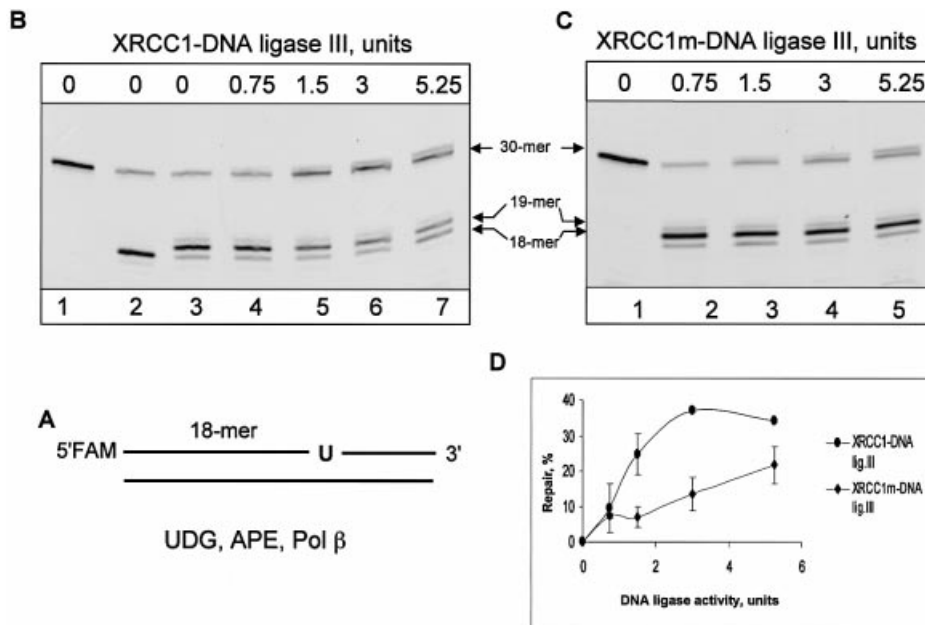


Figure 3. Interaction of XRCC1-DNA ligase IIIα with Pol β is required for efficient ligation in reconstituted BER. (A) Schematic representation of the FAM-5'-labelled oligonucleotide substrate containing uracil (U). A 33 bp duplex oligonucleotide (5 ng, 500 fmol) containing a uracil residue at position 19 was incubated in reaction buffer containing magnesium, dNTPs, 20 ng (645 fmol) of UDG, 10 ng (300 fmol) of APE1, 2 ng (50 fmol) of Pol β and the indicated amount of wild-type (B) or mutated (C) XRCC1-DNA ligase IIIα complex. Untreated substrate was loaded on lane 1 of (B) and (C), and the reaction loaded on lane 2 of (B) contained only UDG and APE1. Following incubation for 20 min at 37°C, reactions were stopped by addition of an equal volume of gel loading buffer and after incubation at 90°C for 2 min, the reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel. (D) Graphical representation of three independent experiments.

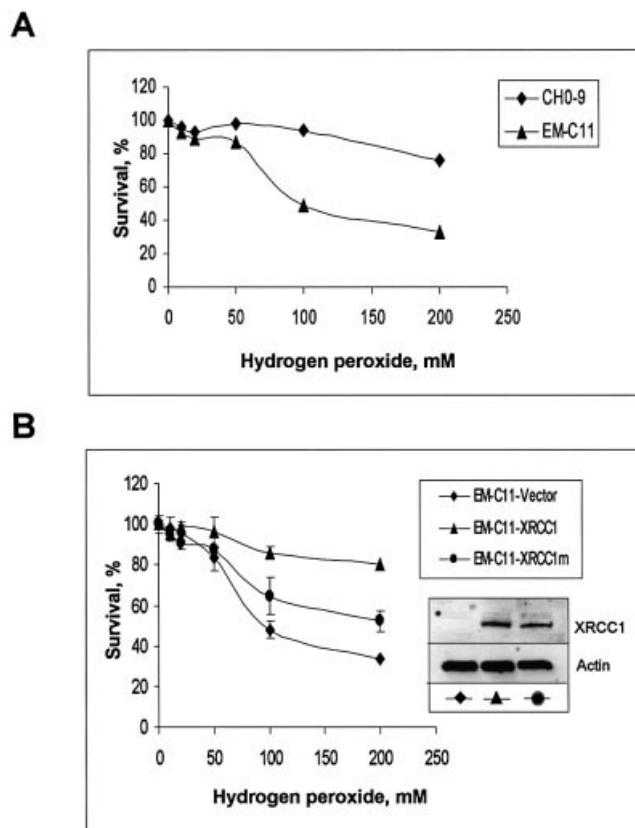


Figure 4. Reduced survival after hydrogen peroxide treatment of EM-C11 cells transformed with plasmid expressing mutated XRCC1. (A) Survival of wild-type (CHO-9) cells and XRCC1-deficient (EM-C11) cells after exposure to hydrogen peroxide. (B) Survival of EM-C11 cells transfected with expression plasmid harbouring wild-type XRCC1, V86R mutant XRCC1 or an empty vector after exposure to hydrogen peroxide. Expression of the XRCC1 gene in the transfected cell lines used for survival experiments was verified by running 30 μ g whole cell extract proteins on a 10% SDS-polyacrylamide gel followed by transfer onto a PVDF membrane, and immunoblot analysis with the indicated antibodies.

BER (Fig. 3C and D). We thus concluded that interaction of the XRCC1–DNA ligase III α complex with Pol β is required for efficient DNA ligation.

Disruption of XRCC1–DNA ligase III α –Pol β interaction increases cell sensitivity to hydrogen peroxide

Finally, to address the biological role of this mutation, we compared sensitivity to hydrogen peroxide between wild-type CHO-9 cells, EM-C11 cells and EM-C11 cells transfected with empty plasmid vector and with plasmid vector carrying wild-type or mutant XRCC1 gene. Western blot analysis revealed that both clones transfected with the plasmid vector containing wild-type or mutant XRCC1 express similar levels of XRCC1, and no XRCC1 was detected in the EM-C11 cells transfected with the empty vector (Fig. 4B). In a clonogenic survival assay, we found that EM-C11 cells were approximately 2-fold more sensitive to killing by hydrogen peroxide than CHO-9 cells (Fig. 4A) and transfection of the EM-C11 cells with the vector carrying wild-type XRCC1, but not empty vector, completely rescued the EM-C11 cells

(Fig. 4B). However, cells carrying mutated XRCC1 remained sensitive to hydrogen peroxide, although not as much as the EM-C11 cells (Fig. 4B).

DISCUSSION

Unless BER enzymes are organized in a preassembled DNA repair complex, BER should be a highly coordinated process to avoid accumulation of repair intermediates, since some of them, such as single strand breaks introduced by APE1 during the course of repair, are a major threat to genome integrity and should be repaired promptly. Recently, several groups proposed the so-called ‘passing the baton’ model for BER (1,29,30). According to this model, the complex of a repair protein with damaged DNA formed at one step of the repair is recognized by the enzyme involved in the next step. Thus, a DNA repair intermediate is ‘handed’ from one repair protein to another without exposure to a cellular milieu. In this model, interactions of XRCC1 protein with other BER proteins were suggested to play an important role. XRCC1 interacts with several enzymes implicated in BER including Pol β (15,17), PARP-1 (17,19) and DNA ligase III α (5–7). While the *in vivo* effects of disrupting the interactions with PARP-1 and DNA ligase III α have been characterized previously (21–23), the biological importance of the Pol β interaction had not been assessed until now. XRCC1 interacts with the palm-thumb domain of Pol β via its N-terminal domain (NTD) and this interaction is therefore distinct from the sites of interaction with either PARP-1 or DNA ligase III α (31). Recently, NMR chemical shift mapping of the XRCC1 NTD–Pol β interaction interface has identified several key residues involved in this protein–protein interaction and their importance has been confirmed by site-directed mutagenesis (27,32). We selected one of the XRCC1 mutants (V86R) for further study since it has been shown to completely abolish binding of the XRCC1 NTD to Pol β (27). The mutation is located in the five-stranded β -sheet of the core β -sandwich (33) and disrupts a key hydrophobic interaction with the Pol β thumb loop without, crucially, affecting the folding of the NTD (27).

In this study, we provide direct evidence of a biological role for the interaction of XRCC1–DNA ligase III α with Pol β by analysing the relative efficiency of wild-type and non-interacting mutant XRCC1–DNA ligase III α heterodimer in BER reactions reconstituted with purified human proteins. We first demonstrated that the V86R mutation does not affect the DNA ligase activity of the mutated XRCC1m–DNA ligase III α heterodimer as measured with a nicked oligonucleotide duplex substrate (Fig. 1). We then used similar amounts of DNA ligase activity in reconstituted BER reactions and found that the XRCC1–DNA ligase III α heterodimer, which does not interact with Pol β , has reduced ability to support BER (Fig. 3). We further demonstrated that this reduced ability of mutated heterodimer to function as a DNA ligase in BER is correlated with a reduced ability of the mutated XRCC1 gene to rescue the DNA damage-sensitive phenotype of XRCC1-deficient cells (Fig. 4). Taken together, our data provide the first evidence that interaction of Pol β with XRCC1–DNA ligase III α heterodimer plays an important role in cellular defence against DNA damage by supporting efficient BER.

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