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An Interaction between the Mammalian DNA Repair Protein XRCC1 and DNA Ligase III

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***XRCC1*, the human gene that fully corrects the Chinese hamster ovary DNA repair mutant EM9, encodes a protein involved in the rejoining of DNA single-strand breaks that arise following treatment with alkylating agents or ionizing radiation. In this study, a cDNA minigene encoding oligohistidine-tagged XRCC1 was constructed to facilitate affinity purification of the recombinant protein. This construct, designated pcD2EHX, fully corrected the EM9 phenotype of high sister chromatid exchange, indicating that the histidine tag was not detrimental to XRCC1 activity. Affinity chromatography of extract from EM9 cells transfected with pcD2EHX resulted in the copurification of histidine-tagged XRCC1 and DNA ligase III activity. Neither XRCC1 or DNA ligase III activity was purified during affinity chromatography of extract from EM9 cells transfected with pcD2EX, a cDNA minigene that encodes untagged XRCC1, or extract from wild-type AA8 or untransfected EM9 cells. The copurification of DNA ligase III activity with histidine-tagged XRCC1 suggests that the two proteins are present in the cell as a complex. Furthermore, DNA ligase III activity was present at lower levels in EM9 cells than in AA8 cells and was returned to normal levels in EM9 cells transfected with pcD2EHX or pcD2EX. These findings indicate that XRCC1 is required for normal levels of DNA ligase III activity, and they implicate a major role for this DNA ligase in DNA base excision repair in mammalian cells.**

The Chinese hamster ovary (CHO) cell mutant EM9 is hypersensitive to ethyl methanesulfonate (EMS) (10-fold) and ionizing radiation (1.8-fold), and it is unable to grow in medium containing chlorodeoxyuridine (CldUrd) under conditions in which 20% of genomic Thy is replaced by chlorouracil during DNA replication (9, 29). EM9 repairs γ-ray and EMS-induced single-strand breaks at a reduced rate and exhibits a 10-fold increase in the occurrence of sister chromatid exchange (SCE) (27, 28). The sensitivity of EM9 to alkylating agents is suggestive of a defect in the base excision repair pathway, which involves sequential action by DNA glycosylase, apurinic-apyrimidinic endonuclease, deoxyribose-phosphodiesterase, DNA polymerase, and DNA ligase activities (17). The reduced rate of single-strand break rejoining suggests that the defect in EM9 lies within a postincision step of this pathway. EM9 is phenotypically similar to cells derived from individuals with Bloom's syndrome (BS), a cancer-prone autosomal recessive disorder characterized by high SCEs (10-fold) and sensitivity to alkylating agents (4, 14, 15). Although chromosome localization and somatic cell hybrid complementation studies indicate that the EM9 and BS defects are genetically distinct (18, 21, 23), the two gene products most likely function in the same, or a closely related, biochemical pathway. The phenotype of both EM9 and BS cells might be explained by a defect in one or more of the three DNA ligases so far identified in mammalian cells, which are designated DNA ligases I, II, and III, respectively (24, 31). DNA ligase I plays a major role in DNA replication but also appears to be involved in the repair of DNA damage caused by a variety of agents, including alkylating agents (reference 1 and references therein), and DNA ligase II has been proposed to play a role both in DNA repair and DNA recombination (31). As yet, no specific role has been identified for DNA ligase III (31). Altered DNA ligase activity in BS cells has been observed in several studies, in which it was proposed that the activity of DNA ligase I was affected (5, 6, 33, 34), but no major abnormality in DNA ligase activity was found in the one reported study in which EM9 was examined (7). However, only DNA ligase II activity was conclusively shown to be normal in EM9 in this study, since DNA ligase I and III activities were not distinguished from each other. Thus, it is possible that a specific defect in one of these enzymes was masked by the combined level of DNA ligase I and III activities that was measured in the study.

The human gene that corrects the repair defect in EM9 has been cloned and is designated *XRCC1* (28). The gene is 33 kb in length and encodes a 2.2-kb transcript and a corresponding protein of 633 amino acids (69.5 kDa). To isolate XRCC1 protein and address its biochemical role in DNA strand break repair, fully functional *XRCC1* cDNA minigenes have been constructed to allow overexpression of XRCC1 in mammalian and prokaryotic cells (3). In this work, we describe the expression of oligohistidine-tagged XRCC1 in EM9 cells and present results suggesting that a DNA ligase activity is indeed affected in EM9. This study demonstrates that XRCC1 is required for normal levels of DNA ligase III activity and strongly suggests that these two proteins are physically associated within the cell.

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MATERIALS AND METHODS

Cell lines and culture conditions. Isolation of the *XRCC1* CHO mutants EM9 and EM-C11, as well as the conditions

used for the routine propagation of these cell lines and their transfection derivatives, have been described elsewhere (27, 28, 37). *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.) was used for routine maintenance and manipulation of plasmids; the expression hosts for pET16BX were *E. coli* HMS174(DE3) and BL21(DE3) (Novagen, Madison, Wis.) (25, 26). The DE3 lysogen present in these cell lines encodes T7 RNA polymerase under the control of the P_{tac} inducible (by isopropylthiogalactopyranoside [IPTG]) promoter. Unless stated otherwise, *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C (22).

XRCC1 expression constructs and oligonucleotides. The mammalian expression construct pcD2EHX contains the full-length *XRCC1* open reading frame (ORF) and was constructed as described previously for pcD2EX, using a pair of complementary oligonucleotides to complete the coding region at the 5′ end (3). pcD2EHX differs from pcD2EX only by the presence of an additional 18 bp that are located near the 5′ terminus of the *XRCC1* ORF and which encode a hexahistidine tag to facilitate affinity purification (Fig. 1). pcD2EHCX was constructed in the same way, but by using a pair of oligonucleotides that contained an additional 27 bp located immediately downstream of the histidine tag. The 27-bp region encodes a well-defined nine-amino-acid epitope from the influenza virus hemagglutinin protein, which is recognized by monoclonal antibody 12CA5 (35). The complementary oligonucleotides used to construct pcD2EHX and pcD2EHCX were generated on an automated synthesizer and were purified with NENSORB cartridges as directed by the manufacturer (DuPont/NEN Research Products, Boston, Mass.).

The prokaryotic expression construct pET16BX was generated by ligating an oligonucleotide duplex encoding the first 37 bp of the *XRCC1* ORF to a truncated cDNA fragment encoding the remainder of the *XRCC1* ORF and by inserting the resulting fragment into the *Nco*I site (ATG cloning site) of the expression vector pET16b (Novagen). No additional amino acids are present in the XRCC1 protein expressed from this construct (Fig. 1). For expression of XRCC1 from pET16BX, 10- to 20-ml starter cultures containing ampicillin (0.1 mg/ml) were inoculated with HMS174(DE3) or BL21 (DE3) cells harboring pET16BX (from a fresh colony or directly from frozen stocks), grown to mid-log phase (optical density at 600 nm of 0.6 to 1.0), and placed at 4°C overnight. Cells from the starter culture were pelleted and resuspended in an equal volume of medium (plus ampicillin), and aliquots were used to inoculate 0.1- to 1-liter cultures (plus ampicillin) at a 1:100 dilution. XRCC1 expression was induced in mid-log phase cells (optical density of ~0.6) by the addition of IPTG to 1 mM.

For specific radiolabeling of XRCC1, BL21(DE3) cells harboring pET16BX were cultured as described above (with the exception that M9 broth [25] was used instead of LB) and were treated with rifampin (Sigma, St. Louis, Mo.) at 0.2 mg/ml 30 min after induction to inhibit *E. coli* RNA polymerase, thus allowing selective expression of XRCC1 from the T7 promoter present in pET16BX (25, 26). Cell samples (0.5 ml) were subsequently pulse-labeled for 15 min with 5 μCi of [^{35}S]methionine (1,000 Ci/mmol, 10 mCi/ml; Amersham Corp., Chicago, Ill.) at the times indicated. Pelleted cells from these samples were frozen at −20°C until needed, when they were lysed by the addition of hot sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer, and aliquots were subjected to electrophoresis through SDS–7.5% polyacrylamide gels. Gels were fixed in 10% acetic acid for 10 min, stained with Coomassie blue,

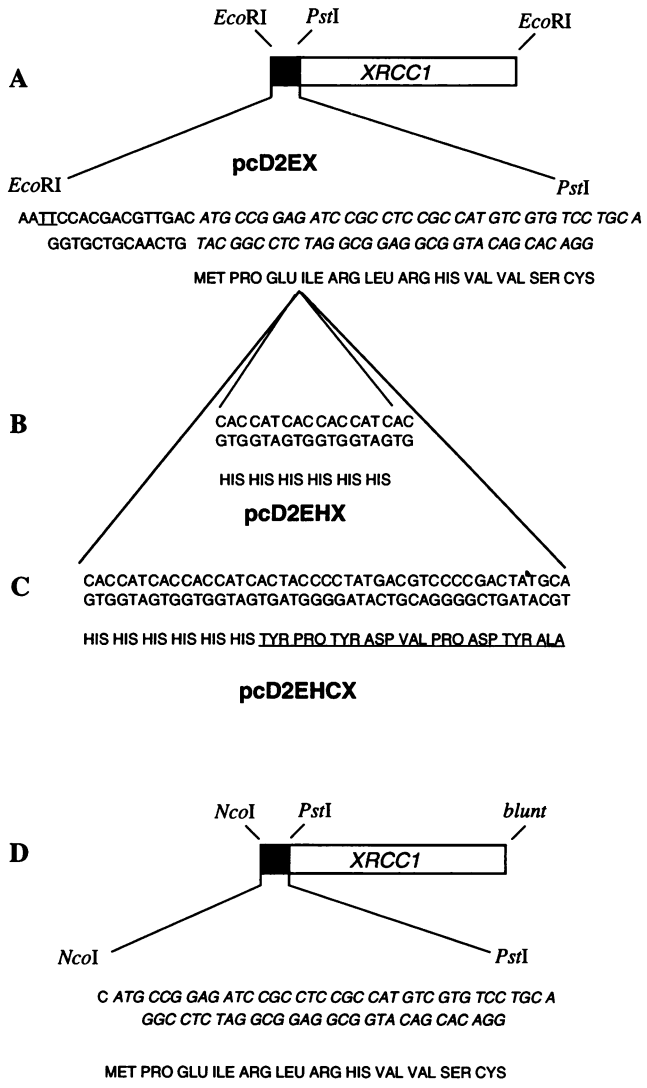

FIG. 1. Schematic showing the construction of the *XRCC1* expression constructs used in this study. (A) The oligonucleotide duplex used previously to reconstruct the full-length *XRCC1* ORF present in pcD2EX. The upper oligonucleotide encodes 17 bases of leader sequence and the first 37 bases of the ORF (italics). The 17-base leader sequence differs from the native *XRCC1* leader sequence by two bases at the 5′ end of the oligonucleotide, where GpC was replaced by TpT in the oligonucleotide to generate an *Eco*RI terminus (underlined). (B) A second oligonucleotide duplex was produced that in addition encoded a hexahistidine region situated after the third amino acid. The oligonucleotide duplex was ligated to the 2.15-kb *Pst*I-*Eco*RI fragment shown in panel A, which spans the remainder of the *XRCC1* ORF, and the resulting *Eco*RI *XRCC1* cDNA cassette was inserted into the *Eco*RI site of the expression vector pcD2E to generate the expression construct pcD2EHX. (C) pcD2EHCX was constructed as described above, using an oligonucleotide duplex that additionally encoded the epitope recognized by monoclonal antibody 12CA5 (underlined). (D) The oligonucleotide duplex shown, which encodes the first 37 bp of the *XRCC1* ORF, was ligated essentially to the *XRCC1* *Pst*I-*Eco*RI fragment shown in panel A (but in which the *Eco*RI terminus was converted to a blunt end with Klenow fragment), and the resulting fragment (*Nco*I-blunt) was inserted into the bacterial expression vector pET16b at the ATG cloning site to generate the XRCC1 expression construct pET16BX.

and subsequently dried and exposed to X-Omat autoradiographic film (Kodak, Rochester, N.Y.).

The nucleotide sequence of the modified 5′ region of *XRCC1* in each of these constructs was confirmed by dideoxynucleotide termination, using Sequenase enzyme as directed by the manufacturer (United States Biochemicals, Cleveland, Ohio).

Transfection of plasmids by electroporation. Five micrograms of plasmid DNA (pcD2EX, pcD2EHX, or pcD2E HCX) and 1×10^7 to 2×10^7 EM9 cells were electroporated in 1 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (20 mM HEPES [pH 7.05], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose), using a Gibco BRL Cell Porator (300 V, 1,600 μF). After electroporation, cells were incubated for 24 to 48 h in fresh medium and subsequently replated into 10-cm-diameter petri dishes in medium containing Geneticin (G418 sulfate; Gibco BRL, Grand Island, N.Y.) at 1.5 mg/ml for determining transfection frequencies and in medium that additionally contained CldUrd for determining the frequency of CldUrd resistance (9, 29) and for isolating transformants for further study. In this selective medium, ~20% of genomic Thy is replaced by chlorouracil during replication, resulting in DNA damage that is fully toxic to EM9 but which does not affect wild-type cells. After ~10 days, individual colonies were isolated by using Pipetman tips, and colony pools were isolated by using trypsin-EDTA.

SCE determinations. Suspension cultures were inoculated at a density of 10^5 cells per ml and grown in the presence of 10 μM bromodeoxyuridine for 23 h, after which Colcemid was added to 0.1 μg/ml. After an additional 5 h, the cells were harvested by standard techniques and prepared for SCE analysis as described previously (19, 20). Fifty well-spread second-division cells with clearly differentiated sister chromatids were scored from each culture.

CHO cell extract preparation. Three-liter suspension cultures of AA8, EM9, and EM9 transformants were grown in spinner flasks to a cell density of 3×10^5 to 4×10^5 cells per ml and pelleted by centrifugation; after resuspension in 50 ml of phosphate-buffered saline containing protease inhibitors (0.5 μg each of leupeptin, pepstatin, chymostatin, and *N*α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK] per ml, 2 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride [Boehringer Mannheim, Indianapolis, Ind.]), the cultures were aliquoted, pelleted as described above, and stored at −80°C for up to 1 month.

For extracts containing total DNA ligase activity, cell pellets (6×10^8 to 8×10^8 cells) were thawed on ice and resuspended in 0.75 to 1.0 ml of extraction buffer (50 mM Tris-Cl [pH 7.5], 0.5 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) containing protease inhibitors as specified above. Cell suspensions were sonicated (six 5-s bursts, 30-s intervals on ice), and after 1 h on ice, cell debris was removed by centrifugation for 30 min (27,000 × *g*, 4°C). The supernatant (typically 1.7 ml) was added to an equal volume of equilibrated DE52 anion-exchange resin (Whatman, Maidstone, England) and incubated on ice for 1 h with occasional mixing. Following the removal of resin-bound nucleic acids by centrifugation (as described above), any particulate matter remaining in the supernatant was pelleted in a microcentrifuge at 4°C. Aliquots of 0.4 ml of the supernatant (typically 1.5 ml total, with protein at 15 to 20 mg/ml) were subject to high-pressure liquid chromatography through two gel filtration columns arranged in series (300 by 75 mm; TSK-40; Bio-Rad, Richmond, Calif.) and eluted in extraction buffer minus protease inhibitors (4°C) at a flow rate of 1 ml/min, with 0.5-ml fractions collected. Fractions were assayed for the ability to form adenylylated DNA ligase polypeptides as described below. Protein concentrations were determined by Coomassie blue dye binding (2), using a calibration curve standardized with bovine serum albumin (BSA).

For immobilized metal affinity chromatography (IMAC), cell pellets (3×10^8 to 4×10^8 cells) were thawed on ice, resuspended in 2 ml of sonication buffer (40 mM HEPES-NaOH [pH 8.0], 0.5 M NaCl, 5 mM 2-mercaptoethanol, 0.8 mM imidazole, 10% glycerol) plus protease inhibitors, and sonicated as described above. Extracts were gently stirred on ice for 30 min, and cell debris was removed by centrifugation as described above. The supernatant (with protein at 7 to 10 mg/ml) was applied to a 1-ml bed volume of equilibrated Ni^{2+} nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Chatsworth, Calif.) and gently stirred on ice for 1 h. The slurry of cell extract and Ni-NTA agarose was then applied to a 5-ml column, and the flowthrough was collected. The column was then washed with 5 volumes (five times with 1 ml each time) of sonication buffer minus protease inhibitors and 5 volumes of wash buffer (40 mM HEPES-NaOH [pH 7.0], 0.1 M NaCl, 5 mM 2-mercaptoethanol, 0.8 mM imidazole, 10% glycerol). Proteins weakly bound to the Ni-NTA agarose were then eluted with 20 volumes of wash buffer containing 8 mM imidazole and 20 volumes of wash buffer containing 32 mM imidazole, with 5-ml fractions collected. Proteins strongly bound to the Ni-NTA agarose (8, 10, 12) were eluted with 10 volumes of wash buffer containing 80 mM imidazole and 10 volumes of wash buffer containing 250 mM imidazole. Twenty fractions of 1 ml each were collected and stored at −80°C as aliquots. DNA ligase assays were conducted as described below. For later experiments (2EHCXP expression), protein extract from 2×10^9 cells was loaded onto a 0.35-ml Ni-NTA agarose column as described above and washed three times with 5 ml of sonication buffer (containing 1 mM imidazole), four times with 5 ml of wash buffer containing 8 mM imidazole, four times with 5 ml of wash buffer containing 32 mM imidazole, and three times with 5 ml of wash buffer containing 250 mM imidazole. Five-milliliter fractions were collected for the early washes, and 1.5-ml fractions were collected for the 250 mM imidazole washes.

Formation of adenylylated DNA ligase intermediates. Reactions (30 μl) were conducted essentially as described previously (31) for 15 min at room temperature and subsequently stopped by the addition of SDS-PAGE loading buffer. Briefly, reaction mixtures contained 60 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg of BSA per ml, 1.5 or 10 μCi of [α-^{32}P]ATP (3,000 Ci/mmol, 10 mCi/ml; Amersham), and indicated amounts of either purified calf thymus DNA ligase I (31) or extract from gel filtration or Ni-NTA chromatography. In experiments in which the substrate specificity of adenylylated polypeptides was examined, 30-μl aliquots were removed from 150-μl reactions after 15 min, and either reactions were terminated as described above or the aliquots were incubated for a further 15 min alone or in the presence of 2 μg of one of the oligonucleotide substrates oligo(dT)$_{16}$ · poly(dA) and oligo(dT)$_{16}$ · poly(rA) before termination. Reaction samples were incubated for 5 min at 85 to 90°C and subjected to electrophoresis through SDS–6.5 or 7.5% polyacrylamide gels as indicated. Gels were fixed in 10% acetic acid for 10 min, dried onto chromatography paper (Whatman 3MM), and exposed to autoradiographic film (Kodak) at room temperature.

For assaying the conversion of 5′-^{32}P-labeled oligo(dT)$_{16}$

phosphomonoesters to ligated alkaline phosphatase-resistant diesters, reactions were conducted as described above but with radiolabeled ATP omitted and cold ATP present at 1 mM and with 5′-^{32}P-labeled oligo(dT)$_{16}$ · poly(dA) or 5′-^{32}P-labeled oligo(dT)$_{16}$ · poly(rA) present throughout the incubation. After 20 min, acid-precipitable radioactivity that was resistant to calf intestinal phosphatase (CIP; Boehringer Mannheim) was determined as described previously (32), using 5 U of CIP for 30 min at 37°C per reaction. Acid-precipitable material collected on GF/C filters (Whatman) was counted in the presence of Universol liquid scintillant (ICN Biomedical Inc., Costa Mesa, Calif.). For analyzing ligated multimers of 5′-^{32}P-labeled oligo(dT)$_{16}$ on 20% denaturing polyacrylamide gels (containing 8 M urea), reactions were performed as described above and stopped by the addition of 20 μl of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol). Following electrophoresis (Bio-Rad Protean II vertical electrophoresis unit), gels were fixed in 10% acetic acid and placed into a heat-sealable plastic bag or dried onto chromatography paper (3 MM; Whatman). Gels and filters were exposed to autoradiographic film at room temperature.

Duplex oligonucleotides were prepared by mixing equal amounts (by weight) of oligo(dT)$_{16}$ with either poly(dA) or poly(rA) in TE (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]), heating for 10 min at 85 to 90°C, and cooling slowly to room temperature. For long-term storage of oligonucleotide duplexes, NaCl was added to 0.1 M. For experiments requiring labeled oligonucleotide substrate, 10 μg of 5′-^{32}P-labeled oligo(dT)$_{16}$ was prepared by successive treatments with 5 U of CIP (1 h, 37°C) and subsequently 30 to 50 U of T4 polynucleotide kinase (Promega, Madison, Wis.) in the presence of 10 μM ATP, 100 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham), and 2 mM K_2HPO_4 to inhibit CIP (1 h, 37°C). Labeled oligonucleotide was annealed to either poly(dA) or poly(rA) as described above.

Immunodetection of epitope-tagged XRCC1. Protein samples were fractionated by SDS-PAGE (7.5% polyacrylamide gels) and electroblotted, using a Bio-Rad minigel apparatus, onto Immobilon polyvinylidene difluoride membranes (Millipore). The Immobilon Western blot (immunoblot) was processed as directed by the manufacturer except that blocking was for 70 min with 5% nonfat dry milk at room temperature. Affinity-purified monoclonal antibody 12CA5 (Ian Wilson, The Scripps Research Institute, La Jolla, Calif.) was used at 1 μg/ml for 2 h at room temperature, and alkaline phosphatase-conjugated anti-mouse goat immunoglobulin G secondary antibody was used for 2 h at room temperature as directed by the manufacturer (Bio-Rad). Immunodetected polypeptides were visualized by using BCIP/NBT Stable Mix (Gibco BRL) as directed by the manufacturer.

RESULTS

Evidence for a biochemical interaction between XRCC1 and DNA ligase III. We previously constructed the full-length *XRCC1* minigene pcD2EX by ligating a truncated *XRCC1* cDNA to an oligonucleotide duplex encoding the missing N-terminal amino acids and inserting the completed ORF into the mammalian expression vector pcD2E (3). We have now repeated this approach by using a second oligonucleotide duplex to generate pcD2EHX, which differs from pcD2EX by the inclusion of a hexahistidine domain near the 5′ terminus of the *XRCC1* ORF (Fig. 1). Plasmid pcD2EHX was transfected into EM9, and both single transfectants (2EHX1, 2EHX2, etc.) and pooled transfectants (2EHXP1 and 2EHXP2) were examined for SCEs (Table 1). Similarly to pcD2EX (3), pcD2EHX restored SCEs to the normal level in all of the transfectants examined, suggesting that the presence of the histidine tag was not detrimental to XRCC1 activity. Total cell extract prepared from the transfectant 2EHX1 (and 2EHXP1; results not shown) was then subjected to IMAC to purify His-tagged XRCC1. Analysis of fractions eluted following the addition of high concentrations of imidazole (32 to 250 mM) revealed the presence of DNA ligase activity, as determined by the ability of these fractions to form an adenylylated DNA ligase intermediate that migrated during SDS-PAGE as a 100-kDa polypeptide (Fig. 2A). Most of the DNA ligase activity eluted under conditions in which oligohistidine-tagged proteins elute (imidazole at 80 to 250 mM) (8, 10, 12), suggesting that the DNA ligase activity was coeluting with His-tagged XRCC1. In support of this conclusion, the elution of DNA ligase activity in these fractions was dependent on the presence of His-tagged XRCC1, since DNA ligase activity was not detected in IMAC fractions of extract from 2EX1 (a pcD2EX transfectant of EM9 expressing untagged XRCC1), AA8, or untransfected EM9 cells (results not shown), even though the level of DNA ligase activity was similar in 2EHX1, 2EX1, and AA8 cell extracts prior to IMAC (see Fig. 6B). This result was confirmed by experiments in which the most active IMAC fraction from 2EHX1 (fraction 80-3) was compared with the analogous fractions from 2EX1, AA8, and EM9 cell extracts in reactions in which [α-^{32}P]ATP was used at 110 nM (rather than 16.6 nM, which was used in earlier experiments) to increase the sensitivity of the assay (Fig. 2B). A small amount of background DNA ligase activity was observed in the latter three extracts under these reaction conditions, but much higher levels of adenylylated DNA ligase polypeptide were formed by 2EHX1 extract, consistent with the notion that expression of His-tagged XRCC1 is required for enrichment of DNA ligase activity by IMAC.

To demonstrate physically that the 100-kDa DNA ligase activity coelutes with His-tagged XRCC1, we repeated these experiments with an EM9 transfectant (2EHCXP) that harbors the cDNA minigene pcD2EHCX (Fig. 1C). pcD2EHCX encodes His-tagged XRCC1 protein that additionally contains a well-defined epitope from the influenza virus hemagglutinin protein at the N terminus (35). We were thus able to

TABLE 1. SCE frequencies in pcD2EHX transfectants of EM9

Cell line	DNA construct	Mean no. of SCEs/cell (SE)[a]	% Correction[b]
AA8		8.6 (0.4)	100
EM9		112.5 (2.6)	0
2EGP1[c]	pcD2E	96.6 (3.1)	15.3
2EHXP1	pcD2EHX	7.8 (0.4)	100.8
2EHXP2	pcD2EHX	7.8 (0.4)	100.7
2EHX1	pcD2EHX	8.6 (0.4)	100.0
2EHX2	pcD2EHX	8.6 (0.4)	100.0
2EHX3	pcD2EHX	8.0 (0.4)	100.6
2EHX4	pcD2EHX	11.1 (1.8)	97.6[d]

[a] Value is based on measurements from 50 second-division cells from each culture in a single experiment.

[b] The degree of correction conferred by pcD2E and pcD2EHX was calculated by the following formula: % correction in cell line Y = (SCE_{EM9} − SCE_Y) × 100/(SCE_{EM9} − SCE_{AA8}).

[c] Letter "P" denotes a cell line consisting of pooled transfectants.

[d] Four cells displayed >40 SCEs, suggesting either instability or a contaminated clone.

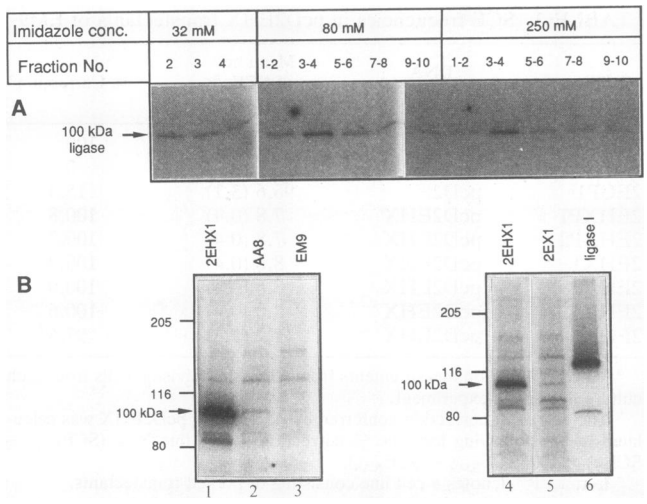

FIG. 2. Enrichment of DNA ligase activity by IMAC. (A) 2EHX1 cell extract was fractionated by IMAC, and aliquots (6 μl) of single or paired fractions exhibiting DNA ligase activity (those eluted following the addition of 32 to 250 mM imidazole) were incubated with [α-^{32}P]ATP (16.6 nM) to form adenylate-DNA ligase intermediates. Reactions were stopped, and samples were subjected to SDS-PAGE and autoradiography. The IMAC fractions each provided ~0.2 μg of protein, except for fractions 32-2, 80 1-2, and 80 3-4, which provided 0.5, 0.3, and 0.4 μg, respectively. (B) Formation of adenylate-DNA ligase intermediates in reactions containing IMAC fraction 80-3 (6 μl; 0.4 μg of protein) from the cell lines indicated and [α-^{32}P]ATP at 110 nM or in lane 6, purified calf thymus DNA ligase I (0.1 ng) and [α-^{32}P]ATP at 16.6 nM. Size markers are indicated in kilodaltons.

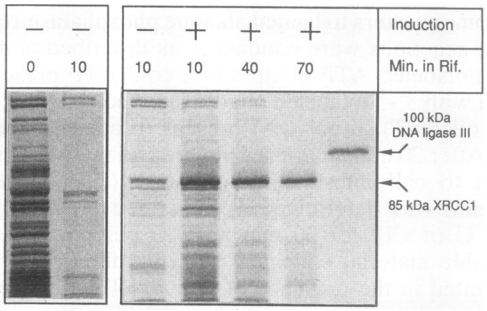

FIG. 3. Selective labeling of human XRCC1 expressed in *E. coli*. Aliquots (0.5 ml) of uninduced or induced (with 1 mM IPTG for 30 min) mid-log phase BL21(DE3) cells harboring pET16BX were incubated in the presence (+) or absence (−) of rifampin (Rif.; 0.2 mg/ml) for 10 to 30 min to inhibit bacterial RNA polymerase. XRCC1 expressed from pET16BX by T7 RNA polymerase was detected by pulse-labeling with [^{35}S]methionine (5 μCi/0.5-ml aliquot) for 15 min. Pulse-labeled cells were pelleted and lysed with 0.1 ml of hot SDS-PAGE loading buffer, and 10- to 30-μl aliquots (containing equivalent amounts of total protein) were subject to SDS-PAGE. The samples loaded onto the gels shown in lanes 2 and 3 are identical but were subjected to electrophoresis for different time periods and so are not perfectly aligned. The IMAC-purified 100-kDa DNA ligase (subsequently identified as DNA ligase III; see text) was adenylylated as described for Fig. 2A and was included for comparison (far-right lane). Following electrophoresis, gels were fixed, stained with Coomassie blue (to confirm that equal amounts of protein had been loaded in all lanes), dried, and subjected to autoradiography.

examine IMAC-fractionated extract from 2EHCXP for coelution of DNA ligase activity and epitope-tagged XRCC1 protein. Immunoprobing of Western blots containing 2EHCXP IMAC fractions with the epitope-specific antibody 12CA5 was expected to identify a polypeptide of ~85 kDa, since this is the apparent molecular mass, following SDS-PAGE, of rat tissue XRCC1 (36) and also human XRCC1 expressed in *E. coli*. The latter was demonstrated by selectively labeling XRCC1 expressed from plasmid pET16BX (Fig. 1D) with [^{35}S]methionine (Fig. 3). The anomalously high apparent molecular mass of XRCC1 following SDS-PAGE is probably a result of XRCC1 being relatively proline rich (31) (XRCC1 is predicted to be 69.5 kDa from the length of the cDNA ORF).

Antibody 12CA5 detected two polypeptides on immunoblots of the 2EHCXP IMAC fraction that exhibited peak DNA ligase activity (Fig. 4, lane 6). The minor band migrated as a polypeptide of ~150 kDa and was present in the negative controls (IMAC fractions with peak DNA ligase activity from two independent fractionations of 2EHX1 extract). This minor band was therefore unrelated to epitope-tagged XRCC1 but served as an internal control for the quality and amount of protein loaded. However, the major polypeptide detected by the antibody was present only in IMAC-fractionated extract from 2EHCXP (compare lanes 1 and 2 with lane 6) and migrated as an 85-kDa polypeptide, identifying it as epitope-tagged XRCC1. Elution of XRCC1 protein from the IMAC column occurred during the 250 mM wash, with no protein detected in the 32 mM wash (compare lanes 4 and 6) and was coincident with the elution of peak DNA ligase activity. Interestingly, epitope-tagged XRCC1 was not detectable in the extract prior to IMAC purification (lane 3). Although only 2 μg of extract protein was used in this experiment, we observed similar results when 10-fold more protein was used (results not shown), suggesting that the level of XRCC1 protein expressed from the minigene in 2EHXCP is very low. Clearly, therefore, significant purification of His-tagged XRCC1 was achieved during IMAC. The coelution of His-tagged XRCC1 and DNA ligase activity during IMAC indicates either that purified His-tagged XRCC1 stimulates the activity of DNA ligase that is carried over nonspecifically during IMAC (Fig. 2B, lanes 1 to 3) or that XRCC1 and the DNA ligase are physically associated.

The three mammalian DNA ligases currently identified are designated DNA ligases I, II, and III. Since they migrate during SDS-PAGE as adenylylated polypeptides of 125, 72, and 100 kDa, respectively (31), the 100-kDa DNA ligase activity enriched by IMAC was most likely either DNA ligase III or a proteolytic fragment of DNA ligase I. However, none of the adenylylated polypeptides formed by IMAC fraction 80-3 comigrated with the two major adenylylated polypeptides formed by the purified calf thymus DNA ligase I control (Fig. 2B, lanes 4 to 6), which were the full-length polypeptide (125 kDa) and its most common active proteolytic fragment (85 kDa) (30), suggesting that DNA ligase I activity was largely absent from the IMAC fraction and therefore, that the enriched activity was DNA ligase III. This conclusion was confirmed by adding oligo(dT)$_{16}$ · poly(dA) or oligo(dT)$_{16}$ · poly(rA) to aliquots of preformed DNA ligase-adenylate intermediates to examine the substrate specificity of the IMAC-enriched DNA ligase activity. While only oligo(dT)$_{16}$ · poly(dA) dissociated the adenylate group from the DNA ligase I control, both DNA substrates dissociated the adenylate group from the affinity-purified polypeptide (Fig. 5A). These substrate specificities are characteristic of DNA ligase I and DNA ligase III,

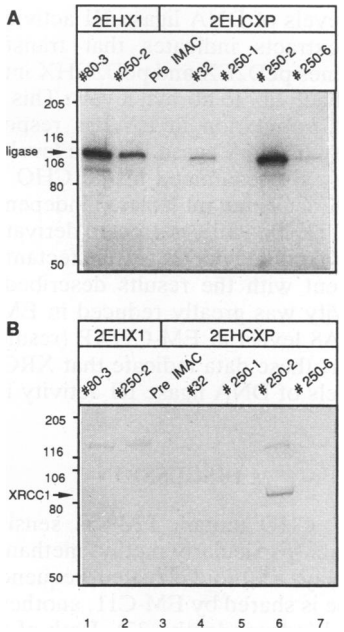

FIG. 4. Copurification of His-tagged XRCC1 and DNA ligase activity during IMAC. Equivalent amounts (by volume) of IMAC fractions collected after the addition of the indicated imidazole washes to column-bound 2EHCXP proteins were either incubated with [α-^{32}P]ATP to form DNA ligase-adenylate intermediates, fractionated by SDS-PAGE, and subjected to autoradiography (A) or subjected to SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane, and immunostained following incubation with primary monoclonal antibody 12CA5 and alkaline phosphatase-conjugated anti-mouse immunoglobulin G secondary antibody (B). (A) Lane 1, 2EHX1 IMAC fraction (6 μl; 1.5 μg of protein of third 1-ml fraction collected after addition of 80 mM imidazole, i.e., fraction with peak DNA ligase activity); lane 2, 2EHX1 IMAC fraction from independent separation (3 μl; 1.5 μg of protein of second 1.5-ml fraction collected after addition of 250 mM imidazole, i.e., fraction with peak DNA ligase activity); lane 3, 1.5 μg of unfractionated 2EHCXP cell extract protein (pre-IMAC); lane 4, 4.5 μl (1.5 μg of protein) of first 5-ml 2EHCXP IMAC fraction collected after addition of 32 mM imidazole; lanes 5 to 7, 1.5 μl of first (0.05 μg of protein), second (1.5 μg of protein), and sixth (0.12 μg of protein) 1.5-ml 2EHCXP IMAC fractions collected after the addition of 250 mM imidazole, respectively. (B) Lanes as for panel A but four times the amount of protein loaded. Molecular weight markers (Bio-Rad) were included in each electrophoretic separation. Sizes are indicated in kilodaltons.

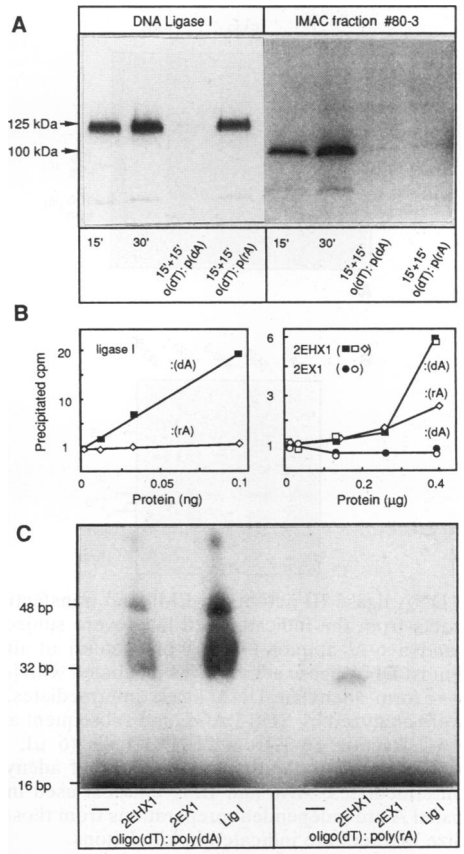

FIG. 5. Substrate specificity of the DNA ligase activity enriched by IMAC. (A) Reaction mixtures (150 μl) containing purified DNA ligase I (2.5 ng) or 2EHX1 IMAC fraction 80-3 (2 μg of protein) were incubated with [α-^{32}P]ATP (16.6 nM) to form adenylate-DNA ligase intermediates. After 15 min, 30-μl aliquots of each reaction were removed and incubated for a further 15 min in the presence or absence of 2 μg of oligo(dT)$_{16}$ · poly(dA) or oligo(dT)$_{16}$ · poly(rA). Reactions were stopped and subjected to SDS-PAGE and autoradiography. (B) Ligated products from 30-μl reactions (15 min) containing the indicated amount of DNA ligase I or protein from 2EHX1 or 2EX1 IMAC fraction 80-3 and either [α-^{32}P]oligo(dT)$_{16}$ · poly(dA) or [α-^{32}P]oligo(dT)$_{16}$ · poly(rA) [:(dA) or :(rA)] were analyzed by measuring the formation of acid-precipitable material. Open and closed symbols represent repeat experiments. The vertical axis represents the fold increase in number of acid-precipitable counts present relative to background (~2,000 cpm, or 0.33 fmol of α-^{32}P-labeled oligonucleotide, estimated from reactions conducted in parallel in the absence of protein). Each 10-fold increase over background indicates the formation of 3.6 × 10^8 (6 fmol) phosphodiester bonds. (C) Ligated products from reactions conducted as described for panel B (but with 0.1 ng of DNA ligase I or 0.4 μg of protein from 2EHX1 or 2EX1 IMAC fraction 80-3) were analyzed by electrophoresis through 20% denaturing polyacrylamide gels and subsequent autoradiography. Sizes of ligated multimers are shown on the left. The first lane in each group represents the reaction mixture incubated in the absence of protein.

respectively (31). We next confirmed that the DNA ligase III enriched by IMAC was capable of completing ligation events, by showing that the enzyme converted 5′-[α-^{32}P] oligo(dT)$_{16}$ · poly(dA) to multimers of higher molecular weight that were acid precipitable and separable by PAGE (Fig. 5B and C, respectively). Little or no DNA ligase activity was detected in the analogous IMAC fraction from 2EX1 extract, a result that is consistent with the results described above. Much shorter exposures of the gel depicted in Fig. 5C revealed that equal amounts of unligated substrate were present in all lanes, excluding the possibility that nuclease activity was affecting the experiment (results not shown). The formation of ligated multimers by the 2EHX1 IMAC fraction in reactions employing the oligo(dT)$_{16}$ · poly(rA) substrate, albeit to a lesser extent than with the oligo(dT)$_{16}$ · poly(dA) substrate, further supported the notion that the enriched DNA ligase activity was DNA ligase III (Fig. 5B and C).

Reduced DNA ligase III activity in the *XRCC1* mutant EM9. To determine whether the apparent ability of XRCC1 to activate and/or physically interact with DNA ligase III has biological significance, we investigated whether DNA ligase III activity was altered in EM9. Cell extracts from AA8, EM9, 2EX1, and 2EHX1 cells were fractionated by gel

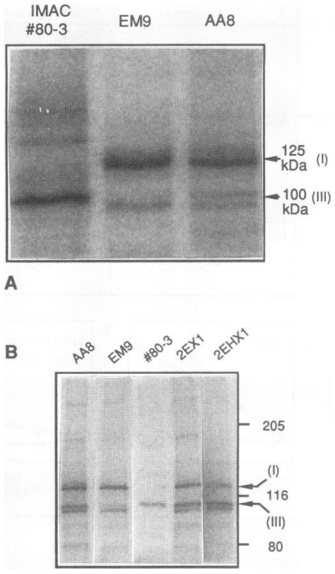

FIG. 6. DNA ligase III activity in EM9 and transfection derivatives. Extracts from the indicated cell lines were subjected to gel filtration, and a 6-μl aliquot (~3 μg of protein) of the fraction containing most DNA ligase activity was incubated with [α-^{32}P]ATP (16.6 nM) to form adenylate-DNA ligase intermediates. Reaction products were analyzed by SDS-PAGE and subsequent autoradiography. IMAC fraction 80-3 from 2EHX1 cells (6 μl; 0.4 μg of protein) was included to identify the positions of adenylate-DNA ligase III intermediates. AA8 and EM9 extracts used in reactions shown in panel A are independent preparations from those shown in panel B. Size markers are indicated in kilodaltons.

filtration and subsequently examined for adenylate-DNA ligase formation. Fractionated extract from AA8 contained three major adenylylated polypeptides that migrated during SDS-PAGE as approximately 100- and 125-kDa species (Fig. 6A). The 125-kDa polypeptide and the larger of the two 100-kDa polypeptides were interpreted as DNA ligases I and III, respectively, on the basis of their size, and in the case of the latter polypeptide because it comigrated with the DNA ligase III activity enriched by IMAC (30, 31) (Fig. 6A). This interpretation was confirmed by experiments in which the substrate specificity of the adenylylated polypeptides was examined as described for Fig. 5A (results not shown). The smaller 100-kDa adenylylated polypeptide was either a proteolytic fragment of ligase I or ligase III or an unrelated protein, since the adenylylated polypeptide was not active on either of the oligonucleotide substrates used (results not shown). We did not observe any major adenylylated reaction products migrating as 72-kDa polypeptides, which is the size expected for adenylylated DNA ligase II. Some minor products were observed around this size range, but we could not determine whether they were DNA ligase II polypeptides or proteolytic fragments of DNA ligase I or III. Very low levels of DNA ligase II activity have been reported previously in such assays (5) and may reflect the high K_m of DNA ligase II for ATP (~15 μM) compared with DNA ligases I and III (0.6 to 2 μM) (31).

The amounts of adenylylated DNA ligase I (125 kDa) and adenylylated proteolytic fragment (~100 kDa) formed by EM9, 2EX1, and 2EHX1 extracts were similar to that formed by AA8 extract (Fig. 6A and B). In contrast, however, DNA ligase III activity was consistently found at a lower level in EM9 extract than in AA8 extract. The presence of AA8 levels of DNA ligase III activity in 2EX1 and 2EHX1 cell extracts indicates that transfection of the *XRCC1* minigenes pcD2EX and pcD2EHX into EM9 returns DNA ligase III activity to normal levels. This result suggests that the *XRCC1* mutation in EM9 is responsible for the reduced activity of DNA ligase III. The level of DNA ligase III activity was also examined in the CHO cell lines EM-C11, another *XRCC1* mutant isolated independently of EM9 (37), and EM-C11XP, a fully corrected derivative of EM-C11 consisting of five pooled pcD2EX transfectants (unpublished data). Consistent with the results described above, DNA ligase III activity was greatly reduced in EM-C11 and was returned to AA8 levels in EM-C11XP (results not shown). Taken together, these data indicate that XRCC1 is required for normal levels of DNA ligase III activity in CHO cells.

DISCUSSION

The *XRCC1* CHO mutant EM9 is sensitive to simple alkylating agents (particularly methyl methanesulfonate and EMS) and displays a 10-fold elevated frequency of SCE (27). This phenotype is shared by EM-C11, another *XRCC1* CHO mutant isolated independently (37). Both of these cell lines rejoin DNA single-strand breaks arising from ionizing radiation and EMS at a reduced rate, suggesting a role for XRCC1 protein in the final stages of a base excision repair pathway. EM9 cell extracts were previously examined for abnormalities in several enzymes with putative roles in the repair of DNA strand breaks, all of which appeared normal (7, 11, 16).

In the work described here, we have shown that His-tagged XRCC1 and DNA ligase III activity copurify during IMAC, demonstrating that the two proteins biochemically and/or physically interact. A low level of background DNA ligase III activity was present following affinity chromatography of extract derived from cells not expressing His-tagged XRCC1 (2EX1, AA8, and EM9 cells), suggesting that DNA ligase III may have some intrinsic affinity for the Ni-NTA agarose. Such proteins appear to be relatively abundant in mammalian cells (8, 12), a notion supported by our estimate that under the conditions described here, 1 to 5% of the applied cell protein binds to the affinity matrix and elutes in the early 80 and 250 mM imidazole IMAC fractions (results not shown). Given the carryover of low levels of background DNA ligase III activity during IMAC, it was considered possible that the high level of DNA ligase III activity present in IMAC fractions of 2EHX1 cell extract resulted from an ability of the enriched (His-tagged) XRCC1 protein present in these fractions to stimulate the background DNA ligase III activity. However, the active 2EHX1 IMAC fractions failed to stimulate the DNA ligase III activity present in gel filtration fractions of AA8 or EM9 extract, suggesting that this interpretation was unlikely (results not shown). A more likely explanation for the high level of DNA ligase III activity present in IMAC fractions of 2EHX1 cell extract is that DNA ligase III and XRCC1 are physically associated within the cell, possibly as components of a multiprotein complex, such that DNA ligase III copurifies with His-tagged XRCC1 during IMAC. With respect to this possibility, a mammalian protein complex (RC-1) of 550 to 600 kDa that repairs double-strand breaks and deletions by homologous recombination was recently identified (13). This complex appears to contain at least DNA polymerase ε, low levels of 5′–3′ and 3′–5′ exonucleases, and DNA ligase III. It will be of considerable

interest to determine whether this complex also contains XRCC1.

In addition to the results described above, a biochemical relationship between XRCC1 and DNA ligase III was further demonstrated by the observation that DNA ligase III activity was reduced in EM9 cells and was returned to normal levels in EM9 cells transfected with the expression plasmids pcD2EX or pcD2EHX. These results indicate that XRCC1 is required for normal levels of DNA ligase III activity in mammalian cells. It is possible that there are other DNA ligase III abnormalities in EM9 that are not detected by the assay used in this work. For example, although the residual DNA ligase III activity in EM9 can apparently complete ligation events, as suggested by the observation that the adenylate-DNA ligase III intermediates are dissociated by the addition of oligo(dT)$_{16}$ · poly(dA) (results not shown), it is possible that the ability of the enzyme to interact with other repair proteins is affected by the *XRCC1* mutation. It is also possible that XRCC1 is required to activate or modify other cellular proteins, such that altered DNA ligase III may be only one of several abnormalities that contribute to the EM9 defect in base excision repair. However, the inability of EM9 cells to complete the final ligation step in this pathway is consistent with the DNA ligase defect observed in this work, suggesting a major role for DNA ligase III in base excision repair in mammalian cells.

Hypersensitivity to (m)ethylating agents and greatly elevated SCEs are also phenotypes characteristic of cells from individuals with BS (4, 14, 15), a disorder in which DNA ligase activity exhibits altered properties (5, 6, 33, 34). If altered DNA ligase I activity is responsible for the phenotype of BS cells, as originally proposed, then the similarity of this phenotype with that of EM9 may indicate that both DNA ligase I and DNA ligase III play a role in DNA base excision repair. Alternatively, however, the recent discovery of DNA ligase III raises the possibility that it is this protein that is responsible for the altered DNA ligase activity observed in BS cells, suggesting that BS and EM9 cells may share a common biochemical defect.

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