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Characterization of the XRCC1–DNA ligase III complex *in vitro* and its absence from mutant hamster cells

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ABSTRACT

The human DNA repair protein XRCC1 was overexpressed as a histidine-tagged polypeptide (denoted XRCC1-His) in *Escherichia coli* and purified in milligram quantities by affinity chromatography. XRCC1-His complemented the mutant Chinese hamster ovary cell line EM9 when constitutively expressed from a plasmid or when introduced by electroporation. XRCC1-His directly interacted with human DNA ligase III *in vitro* to form a complex that was resistant to 2 M NaCl. XRCC1-His interacted equally well with DNA ligase III from Bloom syndrome, HeLa and MRC5 cells, indicating that Bloom syndrome DNA ligase III is normal in this respect. Detection of DNA ligase III on far Western blots by radiolabelled XRCC1-His indicated that the level of the DNA ligase polypeptide was reduced ~4-fold in the mutant EM9 and also in EM-C11, a second member of the XRCC1 complementation group. Decreased levels of polypeptide thus account for most of the ~6-fold reduced DNA ligase III activity observed previously in EM9. Immunodetection of XRCC1 on Western blots revealed that the level of this polypeptide was also decreased in EM9 and EM-C11 (>10-fold), indicating that the XRCC1–DNA ligase III complex is much reduced in the two CHO mutants.

INTRODUCTION

The mutant Chinese hamster ovary cell lines EM9 and EM-C11 are hypersensitive to agents that induce DNA base damage, particularly the simple alkylating agents methyl- and ethylmethanesulphonate (1–4). EM9 and EM-C11 exhibit 10-fold elevated frequencies of spontaneous sister chromatid exchange (SCE) and are unable to efficiently rejoin DNA single-strand breaks that arise from treatment with alkylating agents and ionizing radiation (1,4). These phenotypes suggest a defect in DNA base excision repair in EM9 and EM-C11 at a step subsequent to enzymatic incision of the phosphodiester bond at the damaged nucleoside, but prior to or at DNA ligation. The human gene which fully corrects the repair defect in the mutants has been cloned and is denoted *XRCC1* (2). Consistent with its proposed role in single-strand break rejoining, XRCC1 protein is physically associated in mammalian cells with DNA ligase III (5). A human cDNA encoding DNA ligase III has been cloned and reveals the presence of a putative zinc finger near the N-terminus of the polypeptide (6). The zinc finger exhibits homology (30% identity, 50–60% similarity) with the two zinc fingers present in human poly(ADP-ribose) polymerase, both of which are involved in binding DNA strand breaks *in vitro* (7–9).

The biochemical role of XRCC1 within the XRCC1–DNA ligase III complex is unknown. The formation of DNA ligase III–adenylate intermediates is reduced 6-fold in EM9 and EM-C11, suggesting that XRCC1 is required for normal levels of DNA ligase III activity (5,10). However, none of the previously characterized eukaryotic, viral or prokaryotic DNA ligases require an associated polypeptide for activity and the extensive homology between the active sites of these enzymes and DNA ligase III suggests that this will also be the case for the latter enzyme. It is possible that the reduced formation of DNA ligase III–adenylate intermediates in EM9 results from a reduced amount of polypeptide, rather than reduced specific activity. There are several reports of multiprotein complexes in which a mutation in one component reduces the level of an associated component (11–15). To facilitate further studies to define the biochemical role(s) of XRCC1 we have overexpressed and affinity purified recombinant protein from *Escherichia coli*, isolated monoclonal antibodies specific for XRCC1 and demonstrated biological activity of the recombinant polypeptide following its introduction into EM9 cells by electroporation. We also report that the formation of reduced DNA ligase III–adenylate intermediates in EM9 and EM-C11 results largely from decreased levels of DNA ligase III protein and that the amount of XRCC1–DNA ligase III complex is greatly reduced in these cells. In addition, we have characterized recombinant XRCC1 for its ability to interact with human DNA ligase III from normal human cells and Bloom syndrome (BS) cells *in vitro*.

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MATERIALS AND METHODS

Mammalian cell lines

The CHO cell lines AA8, EM9 and EM-C11 have been described previously (1,4) and derivatives expressing human XRCC1 (EM9-XP and EM9-X1 to EM9-X5) were derived as described below. HeLa S3 cells, MRC5 human fibroblasts and the Bloom syndrome cell line GM08505 were obtained from the Imperial Cancer Research Fund (Cell Production) at the Clare Hall laboratories.

DNA constructs for expression of recombinant XRCC1 in mammalian cells

The mammalian expression construct pcD2EXH (Fig. 1A) encodes human XRCC1 protein that is affinity-tagged at the C-terminus with 10 histidine residues (denoted XRCC1-His) and which is transcribed from the SV40 early promoter present in the expression vector pcD2E (16). pcD2EXH was constructed by ligating a *Bst*XI–*Kpn*I 63mer duplex oligonucleotide (encoding eight amino acids present at the C-terminus of native XRCC1 plus the decahistidine tag) with a 0.8 kb *Sma*I–*Bst*XI fragment of the *XRCC1* open reading frame (ORF) from pcD2EX (5,17). The resulting *Sma*I–*Kpn*I fragment encoded the C-terminal half of XRCC1 plus the decahistidine tag encoded by the oligonucleotide. This fragment was ligated to a 5.6 kb *Sma*I–*Kpn*I fragment from pcD2EX containing the expression vector pcD2E and the N-terminal half of the *XRCC1* ORF (Fig. 1A). pcD2EXH was introduced into mutant EM9 cells by electroporation and DNA repair-proficient transfectants were selected as previously described in medium containing G418 (1.5 mg/ml) and chlorodeoxyuridine (5,17). The cell line EM9-X is a pooled population of 10 transfectants, whereas EM9-X1–EM9-X5 are independent transfectants.

DNA constructs for expression of recombinant XRCC1 in *E.coli*

Human XRCC1-His protein was expressed in the *E.coli* strain BL21(DE3) using the construct pET16BXH (Fig. 1B). To prepare this construct the *XRCC1*-His ORF was inserted into the expression vector pET16b at the *Nco*I site (Novagen, Madison, WI) by ligating the 2 kb *Dra*III–*Eco*RI fragment from pcD2EXH (see above), which encoded all but the N-terminus of XRCC1-His, to a 5.8 kb *Dra*III–*Esp*I fragment from the XRCC1 expression construct pET16BX (5). The latter fragment contained the pET16b vector plus the sequence encoding the N-terminus of XRCC1. The 3′ recessed *Eco*RI and *Esp*I termini were filled in with Klenow fragment to facilitate ligation.

The bacterial expression construct pET16BHX (Fig. 1C), which encodes XRCC1 containing an N-terminal decahistidine tag (His-XRCC1), was constructed by inserting the 2.1 kb *Eco*RI cassette from pcD2EX (containing the *XRCC1* ORF plus 27 bp of leader sequence; 5,16,17) in-frame into the *Nde*I site of pET16b (Fig. 1C). The encoded XRCC1 polypeptide thus contained an N-terminal tag of 19 amino acids comprised of 10 histidine residues encoded by the vector and 9 amino acids encoded by the 27 bp leader sequence. The 3′ recessed termini generated by *Eco*RI and *Nde*I were filled in with Klenow fragment to facilitate ligation.

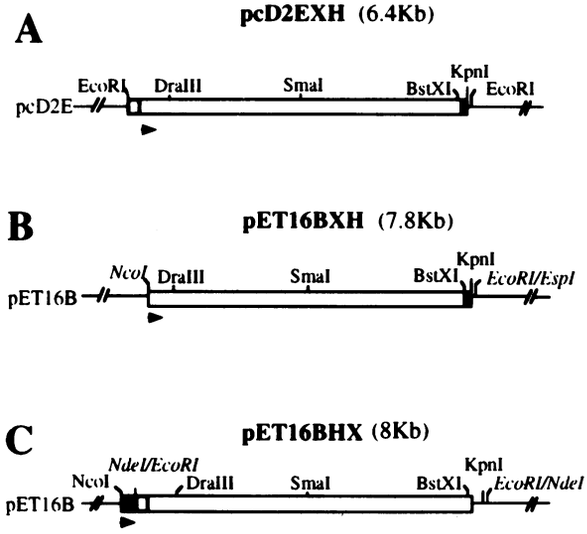


Figure 1. XRCC1 expression constructs. (**A**) pcD2EXH, a derivative of the pcD2E vector (16) encoding XRCC1-His (C-terminal tag) for expression in mammalian cells. (**B**) pET16BXH, a construct encoding XRCC1-His for expression in *E.coli*. (**C**) pET16BHX, a construct encoding His-XRCC1 (N-terminal tag) for expression in *E.coli*. Open bars, *XRCC1* ORF; solid bars, position of decahistidine tag; shaded bars, genomic *XRCC1* leader sequences; solid lines, vector sequences; arrows, beginning of ORF present in each construct. Restriction sites used during subcloning are shown and those altered during cloning are italicized. The size of the plasmid constructs are indicated in parentheses.

Purification of XRCC1 from *E.coli* by immobilized metal–chelate affinity chromatography (IMAC)

For expression of recombinant XRCC1 proteins 30 ml starter cultures containing ampicillin (0.1 mg/ml) were inoculated with BL21(DE3) cells harbouring the relevant plasmid (from frozen stocks), grown to mid-log phase (OD_{600} 0.6–1.0) and placed at 4°C overnight. Pelleted cells from the starter culture were used to inoculate 2 × 1 litre cultures (containing ampicillin) at a 1:100 dilution. XRCC1 expression was induced in mid-log phase cells (OD ~0.6) by the addition of IPTG to 1 mM. After 90 min cells were harvested and resuspended in 40 ml ice-cold sonication buffer (50 mM Hepes–NaOH, pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 10% glycerol). Cell suspensions (4 × 10 ml) were quick frozen, thawed on ice and imidazole, DTT and phenylmethylsulfonyl fluoride (PMSF) added to 1 mM. Cells were broken by sonication on ice (4 × 30 s with 30 s cooling intervals on ice) and cellular debris removed by centrifugation (14 000 *g*, 20 min, 4°C). The supernatant was added to a 3 ml bed volume of Ni–NTA–agarose (Qiagen, Chatsworth, CA) and stirred on ice for 1 h. The slurry was loaded into a 5 ml disposable column (Qiagen) at a flow rate of 2–3 ml/min and the flow-through (~40 ml) collected. The column was washed with 6 column volumes (CV; 2 × 10 ml) of sonication buffer and 9 CV (3 × 10 ml) of wash buffer (50 mM Hepes–NaOH, pH 7.0, 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) containing 40 mM imidazole, pH 8, at a flow rate of 0.5 ml/min, with 10 ml fractions collected. The column was washed further with 4 CV (1 × 15 ml) of wash buffer containing 80 mM imidazole and 4 CV of wash buffer containing 250 mM imidazole, with 1.5 ml fractions

collected. Equivalent proportions (by volume) of each fraction were analysed by SDS–PAGE. Protein concentrations were determined by the method of Bradford (18), using bovine serum albumin (BSA) as a standard. Typically XRCC1-His was purified at a yield of ~1 mg/l cells.

Mammalian cell extracts

Total cell extract from MRC5 fibroblasts, HeLa cells and the transformed BS cell line GMO8505 was prepared from 0.1–1.0 × 10^8 cells. Cell pellets were resuspended in 50 mM Tris–HCl, pH 8, 0.2 M NaCl, 0.1 mM EDTA and 1 mM DTT at a density of 1 × 10^8 cells/ml, protease inhibitors added (aprotinin at 2 μg/ml, PMSF at 1 mM and leupeptin, chymostatin, pepstatin and TLCK at 0.5 μg/ml) and the cell suspensions sonicated on ice (3 × 3 s bursts). Cell debris was pelleted in a microfuge (20 min at 4°C). The protein concentration of the clarified supernatant was typically 5.0–7.5 mg/ml. HeLa crude nuclear extract and partially purified HeLa DNA ligase III were kindly provided by P.Robins and M.Shivji. Partially purified DNA ligase III was prepared by successive fractionation of HeLa crude nuclear extract through phosphocellulose and single-stranded DNA–cellulose, as described (19).

Protein electroporation

EM9 cells (2 × 10^6) were electroporated in 1 ml Hepes-buffered saline in the absence or presence of the indicated amounts of protein as previously described for the electroporation of plasmid DNA (5,17). After electroporation 7 × 10^3 cells were plated into 10 cm dishes and incubated at 37°C for 2–21 h, as indicated in the text, after which plates were treated in triplicate with EMS for 1 h at the doses indicated. Untreated and treated cells were then rinsed with phosphate-buffered saline and incubated in fresh medium for ~10 days to allow formation of macroscopic colonies. Survival was calculated by dividing the mean number of colonies present on treated dishes by the mean number of colonies present on untreated dishes. Typically 200–300 colonies were present on the control dishes not treated with EMS.

SCE measurements and survival curves

SCE frequencies and percentage correction values were calculated as described previously (5,17). For survival curves 500 cells (wild-type AA8, EM9 or EM9-X cells) were plated into 10 cm Petri dishes and were treated 4 h later with EMS for 1 h at the indicated concentration. Treated cells and untreated control cells were then incubated in fresh media for 10 days to allow formation of macroscopic colonies. Survival was calculated as described above. Untreated control dishes typically contained 300–400 colonies.

Affinity precipitation of XRCC1-His

Forty microlitre reactions containing DNA ligase III from crude HeLa nuclear extract (50 μg total protein) or partially purified HeLa DNA ligase III (20 μg total protein), were incubated with 0–1 μg recombinant human XRCC1-His in buffer A (50 mM Tris–HCl, pH 8, 0.1 M NaCl, 2% glycerol, 1 mM DTT, 25 mM imidazole, pH 7.5). After 20 min at room temperature reactions were diluted 5-fold by addition of 160 μl buffer A and a 25 μl bed volume of NTA–agarose (Qiagen) was added. Reactions were incubated on ice with frequent gentle mixing. After 20 min a 35 μl sample (31 μl reaction mixture + 3.8 μl NTA–agarose) was removed, imidazole added to 250 mM final concentration and the aliquot stored on ice ('pre-affinity purification' sample). The NTA–agarose in the remaining reaction mixture was gently pelleted by centrifugation for 1 s at the lowest microfuge setting. The supernatant containing non-adsorbed material was removed (flow-through sample, FT) and the NTA–agarose beads washed with 3 × 190 μl buffer A. XRCC1-His was finally eluted from the NTA–agarose beads by 2 × 190 μl washes with buffer A containing 250 mM imidazole. Aliquots of 25 μl of the supernatants from each of the samples were examined for recovery of XRCC1-His protein (silver staining) and for DNA ligase III activity by adenylylation reactions.

To examine the interaction between BS DNA ligase III and XRCC1, binding reactions (250 μl) containing crude human cell extract from MRC5, HeLa or BS cells (250 μg total protein) were incubated with or without 5 μg recombinant human XRCC1-His at room temperature in 50 mM Tris–HCl, pH 8, 0.2 M NaCl, 0.1 mM EDTA, 1 mM DTT and 12.5 mM imidazole. After 25 min undiluted reactions were processed as described above except that the beads were washed with 4 × 200 μl wash buffer (50 mM Tris–HCl, pH 8, 0.05 M NaCl, 1 mM DTT, 12.5 mM imidazole). NTA–agarose-bound XRCC1-His was eluted by 2 × 50 μl washes with wash buffer containing 250 mM imidazole. Aliquots of 27 μl of the eluates were examined for the presence of DNA ligase III activity by adenylylation reactions.

Immunodetection of XRCC1

Proteins were fractionated through 7.5% SDS–PAGE minigels and electroblotted onto Immobilon P polyvinylidene difluoride membrane (Millipore) in the presence of 25 mM Tris, 192 mM glycine. Membranes were blocked and washed according to the manufacturer's recommendations, with non-fat dried milk (Marvel) used as a blocking agent. Membranes were incubated with hybridoma culture medium containing anti-human XRCC1 monoclonal antibodies (mAb 33-2-5) at 1:100 dilution and subsequently alkaline phosphatase-conjugated goat anti-mouse IgG monoclonal antibody at a dilution of 1:3000 (BioRad) for 2 h at room temperature. Immune complexes were visualized by standard colourimetric procedures.

Immunoprecipitation of XRCC1

Sixty microlitre reactions containing crude HeLa nuclear protein (30 μg), 2 μl hybridoma culture medium (anti-XRCC1 mAb 33-2-5), 40 mM Tris–HCl, pH 7.5, 0.1 M KCl, 0.15 mM EDTA and 2 mM DTT or 200 μl reactions containing 20 μg CHO cell extract protein, 20 μl hybridoma culture medium, 50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 0.2 mM EDTA, 1 mM DTT and 2% glycerol were incubated for 60 min on ice. Five microlitre (bed volume) each of protein A–Sepharose and protein G–Sepharose (Pharmacia LKB) were added and incubations continued for 30 min with frequent mixing. Aliquots (10% v/v) of the final suspension were removed as pre-immunoprecipitation samples (Pre-IP) and the remaining suspension centrifuged in a microfuge for 1 s. Following removal of the supernatant the pelleted protein A/protein G beads were washed with 5 × 100 μl wash buffer (50 mM Tris–HCl, pH 7.5, 25 mM NaCl, 1 mM DTT) and finally resuspended in 20 μl wash buffer. Aliquots were examined for DNA ligase activity by adenylylation.

Far Western blotting

Cell extract proteins fractionated by SDS–PAGE were electroblotted onto nitrocellulose paper and were processed for the far Western technique as described (6), using XRCC1-His phosphorylated by casein kinase II (Boehringer Mannheim) in the presence of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham). Washed and dried blots were analysed on a phosphorimager (Molecular Dynamics).

DNA ligase adenylylation assays

Reactions (30 µl) were incubated for 20 min at room temperature and stopped by addition of SDS–PAGE loading buffer. Reactions contained 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 25–100 mM NaCl, 3 µCi [α-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml; Amersham) and aliquots of affinity-purified, immunoprecipitated or crude cell extract protein. Samples were separated by SDS–PAGE and gels silver stained or analysed on a phosphorimager.

RESULTS

Purification of recombinant human XRCC1-His from *E.coli*

Initial attempts to affinity purify from *E.coli* full-length XRCC1 tagged at the N-terminus with polyhistidine (His-XRCC1) or maltose binding protein (MBP-XRCC1) resulted in co-purification of truncated products (Fig. 2C). Subsequent experiments suggested that the truncated polypeptides were incomplete translation products, rather than products of proteolysis (results not shown). To prevent affinity purification of the incomplete translation products XRCC1 was expressed as a polypeptide containing a C-terminal decahistidine tag (Fig. 1B; pET16BXH). The resulting histidine-tagged polypeptide, denoted XRCC1-His, comprised ~1% total cellular protein 90 min after its expression was induced (Fig. 2A) and was purified by immobilized metal–chelate affinity chromatography (IMAC) largely as a single polypeptide of 85 kDa (Fig 2B and C). Although native XRCC1 is 69.5 kDa, we have previously shown that the full-length polypeptide migrates during SDS–PAGE as a polypeptide of ~85 kDa (5). The slower migration of full-length His-XRCC1 and MBP-XRCC1 during SDS–PAGE (Fig. 2C), compared with XRCC1-His, reflects the extra residues incorporated as a part of the fusion moieties during subcloning (see Materials and Methods).

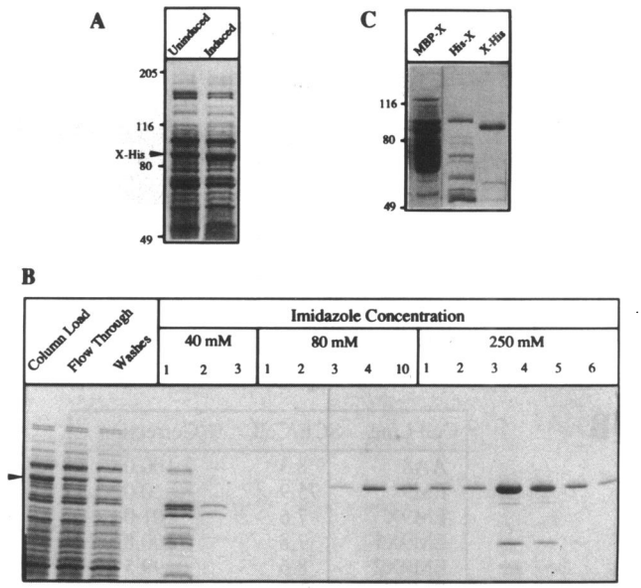
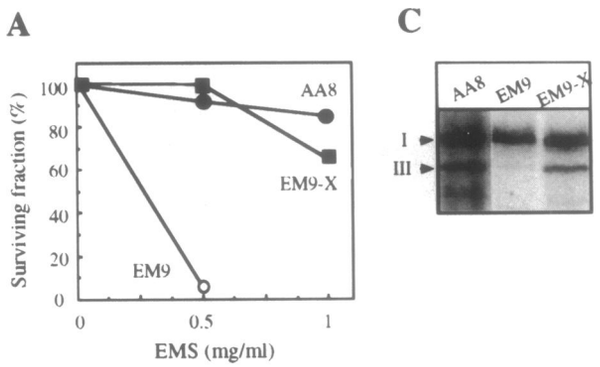


Figure 2. Purification of His-tagged human XRCC1 from *E.coli*. (**A**) Expression of XRCC1-His in *E.coli*. BL21(DE3) cells harbouring pET16BXH were sampled prior to and 90 min after addition of IPTG (1 mM final) and lysed by addition of hot SDS–PAGE loading buffer. Total protein from equivalent cell numbers was fractionated by SDS–PAGE and stained with Coomassie blue. The position of XRCC1-His protein is indicated. (**B**) XRCC1-His was purified from 160 mg soluble cell extract protein by IMAC. Aliquots of 5 µl of the column load and column flow-through, 10 µl of column washes with sonication buffer (Washes), 15 µl of three column washes with 40 mM imidazole and 3 µl of alternate 1.5 ml fractions collected during column washes with 80 and 250 mM imidazole were fractionated by SDS–PAGE and stained with Coomassie blue. The position of XRCC1-His is indicated (small arrow). (**C**) Comparison of IMAC-purified XRCC1 fusion proteins MBP-XRCC1, His-XRCC1 and XRCC1-His. MBP-XRCC1 (5 µg), His-XRCC1 (1 µg) and XRCC1-His (1 µg) were fractionated by SDS–PAGE and stained with Coomassie blue.

Correction of EM9 cells by recombinant XRCC1-His

To test whether the C-terminal histidine tag was detrimental to the activity of XRCC1 we examined XRCC1-His for its ability to correct EM9 cells when expressed constitutively in these cells from the construct pcD2EXH (Fig. 1A; see Materials and Methods). XRCC1-His appeared to fully correct the EM9 phenotypes of sensitivity to killing by EMS (Fig. 3A), high SCE (Fig. 3B) and reduced adenylylation activity of DNA ligase III (Fig. 3C), indicating that the C-terminal tag does not impair XRCC1 activity. We have previously shown that an N-terminal histidine tag similarly does not interfere with XRCC1 activity (5). We next wished to establish an assay by which the biological activity of XRCC1 polypeptides purified from bacteria could be determined *in vitro*. Attempts to complement the DNA ligase III defect *in vitro* by addition of recombinant XRCC1 to EM9 cell extract were unsuccessful (results not shown). Therefore, the ability of recombinant XRCC1 to correct the sensitivity of EM9 cells to EMS was examined following its introduction into the mutant cells by electroporation. EM9 cells were electroporated in the presence or absence of XRCC1-His and then challenged with EMS 4 h later. XRCC1-His conferred significant resistance to the mutagen on EM9 cells, whereas electroporation in the absence of protein or in the presence of BSA did not (Fig. 4, top). The degree of resistance conferred by XRCC1-His following electroporation was close to that conferred by stable expression of XRCC1-His from transfected plasmid pcD2EXH (EM9-X; reproduced from Fig. 3A for comparison). Correction was also seen when His-XRCC1 or MBP-XRCC1 were introduced by electroporation, while MBP protein alone failed to enhance survival. Survival was dependent on the concentration of XRCC1-His during electroporation (Fig. 4, bottom left) over the range examined. Varying the time interval between electroporation and EMS treatment from 2 to 21 h had little effect, suggesting that the electroporated protein was relatively stable following its entry into EM9 cells (Fig. 4, bottom right). These results show that



B

Cell Line	SCE/Cell	% Correction
AA8	8.3	100.0
EM9	75.9	0.0
EM9X	7.6	101.0
EM9X1	7.8	100.8
EM9X2	8.6	99.5
EM9X3	5.9	103.6
EM9X4	9.9	97.6
EM9X5	7.8	100.8

Figure 3. Correction of EMS sensitivity, DNA ligase III activity and SCE in EM9 cells expressing human XRCC1-His. (**A**) AA8 cells (closed circles), EM9 cells (open circles) or EM9-X cells (EM9 cells constitutively expressing XRCC1-His; closed squares) were plated into Petri dishes and 4 h later treated with EMS for 1 h. The fraction of cells surviving relative to untreated dishes is presented. (**B**) Frequencies of SCE were determined from 50 metaphase cells for each of the cell lines AA8, EM9, EM9-X and EM9-X1–EM9-X5. EM9-X is a pooled population of 10 transformants, whereas EM9-X1–EM9-X5 are independent clonal isolates. (**C**) Cell extract protein (0.87 μg) from AA8, EM9 or EM9-X was incubated with [α-^{32}P]ATP to adenylylate DNA ligase polypeptides and fractionated by SDS–PAGE. Dried gels were analysed on a phosphorimager.

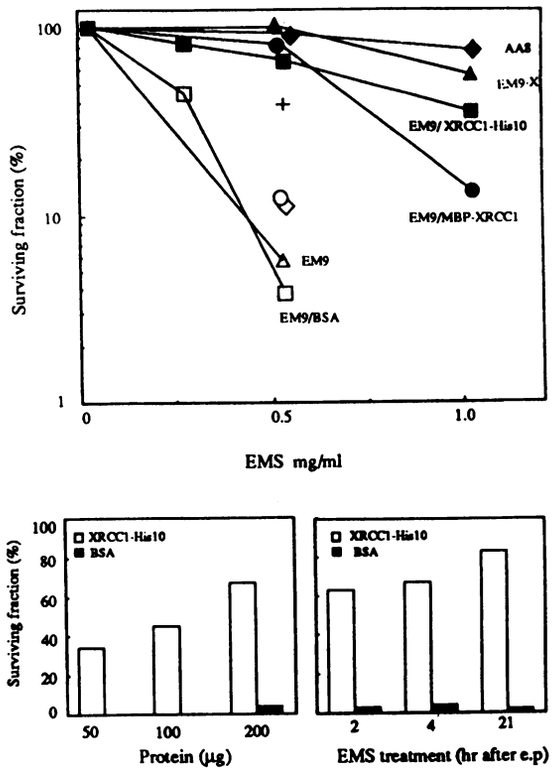


Figure 4. Functionality of recombinant XRCC1. (Upper) EM9 cells (2×10^6) were electroporated in a volume of 1 ml either in the absence of added protein (open diamonds) or in the presence of 200 μg XRCC1-His (closed triangles), 200 μg His-XRCC1 (crosses), 400 μg MBP-XRCC1 (closed circles), 400 μg MBP (open circles) or 200 μg BSA (open squares). Electroporated cells were plated immediately and treated 4 h later with EMS for 1 h. Survival curves in the absence of electroporation for AA8 cells (closed diamonds), EM9 cells (open triangles) and EM9-X cells (closed triangles) were taken from Figure 2 for comparison. (Lower left) Survival of EM9 cells electroporated in the presence of the indicated amount of XRCC1-His (open bars) or BSA (closed bars) and treated with 0.5 mg/ml EMS 4 h later. (Lower right) Survival of EM9 cells electroporated in the presence of 200 μg XRCC1-His (open bars) or BSA (closed bars) and exposed to 0.5 mg/ml EMS at the indicated times after electroporation.

XRCC1-His, His-XRCC1 and MBP-XRCC1 are biologically active proteins upon entry into the cellular environment.

Anti-XRCC1 monoclonal antibodies

Initial attempts to raise anti-XRCC1 mAbs against recombinant His-XRCC1 protein resulted in the isolation of four mouse hybridomas expressing anti-XRCC1 mAbs. Further characterization of one of the mAbs, 33-2-5, revealed that the antibody detected an 85 kDa polypeptide corresponding to XRCC1 on Western blots of human cell extract (Fig. 5A). XRCC1 was predominantly located in the nuclear fraction of cell extract isolated from Hela cells. 33-2-5 was also able to immunoprecipitate recombinant XRCC1-His from solution (data not shown) and to co-immunoprecipitate DNA ligase III from HeLa nuclear extract (Fig. 5B). The characteristic 87 kDa proteolytic fragment of DNA ligase III was also immunoprecipitated, indicating that this fragment contains a binding site for XRCC1.

Interaction between XRCC1-His and DNA ligase III from HeLa, MRC5 and BS cells *in vitro*

We showed previously that human XRCC1 is present in a complex with rodent DNA ligase III when expressed heterologously in CHO cells (5). We therefore wished to characterize purified recombinant XRCC1-His for its ability to interact with human DNA ligase III *in vitro*. A rapid affinity precipitation assay was employed in which small amounts of XRCC1-His and NTA–agarose affinity beads were added to human cell extract to form agarose–XRCC1–DNA ligase III complexes, which were subsequently recovered by centrifugation. DNA ligase molecules present in the complexes were adenylylated by incubation with [α-^{32}P]ATP, fractionated by electrophoresis and subsequently detected by autoradiography. XRCC1-His associated with DNA ligase III when incubated with either the partially purified enzyme (Fig. 6A) or with crude nuclear extract (Fig. 6B), as indicated by the partial depletion of DNA ligase III activity from the bead flow-through (FT) and its recovery in the imidazole eluate (beads). Typically 30–60% of DNA ligase III was recovered by NTA–agarose in the presence of small amounts of exogenous XRCC1-His (1–5% w/w total protein), but was not recovered in

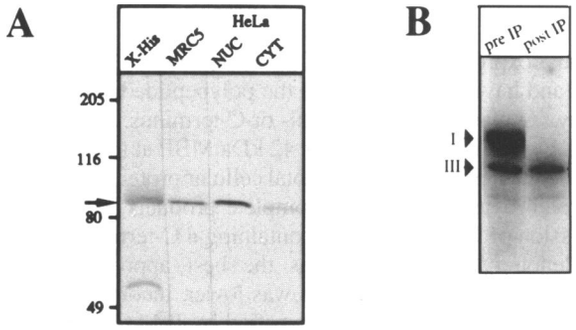


Figure 5. XRCC1-specific monoclonal antibodies. (**A**) Immunoblot of affinity-purified XRCC1-His (0.6 μg), MRC5 cell extract protein (10 μg) and cell equivalent amounts of HeLa nuclear and cytoplasmic protein (5 and 30 μg respectively) with the anti-XRCC1 mAb 33-2-5. (**B**) Immunoprecipitation of DNA ligase III by anti-XRCC1 mAb 33-2-5. HeLa crude nuclear extract (3 μg protein, Pre-IP) and proteins immunoprecipitated from HeLa crude nuclear extract (30 μg protein, Post-IP) by mAb 33-2-5 were assayed for DNA ligases by adenylylation and fractionated by SDS–PAGE. I and III denote adenylylated DNA ligases I and III respectively.

the absence of XRCC1-His (Fig. 6A, lanes 9–12). Consistent with the immunoprecipitation experiments described above, the characteristic 87 kDa proteolytic fragment of DNA ligase III (20) was also recovered by XRCC1 (Fig 6B). Washing preformed XRCC1–DNA ligase III complex with 1 or 2 M NaCl did not disrupt the association of the two proteins, indicating that their interaction is salt stable (Fig. 6C).

The affinity precipitation assay was also employed to examine DNA ligase III from BS cells, which exhibit some phenotypic similarities to EM9 (21,22), for its ability to bind XRCC1. XRCC1-His recovered comparable levels of DNA ligase III activity from crude cell extracts of the BS cell line GM08505 and MRC5 and HeLa cells (Fig. 6D). Since these cell lines displayed similar levels of DNA ligase III activity prior to affinity precipitation with XRCC1-His (results not shown), these data indicate that there is no gross defect in DNA ligase III from BS cells in its ability to interact with XRCC1.

Absence of XRCC1–DNA ligase III complex in CHO mutants EM9 and EM-C11

DNA ligase III activity, as measured by adenylylation, is reduced ~6-fold in the mutant CHO cell lines EM9 and EM-C11, both of which are members of the XRCC1 complementation group (5,10). Reduced DNA ligase III activity in these mutants could result from reduced specific activity of the enzyme or from reduced levels of polypeptide. The inability of recombinant XRCC1 to complement the DNA ligase defect in mutant cell extracts *in vitro* is consistent with the latter possibility, since the synthesis of nascent DNA ligase III polypeptide would be required for complementation. Therefore, we wished to compare the level of DNA ligase III present in wild-type cell extracts with that present in EM9 and EM-C11 cell extracts. We have shown previously that radiolabelled recombinant XRCC1 can be used as a probe to detect DNA ligase III on far Western blots of human nuclear extract (6). This approach was adopted to quantitate the level of DNA ligase III in EM9 and EM-C11 (Fig. 7A, bottom). The amount of DNA ligase III detected in EM9 and EM-C11 cells

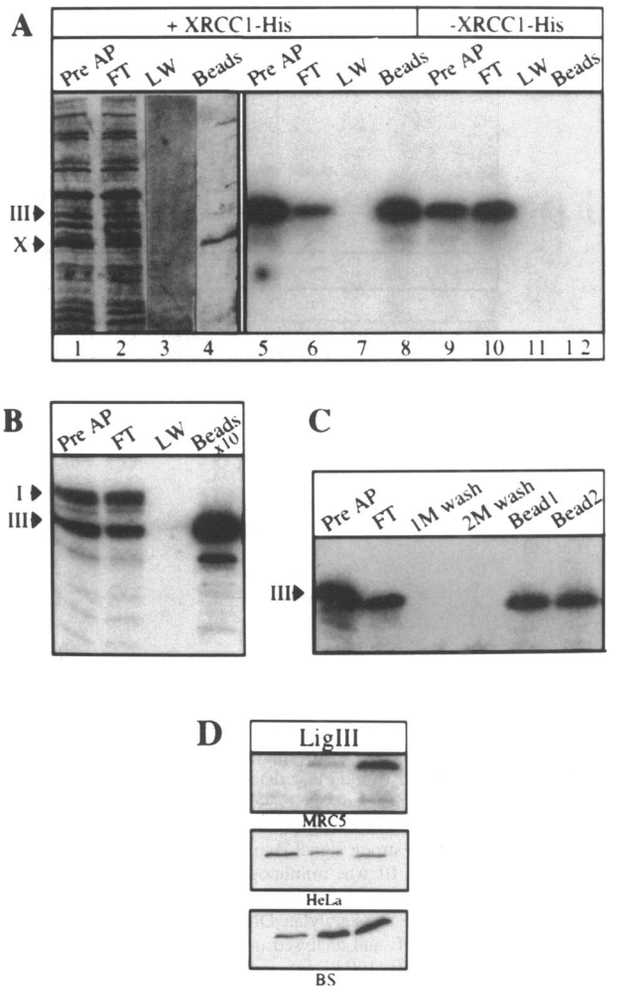


Figure 6. Affinity precipitation of DNA ligase III from human cell extract by XRCC1-His. (**A**) Partially purified HeLa DNA ligase III (20 μg total protein) was incubated with NTA–agarose beads and with (lanes 1–8) or without (lanes 9–12) XRCC1-His (1 μg). An aliquot of this suspension was removed (Pre-AP sample) and the remaining beads pelleted. The supernatant was removed (FT sample, i.e. flow-through) and the beads extensively washed prior to elution of tightly bound proteins with two imidazole washes (250 mM). Aliquots of the Pre-AP and FT samples and of the final bead wash (LW) and imidazole eluate (beads), were incubated with [α-^{32}P]ATP to adenylylate DNA ligase III polypeptides and subjected to SDS–PAGE. Gels were either silver stained to detect XRCC1 polypeptides (lanes 1–4) or were dried and analysed on a phosphorimager (lanes 5–12). The positions of DNA ligase III (III) and XRCC1-His (X) are marked by arrows. (**B**) HeLa crude nuclear extract (200 μg protein) was incubated with XRCC1-His (2 μg) and NTA–agarose beads and processed as described above. Lanes 1 and 2 contain aliquots (10% of total) of the initial suspension (Pre-AP) and supernatant from the pelleted beads (FT) respectively. Lanes 3 and 4 contain all of the last wash (LW) of the remaining beads and the imidazole eluate from these beads (beads ×10) respectively. The positions of DNA ligases I and III are indicated. (**C**) As in (A) except that after Pre-AP and FT samples were taken the NTA–agarose beads were divided and washed with either 1 or 2 M NaCl prior to recovery of bound proteins with 250 mM imidazole. Pre-AP and FT samples and aliquots of the high salt washes (1 M Wash and 2 M Wash) and imidazole eluates (Bead1 and Bead2, for beads washed with 1 or 2 M NaCl respectively) were incubated with [α-^{32}P]ATP and processed as described above. (**D**) Recovery of DNA ligase III from MRC5, HeLa and BS cell extracts by XRCC1-His. Protein from MRC5, HeLa or GM08505 cell extract (250 μg protein) was incubated with XRCC1-His (5 μg) and NTA–agarose beads and processed as described above. XRCC1-His was eluted from the beads with three 250 mM imidazole washes, aliquots of which were analysed for the presence of DNA ligase III as above.

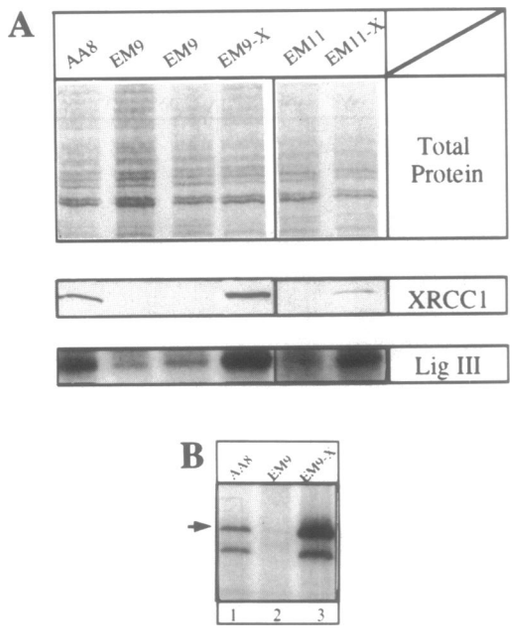


Figure 7. Reduced XRCC1 and ligase III proteins in the CHO mutants EM9 and EM-C11. (**A**) Lanes 1–6 respectively, 10 μg cell extract protein from AA8, EM9 (two preparations), EM9-X (EM9-X), EM-C11 (EM11) and EM-C11X (EM11-X) were fractionated by SDS–PAGE and either stained with Coomassie blue (top), electroblotted onto PVDF membrane for immunodetection of XRCC1 with mAb 33-2-5 (middle) or detection of DNA ligase III by far Western blotting with radiolabeled XRCC1-His (bottom). Lanes 4 and 6 of the middle panel were incubated in visualization solution for a shorter period than the other lanes, due to the strong signal resulting from overexpressed human XRCC1. (**B**) DNA ligase III was immunoprecipitated from AA8, EM9 or EM9-XH cell extract (20 μg total protein) with anti-XRCC1 mAb 33-2-5 and incubated with [α-^{32}P]ATP to adenylylate DNA ligases. Protein samples were fractionated by SDS–PAGE and analysed on a phosphorimager. Full-length adenylylated DNA ligase III (100 kDa) is indicated (arrow).

was reduced ~4-fold compared with wild-type AA8 cells and to corrected mutant cells expressing the human XRCC1 cDNA (EM9-X and EM11-X), suggesting that DNA ligase III polypeptide levels are reduced in the mutants. This notion was subsequently confirmed by immunoblotting with polyclonal antibodies raised against recombinant DNA ligase III (antibody TL-25; results not shown). These data indicate that the reduced formation (6-fold) of DNA ligase III–adenylate intermediates by EM9 cell extract is due largely to reduced levels of the polypeptide. Levels of XRCC1 were also greatly reduced on immunoblots of EM9 and EM-C11 cell extracts, as detected by anti-XRCC1 mAb (Fig. 7A, middle, and results not shown). Quantitative Western blots revealed that the amount of XRCC1 protein was reduced >10-fold in the mutants (results not shown). Reduced levels of XRCC1 and DNA ligase III suggest that the XRCC1–DNA ligase III complex is largely absent from EM9 and EM-C11 cells. This notion was confirmed by co-immunoprecipitation of 10-fold less DNA ligase III activity, as measured by adenylylation, from EM9 cell extract than from AA8 cell extract by the mAb 33-2-5 (Fig. 7B, lanes 1 and 2). The lower of the two immunoprecipitated bands represents the 87 kDa proteolytic fragment of DNA ligase III, again indicating that this fragment contains a binding site for XRCC1.

DISCUSSION

We have expressed the human DNA repair protein XRCC1 in *E.coli* and have affinity purified the polypeptide by virtue of an affinity tag placed at either the N- or C-terminus. Expression of XRCC1 as a fusion protein with 42 kDa MBP at the N-terminus gave the greatest yield (30% of total cellular protein), but most of the protein was purified as incomplete products of translation. Expression of XRCC1 protein containing a C-terminal histidine tag (denoted XRCC1-His) was the best approach, because although the level of expression was lower, incomplete translation products were not affinity purified by IMAC. XRCC1-His complemented the EM9 phenotypes of elevated SCE, elevated sensitivity to EMS and reduced DNA ligase III activity when expressed constitutively from the construct pcD2EXH, indicating that the C-terminal histidine tag is not detrimental to XRCC1 activity. Affinity-purified XRCC1-His also complemented the sensitivity of EM9 cells to EMS when the protein was introduced by electroporation. The recombinant protein was able to confer resistance to EMS for at least 24 h after electroporation, indicating that it was stable in the cellular environment. This assay should allow comparison of the activities of different XRCC1 polypeptides modified *in vitro* by, for example, phosphorylation.

Using an affinity precipitation assay we have shown that recombinant XRCC1-His interacts efficiently with human DNA ligase III in a cell-free extract. The interaction was very stable, as indicated by its resistance to 2 M NaCl. We are currently using this assay to identify the motifs present in XRCC1 and DNA ligase III that are required for interaction. The affinity precipitation assay was also used to compare DNA ligase III from various cell lines for its ability to bind XRCC1. Particular cell lines of interest are those derived from individuals with the cancer-prone disorder BS. Similar to EM9 cells, BS cells exhibit sensitivity to alkylating agents and greatly elevated frequencies of SCE (21,22). In addition, a defect in DNA ligase activity in BS cells has been reported (23,24). A number of possibilities could account for the similarity between these cell lines. For example, although it is genetically distinct from both XRCC1 and DNA ligase III (6,25–27), the wild type BS gene product may modify DNA ligase III to allow interaction with XRCC1 or may be a component of the XRCC1–DNA ligase III complex. The latter possibility is the most likely of the two, since we have shown in this report that DNA ligase III polypeptides present in BS, HeLa and MRC5 cell extracts appear to interact equally well with recombinant XRCC1.

Levels of DNA ligase III polypeptide were found to be reduced ~4-fold in EM9 and EM-C11 cell extracts, thus explaining the 6-fold reduction in DNA ligase III adenylylation observed previously in these cells (5). The level of XRCC1 polypeptide was also greatly decreased in EM9 and EM-C11 cells (~10-fold), suggesting that the XRCC1–DNA ligase III complex is greatly reduced in the mutants. This was confirmed by the observed inability of XRCC1-specific mAbs to co-immunoprecipitate XRCC1 and DNA ligase III activity from EM9 cell extracts. The simplest explanation for the reduced presence of XRCC1–DNA ligase III complex in EM9 and EM-C11 cells is that the putative XRCC1 mutation in these cells destabilizes the complex such that the component subunits are degraded. The phenotypic complementation of EM9 cells by recombinant XRCC1-His most likely reflects association of the protein with nascent DNA ligase III and consequent formation of stable complexes *in vivo*. It is not yet

known whether complementation of EM9 cells by recombinant XRCC1 is simply the result of restoring normal levels of DNA ligase III polypeptide and activity or whether XRCC1 has a more direct role in DNA strand break repair. For example, XRCC1 may specifically target DNA ligase III to DNA damage or may fulfil a role that is not directly related to its association with DNA ligase. The availability of recombinant proteins, specific antibodies and the *in vitro* assays described here should help identify the biochemical role(s) of XRCC1.

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