The RAD51 Family Member, RAD51L3, Is a DNA-stimulated ATPase That Forms a Complex with XRCC2

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Jeremy P. Braybrooke‡, Karen G. Spink§, John Thacker§, and Ian D. Hickson¶

†To whom correspondence should be addressed. Tel.: 44-1865-222417; Fax: 44-1865-222431; E-mail: hickson@icrf.icnet.uk.

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The Rad51 protein in eukaryotic cells is a structural and functional homolog of Escherichia coli RecA with a role in DNA repair and genetic recombination. Several proteins showing sequence similarity to Rad51 have previously been identified in both yeast and human cells. In Saccharomyces cerevisiae, two of these proteins, Rad55p and Rad57p, form a heterodimer that can stimulate Rad51-mediated DNA strand exchange. Here, we report the purification of one of the representative proteins of the RAD51 family in human cells. We demonstrate that the purified RAD51L3 protein possesses single-stranded DNA binding activity and DNA-stimulated ATPase activity, consistent with the presence of “Walker box” motifs in the deduced RAD51L3 sequence. We have identified a protein complex in human cells containing RAD51L3 and a second RAD51 family member, XRCC2. By using purified proteins, we demonstrate that the interaction between RAD51L3 and XRCC2 is direct. Given the requirements for XRCC2 in genetic recombination and protection against DNA-damaging agents, we suggest that the complex of RAD51L3 and XRCC2 is likely to be important for these functions in human cells.

Genetic recombination is an essential process in eukaryotic cells with roles in both mitotic and meiotic processes. During meiosis, genetic recombination is required both for the generation of genetic diversity and for ensuring that segregation of homologous chromosomes occurs faithfully at meiosis I. In the mitotic cell cycle, the major role for recombination is probably in the elimination of DNA damage, including repair of DNA double strand breaks and cross-links (1).

In bacteria, the process of genetic recombination depends upon the RecA protein, which forms nucleoprotein filaments on DNA and promotes DNA strand exchange between homologous sequences (1, 2). Structural and functional homologs of RecA have been isolated from several other organisms, including yeasts and mammalian cells, where the genes have been designated RAD51 (3–8). Strains of Saccharomyces cerevisiae carrying a deletion of the RAD51 gene are viable but are highly sensitive to ionizing radiation and are defective in meiosis (7). The S. cerevisiae Rad51 protein has been purified and shown to form nucleoprotein filaments and promote DNA strand exchange, albeit far less efficiently than does RecA (6, 9, 10). In contrast to yeast, the equivalent gene in mammalian cells (RAD51) has been shown to be essential for embryonic development, as determined by analysis of mice homozygous for a targeted deletion of the RAD51 gene (11, 12).

Efficient genetic recombination in S. cerevisiae requires several genes other than RAD51, including RAD52, RAD54, RAD55, RAD57, and RAD59. These genes belong to the same epistasis group (the RAD52 group) and are therefore considered to operate in the same biochemical pathway (1). This supposition has been validated through biochemical analyses, which have shown, for example, that Rad51p makes specific interactions with Rad52p, Rad54p, and Rad55p (13, 14). Functional homologs of Rad52p and Rad54p have been identified in mammalian cells, and their biochemical properties have been partially characterized (15–18). Rad55p and Rad57p show some primary sequence similarity to RecA but do not appear to perform the same biochemical function(s) as Rad51p (13, 19).

Instead, Rad55p and Rad57p form a heterodimer that is thought to act as a Rad51 accessory factor greatly stimulating the efficiency with which Rad51 catalyzes DNA strand exchange (20). Consistent with this important role in genetic recombination, rad55 and rad57 mutants have a phenotype similar to that of rad51 mutants, including extreme sensitivity to ionizing radiation (1).

To date, no obvious RAD55 and RAD57 orthologs have been identified in human cells. Nevertheless, several genes have been isolated from rodent and human cells that show a limited degree of sequence similarity to RAD51. These genes were identified either through data base searching (RAD51L1, RAD51L2, and RAD51L3, also known as RAD51B, RAD51C, and RAD51D, respectively) or through functional complementation of x-ray-sensitive rodent cell mutants (XRCC2 and XRCC3) (21–28). Although the functional roles of the RAD51L1/L2/L3 proteins can only be surmised at this stage, there is good evidence that the XRCC2 and XRCC3 proteins participate in genetic recombination processes. The mutants of Chinese hamster ovary (XRCC3) or V79 cells (XRCC2) defective in these proteins show mild sensitivity to ionizing radiation but extreme sensitivity to DNA cross-linking agents (29, 30). Both mutants also show an elevated incidence of spontaneous and radiation-induced chromosomal aberrations (30–32). Moreover, the XRCC2 mutant cell line (irs1) displays a 100-fold decrease in the frequency of homologous recombinational repair of DNA double strand breaks (33). Evidence that XRCC3 also operates in the RAD51 pathway comes from the finding that the characteristic nuclear focal pattern of localization for RAD51 is disrupted in XRCC3 mutants (34).

Although the sequence similarities of the human RAD51-like proteins to their yeast counterparts are limited, multiple protein sequence alignments of eukaryotic RAD51 family members suggest that RAD51L3 may be closer in structure to the...
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yeast Rad57p than to the other family members (35). Similarly, the closest human RAD51-like protein to Rad55p is XRCC2. The biochemical functions of these human RAD51-like proteins have not been explored, and therefore as a first step in understanding these functions, we have purified both RAD51L3 and XRCC2 following expression in Escherichia coli. We show that RAD51L3 is a DNA-stimulated ATPase that binds specifically to single-stranded DNA. We present evidence that RAD51L3 forms a complex with XRCC2 in human cells and that this interaction is direct.

MATERIALS AND METHODS

Construction of Plasmids—cDNA for RAD51L3 was amplified from a human testis cDNA library (CLONTECH; HL1142q) using the polymerase chain reaction. The complete open reading frame (ORF) for RAD51L3 was further amplified with primers that incorporate a 5′ EcoRI (5′-AGAGAGGATTCCTCAACATGGGCCTGCTAGGGTC) and a 3′ NotI (5′-AGAGAGGCGGCGGTCGTAGTGCCT) restriction site (underlined in each case). The amplified DNA was cloned into pET30a (Novagen) and pGEX4T-1 (Amerham Pharmacia Biotech) to create pJB3.1 and pJB3.2, respectively. In subsequent cloning of human RAD51L3 gene from several different sources, we found that a polymorphism occurs at nucleotide position 613 (G or A), predicting an amino acid variation at position 165 of the protein (Arg or Gin). This sequence variation has been noted in different nucleotide data base entries (compare Y15572 with GenBank™ AB013341). The cDNA used for these studies (23) carried the nucleotide 613A. The ORF for XRCC2 was cloned into pET30a (Novagen) and pGEX4T-1 (Amerham Pharmacia Biotech) to create pJB1.1 and pJB1.2. Individual colonies were picked and cultured overnight in LB media (Sigma), previously washed with 10 bed volumes of 25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM DTT. RAD51L3 was further purified by heparin affinity chromatography. Disposable columns (Bio-Rad) containing 1 ml bed volume of immobilized heparin-agarose resin (Pierce) were equilibrated with 10 bed volumes of binding buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT). Dia lysed RAD51L3 eluate was loaded onto the resin column and washed with at least 4 h of the same buffer containing 250 mM NaCl, and proteins were eluted with the same buffer containing 600 mM NaCl. Fractions of 0.5 ml were collected, and those containing recombinant RAD51L3 (rRAD51L3), as determined by SDS-PAGE, were dialyzed for 16 h against buffer containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol. Protein samples were stored in aliquots at -20 °C.

Purification of Recombinant RAD51L3-GST and XRCC2-GST Fusion Proteins—Purification of RAD51L3 or XRCC2 was accomplished by cDNA cloning into pET30a (Novagen) or pGEX4T-1 (Amerham Pharmacia Biotech) containing an N- or C-terminal GST fusion tag, respectively. Individual colonies were picked and cultured overnight in LB media (Sigma), previously washed with 10 bed volumes of 25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM DTT. Proteins were purified using heparin agarose resin (Pierce), previously washed with 10 bed volumes of buffer containing 25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM EDTA, 1 mM DTT. The column was subsequently washed with 10 bed volumes of the same buffer before the resin was either boiled in SDS-PAGE loading buffer or used immediately in “pull-down” experiments (see below).

Antibodies—HIIC42 and HIIC48 were generated by immunizing New Zealand White rabbits with 6–8 × 10^6 copies of plasmid DNA encoding XRCC2. The sera were affinity purified against rXRCC2 (Sigma), previously washed with 10 bed volumes of the same buffer prior to elution with 1 M Tris-HCl, pH 8.0. For XRCC2, the sera were affinity purified against rRAD51L3 protein or rXRCC2, respectively. For this, whole cell lysates containing several milligrams of overexpressed recombinant protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane (Hybond C extra, Amersham Pharmacia Biotech). The membranes were stained with Ponceau S and the bands corresponding to rRAD51L3 or rXRCC2 were cut out. Filters were blocked in PBSA containing 5% low fat milk powder, 0.1% Tween 20 for 30 min, before overnight incubation at 4 °C with 2 ml of washes in PBSA plus 1% Tween 20, anti-RAD51L3 or anti-XRCC2 antibodies were eluted in 500 μl of 0.5% SDS in boiling lysis buffer (25 mM Tris-HCl, pH 2.5, 0.5 to 2.0 °C, and the samples were neutralized by the addition of 35 μl of 1 M Tris-HCl, pH 8.0.

ATPase Assays—ATPase activity was determined by the release of 32P, from [γ-32P]ATP. The assay mixture (20 μl) contained 25 μM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 100 μg/ml BSA, 100 mM NaCl, 2.5 mM MgCl2 (or other divalent metal ion) 25 μg/ml (78 μM) nuclease, 0.2 mM ATP, 50 nCi [γ-32P]ATP (3000 Ci/μmol), and 20 μl of extracts. After incubation at 37 °C for 20 min, the reaction was stopped by the addition of 10 μl of 0.5 M EDTA, 1 μl of each reaction was spotted onto CEL 300 PEI/UV254 (Polygram) thin layer chromatography plates, which were rinsed in 100% methanol before separation of 32P from [γ-32P]ATP in buffer containing 0.5 M LiCl, 0.8 M acetic acid. Plates were exposed on PhosphorImager screens, and the percent release of 32P was quantified using ImageQuant software (Molecular Dynamics).

DNA Binding Assays—50-nucleotide single-stranded oligonucleotide, XI-12 (5′-GAGGGTCTCCATGGGCAGCTGAGAGTTTGGGACCACTTCCGAACGGCTAAGAGTGTTTG), was end-labeled with [32P]ATP using standard protocols. BL3 was annealed to BL4 (5′-CGACTAGGATCCGTCAGGCTGCGACGCTGAGATGGGTCGAGAGTGCACCACTGATGGGACAGCTGAGAGTTTGGGACCACTTCCGAACGGCTAAGAGTGTTTG) and 5′-nucleotide oligonucleotide BL3 (5′-AAAATGGAAGAATTGTCCATCTCATTGGGACAGCTGAGATGGGTCGAGAGTGCACCACTGATGGGACAGCTGAGAGTTTGGGACCACTTCCGAACGGCTAAGAGTGTTTG) were 5′-end-labeled with [32P]ATP using standard protocols. The reaction mixture was annealed at 37 °C for 30 min, before incubation at 4 °C for 10 min. After incubation at 37 °C for 30 min, the reaction mixture was spotted onto CEL 300 PEI/UV254 (Polygram) thin layer chromatography plates, which were rinsed in 100% methanol before separation of 32P from [γ-32P]ATP in buffer containing 0.5 M LiCl, 0.8 M acetic acid. Plates were exposed on PhosphorImager screens, and the percent release of 32P was quantified using ImageQuant software (Molecular Dynamics).

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were carried out according to the Invitrogen Hybrid Hunter (version B) protocol. Positive (pHybLex/Zeo-Fos, pYESTrp-Jun) and negative (pHyb-Lex/Zeo-Lamin) controls were used as supplied. Interactions were identified using the β-galactosidase reporter gene in a liquid culture assay using O-nitrophenyl β-d-galactopyranoside as substrate (36).

**Whole Cell and Nuclear Extracts**—Whole cell extracts for Western blotting were prepared by washing cells in PBSA and then boiling in protein loading buffer as described above. Nuclear extracts were prepared from exponentially growing HeLa S3 or WI-38/VA-13 cells. Approximately 2 × 10^6 cells were washed in PBSA, and the pellet was lysed in 5 ml of buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl₂, 10 mM NaCl, 1% Nonidet P-40, 1 mM DTT), supplemented with protease and phosphatase inhibitors (1 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM glucose, 1 phosphate, 10 mM microcystin, 0.1 mM para-nitrophenyl phosphate, 1 mM PMSF, and complete protease inhibitor mixture tablets (Roche Molecular Biochemicals) according to manufacturer’s instructions), on ice for 45 min. Nuclei were harvested at 5000 g for 5 min. The pellet was resuspended in 0.5 ml of TKM buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 25 mM KCl, 1 mM DTT, supplemented with protease and phosphatase inhibitors as described above) to which 0.6 ml of buffer D was added (80 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM NaCl, 1 mM DTT, supplemented with protease and phosphatase inhibitors as above) before incubation on ice for 30 min. The nuclear extract was cleared by centrifugation at 14,000 rpm in a microcentrifuge at 4 °C and used on the day of preparation.

**Western Blotting**—Samples were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Hybond C-extra, Amersham Pharmacia Biotech) using a TE 70 semi-dry transfer unit (Amersham Pharmacia Biotech). The RAD51L3 protein was detected either by a mouse monoclonal anti-histidine tag antibody (Sigma, anti-polyhistidine) or by the IHIC42 and IHIC48 polyclonal antibodies described above. RAD51L3 was detected in cell extracts by IHIC42 used at a dilution of 1:1000 (crude serum) or 1:200 when affinity purified. Affinity purified IHIC48 was used at a dilution of 1:1000. Anti-mouse or anti-rabbit IgG/HRP conjugates (Sigma-Aldrich) were used as secondary antibodies. All subsequent steps were performed at 4°C. Filters were immersed twice in destaining buffer (6 x glycerol:3 x HCl in PBSA) for 10 min and then incubated 6 times for 10 min in destaining buffer diluted 1:1 with PBSA supplemented with 1 mM DTT. Filters were then blocked in PBSA containing 10% powdered milk, 0.1% Tween 20 for 30 min before being incubated with either RAD51L3 or IHIC42 diluted 1:1 with PBSA containing 0.2% milk, 0.1% Tween 20, 1 mM DTT, and 1 mM PMSF for 60 min. Filters were washed 4 times for 10 min in PBSA containing 0.25% powdered milk, 0.1% Tween 20. The second wash contained 0.0001% glutaraldehyde. Conventional Western analysis, as described above, was then performed to detect the presence of RAD51L3 using IHIC42 as the primary antibody. A negative control blot was treated in exactly the same manner omitting the incubation with RAD51L3.

**Results**

**Purification of rRAD51L3 protein.** A, Coomassie Blue stained 12% SDS-polyacrylamide gel of extracts of BL21(ADE3) cells transformed with pB53.1 before (U) and after (I) addition of isopropyl-1-thio-β-D-galactopyranoside. Insoluble inclusion bodies (P) formed greater than 50% of the induced protein. The soluble lysate (S) was purified by nickel chelate chromatography (Ni) and heparin affinity chromatography (Hep). The position of recombinant RAD51L3 protein is indicated on the right. Molecular mass standards are shown in lane M and their sizes in kDa are shown on the left. B, Western blot, using an anti-histidine tag antibody, of the purified rRAD51L3 protein eluted from the heparin column. The position of the immunoreactive protein is indicated on the right.

**Generation of Anti-RAD51L3 and Anti-XRCC2 Antibodies—**Polyclonal anti-RAD51L3 (IHIC42) and anti-XRCC2 (IHIC48) antibodies were raised in rabbits. Using Western blotting, IHIC42 recognized purified His-tagged rRAD51L3 (Fig. 2A, lane 2), and the non-His tagged RAD51L3-GST fusion protein (Fig. 2A, lane 1), but not XRCC2-GST or human RAD51 protein (a gift from Dr S. West, ICRF, London) (Fig. 2A, lanes 3 and 4).

**Purification of rXRCC2—**The XRCC2 cDNA was cloned into pET30a, as described above. Following induction of XRCC2 expression in BL21(ADE3), the vast majority of the recombinant protein was found to be insoluble, with only trace amounts of protein detectable in the soluble lysate. This soluble material, which was insufficient for use in detailed enzymatic analyses, was purified by nickel chelate chromatography. Confirmation that the purified protein was XRCC2 was provided by Western blotting both with an anti-His tag antibody (data not shown) and with an anti-XRCC2 antibody (IHIC48; see below). A chimeric protein comprising XRCC2 fused to GST (XRCC2-GST) was also purified from E. coli for use in antibody validation and pull-down experiments (see “Materials and Methods” and below).

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FIG. 2. Characterization of anti-RAD51L3 and anti-XRCC2 antibodies. A, Western blot of recombinant proteins using affinity purified IHIC42 (anti-RAD51L3) antibodies. Lane 1, RAD51L3-GST; lane 2, His-tagged RAD51L3; lane 3, XRCC2-GST; lane 4, human RAD51. The positions of the RAD51L3-GST and His-tagged RAD51L3 proteins are indicated on the right. B, Western blot of a human WI-38/VA-13 cell extract for RAD51L3 using affinity purified IHIC42 (anti-RAD51L3) antibodies. The position of the RAD51L3 protein is indicated on the right. C, Western blot of recombinant proteins using affinity purified IHIC48 (anti-XRCC2) antibodies. Lane 1, His-tagged XRCC2; lane 2, XRCC2-GST; lane 3, RAD51L3-GST; lane 4, human RAD51; lane 5, GST. The positions of XRCC2-GST and His-tagged XRCC2 are indicated on the right. D, Western blot of a WI-38/VA-13 cell extract for XRCC2 using affinity purified IHIC48 (anti-XRCC2) antibodies. The position of the XRCC2 protein is indicated on the right.

using IHIC42, consistent with the predicted molecular mass of native RAD51L3 (Fig. 2B).

By using Western blotting, IHIC48 detected His-tagged rXRCC2 protein and an XRCC2-GST fusion protein (Fig. 2C, lanes 1 and 2) but not rRAD51L3, human RAD51, or GST itself (Fig. 2C, lanes 3–5). There was considerable degradation of the recombinant XRCC2 proteins leading to detection of multiple bands of lower molecular mass. By using IHIC48, a band of molecular mass 31 kDa was detected in whole cell extracts from WI-38/VA-13 cells, consistent with the predicted size of native XRCC2 protein (Fig. 2D).

rRAD51L3 Is a DNA-stimulated ATPase—All members of the mammalian RAD51-like proteins contain “Walker box” motifs and are, therefore, predicted to be adenine nucleotide-binding proteins (37). rRAD51L3, like human RAD51 itself, was able to hydrolyze ATP. Significant ATPase activity was seen in the absence of DNA, with a small, but reproducible, stimulation of the ATPase activity by both single- and double-stranded DNA (Fig. 3A). Single-stranded DNA was the more efficient cofactor (Fig. 3, A and B). The ATPase activity of rRAD51L3 was dependent upon the presence of a divalent cation, with significant ATPase activity seen at Mg2+ concentrations >0.1 μM (data not shown). Mn2+ could partially substitute for Mg2+ (Fig. 3C). In order to confirm that the observed ATPase activity was directly associated with rRAD51L3, individual eluted fractions from the heparin chromatography column were dialyzed and assayed for ATPase activity as well as being subjected to SDS-PAGE. There was a strong concordance between the level of ATPase activity and the amount of rRAD51L3 protein in each fraction (Fig. 3D) indicating that the ability to hydrolyze ATP is an intrinsic activity of the protein. rRAD51L3 Preferentially BINDS Single-stranded DNA—Members of the RAD51-like family are thought to play a role in homologous recombination and DNA repair and would be predicted, therefore, to bind DNA. By using oligonucleotide substrates (52 nucleotides in length), rRAD51L3 was found to preferentially bind to single-stranded DNA compared with blunt-ended double-stranded DNA (Fig. 4). The binding profiles for two unrelated sequences of single-stranded oligonucleotides were identical (data not shown), suggesting that RADD51L3 is unlikely to show strict sequence preference for binding. DNA binding by RAD51L3 was not dependent upon the presence of divalent metal ions or ATP (data not shown).

RAD51L3 Physically Interacts with XRCC2 in Vivo—The precise functions of, and interactions between, the individual human RAD51-like proteins are not known. In S. cerevisiae, Rad55p and Rad57p form a heterodimer that stimulates Rad51-mediated strand exchange activity (20). It is thought that some of the mammalian RAD51 homologs could, therefore, act in a similar manner. To address this, we analyzed whether RAD51L3 and XRCC2 can physically interact. This was stud-
Next, we asked whether RAD51L3 and XRCC2 form a complex in human cell extracts. To do this, pull-down experiments were performed with extracts from HeLa cells using XRCC2-GST bound to glutathione-agarose matrix. After washing the matrix, any bound complexes were boiled in SDS loading buffer and separated by SDS-PAGE. Western blotting of the fractions with anti-RAD51L3 antibodies (IHIC42) revealed an immunoreactive band of molecular mass 35 kDa, consistent with native RAD51L3 being bound to XRCC2-GST on the matrix (Fig. 6A). This interaction was also apparent in extracts from WI-38/VA-13 cells (data not shown). Interestingly, the RAD51L3 was detected as a doublet, consistent with possible phosphorylation of the protein. Reciprocal pull-down experiments with RAD51L3-GST bound to glutathione-agarose were then performed using extracts from HeLa cells. Western blotting of the pulled down material with anti-XRCC2 antibodies (IHIC48) revealed a band of molecular mass 31 kDa, consistent with native XRCC2 forming a complex with RAD51L3-GST in human cell extracts (Fig. 6B). Taken together, these data indicate that RAD51L3 and XRCC2 exist as (or part of) a complex in human cells.

**rRAD51L3 and XRCC2 Form a Complex in Vitro**—Next, we asked whether the interaction between RAD51L3 and XRCC2 that we had detected in human cell extracts was a direct one and therefore did not require accessory factors. To do this, we used two independent methods to ascertain whether the purified RAD51L3 and XRCC2 proteins can form a complex in vitro. First, we performed pull-down experiments using the recombinant RAD51L3-GST and XRCC2-GST fusion proteins. The XRCC2-GST fusion protein was bound to glutathione-agarose and incubated with recombinant His-tagged RAD51L3. The matrix was then washed, and the bound material Western-blotted using anti-His tag antibodies. The rRAD51L3 protein was retained on the matrix (Fig. 7A), indicating that XRCC2 and RAD51L3 can bind directly to each other. In reciprocal pull-down experiments, a direct interaction was demonstrated between RAD51L3-GST and His-tagged XRCC2 (Fig. 7B). This interaction was maintained at wash concentrations of up to 500 mM NaCl.

Far Western analysis with purified recombinant RAD51L3 and XRCC2-GST was used as a second method to confirm a direct physical interaction between the proteins. This procedure involved the immobilization of XRCC2-GST on nitrocellulose membranes, denaturation, and re-binding of the protein on the membrane before incubation in buffer containing His-tagged RAD51L3. Filters were then washed to remove unbound protein before detection of the RAD51L3 protein by conventional Western analysis. When probed with anti-RAD51L3 antibodies, an immunoreactive band was detected at the position where XRCC2-GST migrates. Several bands of higher mobility were also detected, consistent with interactions between RAD51L3 and breakdown products from XRCC2-GST. There
was no evidence of interaction with GST alone, and no bands were detected on a control blot treated in exactly the same manner apart from incubation of the membrane in PBSA alone rather than with His-tagged RAD51L3 protein (Fig. 7C).

Analysis of the Native Molecular Mass of RAD51L3 and XRCC2 Complexes in Human Cell Extracts—The S. cerevisiae Rad55 and Rad57 proteins form a heterodimer (20). We therefore separated HeLa cell extracts by size-exclusion chromatography to address whether the RAD51L3 and XRCC2 proteins elute in the same fractions from the column, and whether they do so in a size range consistent with the existence of a RAD51L3/XRCC2 heterodimer. The eluted fractions were analyzed by SDS-PAGE and Western blotting with either IHIC42 (anti-RAD51L3) or IHIC46 (anti-XRCC2) antibodies. RAD51L3 and XRCC2 were detected in the same eluted fractions at a molecular mass of approximately 70 kDa, consistent with the formation of a heterodimer in human cells. The positions of the RAD51L3 and XRCC2 proteins are indicated on the right.

Figure 7. rXRCC2 and rRAD51L3 interact directly. Recombinant XRCC2-GST or RAD51L3-GST fusion proteins were bound to glutathione-agarose matrix and purified as described under “Materials and Methods.” Purified proteins were either used in pull-down experiments or far Western analysis. A, XRCC2-GST or GST alone were incubated with His-tagged RAD51L3 before SDS-PAGE and transfer to nitrocellulose membrane. Western blot for His-tagged RAD51L3 using anti-His tag antibodies. Lane 1, rRAD51L3 positive control; lane 2, pull-down with GST; lane 3, pull-down with XRCC2-GST. The position of rRAD51L3 is shown on the right. B, pull-down of rXRCC2 with either GST alone (lane 1) or RAD51L3-GST (lane 2), as detected by Western blotting with anti-His tag antibodies. The position of the rXRCC2 protein is shown on the right. C, far Western blotting analysis. Purified XRCC2-GST and GST were subjected to SDS-PAGE (as indicated above the lanes) and either stained with Coomassie Blue (left panel) or transferred to nitrocellulose membranes (middle and right panels). Proteins on the nitrocellulose membranes were denatured and refolded before incubation either without (middle panel; negative) or with His-tagged RAD51L3 (right panel; positive). After washing, conventional Western blotting was performed using affinity purified IHIC42 to detect the presence of rRAD51L3, as indicated on the right.

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FIG. 8. RAD51L3 and XRCC2 from human cell extracts co-elute on gel filtration chromatography. 200 µl of HeLa nuclear extract was loaded onto a Superose-6 gel filtration column. Individual fractions were collected, subjected to SDS-PAGE, and Western blotted for either RAD51L3 (with IHIC42) or XRCC2 (with IHIC46). The sizes of proteins in individual fractions were determined by running molecular mass standards (β-amylase, 220 kDa; bovine serum albumin, 66 kDa; carboxic anhydrase, 29 kDa, as indicated above the lanes). XRCC2 and RAD51L3 eluted in the same fractions at a native molecular mass of approximately 70 kDa, consistent with the formation of a heterodimer in human cells. The positions of the RAD51L3 and XRCC2 proteins are indicated on the right.

An important feature of all Reca/RAD51-like proteins is the presence of the Walker A and B boxes, which commonly indicate an ATPase function. Accordingly, site-directed mutagenesis of the S. cerevisiae and human RAD51 proteins at highly conserved residues in the Walker A box leads to severe defects in function (7, 38). Similar experiments with S. cerevisiae Rad55p and Rad57p, which form a heterodimer and stimulate the action of Rad51p (20), have shown that mutation of the Walker A box of Rad55p, but not of Rad57p, disables function (13). This finding suggests that ATP hydrolysis may be dispensable for some of the Reca/RAD51 family members. Here, we have shown that purified RAD51L3 has significant ATPase activity in the absence of DNA but that both single-stranded and double-stranded DNA stimulate this activity. The rate of hydrolysis of ATP by RAD51L3 is relatively slow but nevertheless similar to that of human RAD51. ATP hydrolysis by human RAD51 is 2 orders of magnitude slower than the rate shown by E. coli RecA, as noted previously (9, 39–41). It may be that some modification of, or interaction between, the human RAD51-like proteins is required before a high rate of ATP hydrolysis is revealed. It remains to be tested whether interactions among the human RAD51-like proteins will influence this activity of RAD51L3 protein.

An understanding of the DNA binding properties of members of the RAD51 family may help elucidate the roles that they play in the complex process of homologous recombination. The E. coli RecA protein binds preferentially to single-stranded DNA, but the RAD51 proteins from yeast and man bind single- and double-stranded DNA with a similar affinity (9, 40, 42). These differences between prokaryotic and eukaryotic DNA strand exchange proteins may reflect different requirements for cofactors or accessory proteins to promote DNA pairing and exchange. Indeed, in yeast, the Rad55/Rad57 heterodimer has an enhanced affinity for single-stranded DNA, and there is experimental support for a model in which these proteins facilitate the nucleation of Rad51 onto DNA by displacing the single-stranded DNA from single-stranded DNA.
stranded DNA-binding protein, RPA (20). Our finding that single-stranded DNA is the preferred substrate for binding by RAD51L3 is consistent with a similar facilitating role for this protein in human cells.

As noted above, it is known that Rad55p and Rad57p interact to form a heterodimer and that these proteins have some sequence similarities to XRCC2 and RAD51L3, respectively. By using several different experimental approaches, we have shown that the RAD51L3 protein and the XRCC2 protein interact and that this interaction does not require additional accessory factors. First, to assess in vivo the interaction, we showed using the yeast two-hybrid system that XRCC2 interacts strongly with RAD51L3. Second, we showed that the RAD51L3 and XRCC2 proteins physically interact in pull-down experiments, both using purified recombinant proteins and human cell extracts. Finally, far Western analysis with the purified recombinant proteins showed that immobilized XRCC2 interacts with RAD51L3. These data argue strongly for the existence of a direct interaction between the RAD51L3 and XRCC2 proteins in vivo. Our gel filtration analysis is consistent with but does not prove that RAD51L3 and XRCC2 form a heterodimeric complex in human cells. It is significant that no protein was detected in the eluted fractions of a size expected for monomeric XRCC2 and RAD51L3.

Additional studies are required to assess the functional significance of the XRCC2 and RAD51L3 interaction. Based on previous studies of the Rad55 and Rad57 proteins, we anticipate that the proposed heterodimer of XRCC2 and RAD51L3 will be required to promote the formation of RAD51 molecules on single-stranded DNA. The RAD51L3-XRCC2 complex could influence the loading of Rad51 onto DNA in the presence of single-stranded DNA-binding protein (20, 43) or help in the remodeling of chromatin at damaged sites subject to homologous recombination repair (44).

In addition to RAD51L3 and XRCC2, there are currently three other members of the RAD51-like family in human cells as follows: XRCC3, RAD51L1, and RAD51L2. In a recent review article, Thompson and Schild (45) summarized their results of 2-hybrid analyses, which suggested that multiple interactions may occur between RAD51-like proteins, including an interaction between RAD51L3 and XRCC2. This raises the possibility that, rather than acting as a heterodimer in a manner analogous to yeast Rad55p and Rad57p, RAD51L3 and XRCC2 form part of a complex containing multiple RAD51-like proteins and possibly RAD51 itself. However, given the yeast paradigm of a Rad55/Rad57 heterodimer influencing Rad51-catalyzed reactions (20), together with our gel filtration data, it perhaps seems more likely that the RAD51L3/XRCC2 partnership is just one of several possible heterodimeric complexes that participate in genetic recombination reactions in mammalian cells.

The irs1 mutant hamster cell line defective in XRCC2 shows a phenotype similar to that of yeast rad55/rad57 mutants in being x-ray-sensitive and deficient in genetic recombination. However, a striking feature of XRCC2-deficient cells is their extreme sensitivity to DNA cross-linking agents such as mitomycin C (29). This raises the possibility that the RAD51L3-XRCC2 complex is particularly important in those recombination functions that are required for the removal of DNA cross-links. This also suggests a possible scenario in which different combinations of the multiple RAD51-like proteins in human cells impart functionally different roles on RAD51.

In summary, we have purified the human RAD51L3 protein and partially characterized its biochemical properties. We have also shown that RAD51L3 forms a complex with XRCC2 protein in vitro and in vivo. It is clear based on what we currently know about the roles of the RAD51-like proteins, such as from analysis of mutant cell lines lacking XRCC2 or XRCC3, that the correct functioning of the RAD51-like proteins is vital for the maintenance of genome stability in mammalian cells. The goal for the future is to determine how XRCC2 and RAD51L3 participate in recombination reactions in human cells.

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Jeremy P. Braybrooke, Karen G. Spink, John Thacker and Ian D. Hickson

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