hXRCC2 Enhances ADP/ATP Processing and Strand Exchange by hRAD51*

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The assembly of bacterial RecA, and its human homolog hRAD51, into an operational ADP/ATP-regulated DNA-protein (nucleoprotein) filament is essential for homologous recombination repair (HRR). Yet hRAD51 lacks the coordinated ADP/ATP processing exhibited by RecA and is less efficient in HRR reactions in vitro. In this study, we demonstrate that hXRCC2, one of five other poorly understood non-redundant human mitotic RecA homologs (hRAD51B, hRAD51C, hRAD51D, hXRCC2, and hXRCC3), stimulates hRAD51 ATP processing. hXRCC2 also increases hRAD51-mediated DNA unwinding and strand exchange activities that are integral for HRR. Although there does not seem to be a long-lived interaction between hXRCC2 and hRAD51, we detail a strong adenosine nucleotide-regulated interaction between the hXRCC2-hRAD51D heterodimer and hRAD51. These observations begin to elucidate the separate and specialized functions of the human mitotic RecA homologs that enable an efficient nucleoprotein filament required for HRR.

RecA is the prototypical mediator of homologous recombination repair (HRR) in bacteria (for review, see Refs. 1 and 2). The complexity of HRR is dramatically increased in human cells, where six non-redundant mitotic RecA homologs (hRAD51, hRAD51B, hRAD51C, hRAD51D, hXRCC2, and hXRCC3) are required for efficient HRR in vivo (3, 4). Unlike the other human RecA/RAD51 family members, RAD51 seems to be essential for HRR in both mitotic and meiotic cells (5, 6), suggesting a fundamental role in eukaryotic HRR. The RecA/RAD51 family of proteins promotes HRR by catalyzing homologous pairing and strand exchange between parental DNAs (2, 5–7). A number of discrete heteromeric complexes between the human proteins have been identified (8). Whereas hRAD51 and other poorly understood non-redundant human mitotic RecA homologs (hRAD51B, hRAD51C, hRAD51D, hXRCC2, and hXRCC3) seem to possess significant intrinsic DNA binding, ADP/ATP processing, and strand exchange activity (9–14), the function(s) of the remaining human RecA/RAD51 homologs is unknown.

Homology between RecA/RAD51 family members is largely confined to the Walker A/B nucleotide binding domains (2, 7, 15). These peptide motifs allow a number of diverse proteins to coordinate the free energy of nucleotide triphosphate (NTP) binding and hydrolysis into biological processes (15). During HRR, the bacterial RecA protein efficiently synchronizes the binding and hydrolysis of ATP between monomers within a nucleoprotein filament (NPF) that ultimately facilitates unwinding and strand exchange between homologous DNAs (2, 7). However, hRAD51 alone is largely unable to coordinate ATP processing between individual subunits of the NPF, which is manifest in modest strand exchange activity and a dramatically reduced ability to process heterologous (mismatched) DNA sequences, a genetic signature of HRR (9, 10, 16–24). These results have suggested that additional factors are necessary to coordinate the hRAD51 ATPase during HRR in eukaryotic cells.

Hydrolysis of an NTP can be conceptually divided into two phases: 1) γ-phosphate hydrolysis and 2) the release of the hydrolysis products (NDP + Pi) followed by binding of a new NTP (NDP→NTP exchange). NDP→NTP exchange seems to be the rate-limiting step in many NTPase cycles (25–27). The regulation of NDP→NTP exchange by “exchange factors” is one mechanism by which cells may control protein conformational transitions that are coupled to biological function. Such regulated control seems to enhance the efficiency of NTPases (28). Examples of exchange factors and their cognate NTPases include guanine nucleotide exchange factors (GEFs) for G-proteins, profilin for actin, actin for myosin, and β-tubulin for dynein and kinesin (25–27). Biochemical evidence that links exchange factors with ATPases has been previously inferred from single-turnover ATP hydrolysis studies and/or comparison of protein cofactor alterations of ADP/ATP binding (for example, see Refs. 29 and 30). It is noteworthy that there are very few examples in which a direct examination of ADP→ATP exchange has been demonstrated (31, 32).

The human mitotic RecA homolog XRCC2 (x-ray sensitive cross-complementation group-2) was identified based on its ability to complement the sensitivity of the yeast mutator strain irs1 hamster cells to the DNA cross-linking agent mitomycin C (33, 34). Although the biochemical and molecular basis of XRCC2 function is unknown, these studies have suggested that it plays an important role in HRR (35, 36). In this study, we demonstrate that hXRCC2 enhances ATP processing by hRAD51. Unlike other well-characterized RecA/RAD51 homologs, hXRCC2 does not seem to possess significant intrinsic DNA binding, ADP/ATP binding, or ATPase activities. We confirm and purify a stable hXRCC2-hRAD51D heterodimer (37, 38). hRAD51D also does...
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not seem to significantly bind ATP. We find that hRAD51D and the xHRC2-hhRAD51D heterodimer only interact with hRAD51 in the presence of the reaction intermediate mimetic ADP-aluminum fluoride. These results are consistent with a role for hRAD51D in localizing xHRC2 to the active site of the hRAD51 NPF and a unique role for xHRC2 in regulating hRAD51 activities.

MATERIALS AND METHODS

Protein Purification—hRAD51 and hRAD51(I313R) [KR hRAD51] was purified as described previously (20). The cDNA encoding the C-terminal His6-tagged xHRC2 was subcloned into pFastBac Dual vector containing his6-xHRC2. xHRC2 and the xHRC2- hRAD51D heterodimer were overexpressed in High Five insect cells (Invitrogen). Infected cells containing overexpressed xHRC2 or xHRC2-hRAD51D were harvested and resuspended in buffer A (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 20 mM imidazole, and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.8 μg/ml leupeptin, 0.8 μg/ml pepstatin)) followed by rapid freezing in liquid nitrogen. Cell lysates were prepared by thawing cells on ice, passed them through a 25-gauge needle, and clearing debris by ultracentrifugation.

For xHRC2 purification the supernatant was loaded onto a nickel-nitrilotriacetic acid Superflow (Qiagen) column, washed with buffer A, and eluted with a linear gradient of imidazole from 20 mM to 200 mM. Pooled fractions (fraction I) containing xHRC2 were dialyzed against buffer B (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors). Fraction I was loaded on to Mono-S in tandem with a Heparin-Sepharose column (Amersham Biosciences). The flow through (fraction II) was dialyzed against buffer C (5 mM potassium phosphate, pH 6.8, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors). Fraction II was loaded onto a hydroxyapatite column in tandem with Polybuffer Exchanger (PBE) (Pharmacia). The PBE column was disconnected. The remaining hydroxyapatite column, including hXRC2 protein, was washed and eluted with a linear gradient of potassium phosphate from 5 to 200 mM. Pooled fractions (fraction III) were dialyzed against 20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors, snap-frozen, and stored at –80 °C.

ATPase and ATP-S Binding—The ATPase activity was measured in 10 μl buffer X (20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, and 15 mg/ml bovine serum albumin), plus 3 μM (nt or bp) εX174 ssDNA or εX174 dsDNA RFI (unless otherwise specified) and the indicated concentration of hRAD51D or hXRCC2 in 20 μl buffer X supplemented with 2 mM Mg(OAc)₂ and subsequently concentrated in the figure legends. The reactions were incubated at 37 °C for 30 min until otherwise indicated. Protein-DNA complexes were resolved by 4% nondenaturing PAGE in 1× Tris-borate/EDTA buffer. Gels were dried and exposed to PhosphorImager screens (Amersham Biosciences) to visualize the products as described previously (31).

ATP Binding—ADP and ATP concentrations in each reaction were measured in 10 μl of buffer X supplemented with 2 mM Mg(OAc)₂, 3 μM (nt or bp) εX174 ssDNA or εX174 dsDNA RFI (unless otherwise specified), the indicated concentration of ADP in Fig. 3 containing 2 μM [³²P]ADP. Reactions were incubated at 37 °C for 1 h and placed on ice for 10 min. The solution was filtered through a nitrocellulose membrane (0.45 μm) with 4 ml of ice-cold buffer A supplemented with 2 mM Mg(OAc)₂. Filters were air-dried, incubated over-night in scintillation fluid, and the amount of radioactivity retained on the filters was determined as described previously (31). The ADP—ATP exchange was measured in buffer X supplemented with 2 mM Mg(OAc)₂ plus indicated ADP/[³²P]ADP. 0.6 μM hRAD51D was pre-incubated with ADP and εX174 DNA at 37 °C for 15 min in a final volume of 10 μl. ADP—ATP exchange was initiated by adding the indicated concentration of xHRC2, and 1 mM ATP in buffer X supplemented with 2 mM Mg(OAc)₂ (final volume, 30 μl), and incubation was continued at 25 °C. Reactions were stopped at indicated times in Fig. 3 by dilution and immediate filtration through a nitrocellulose membrane (HAWP; Millipore). Radioactivity retained on the filters was quantitated as described previously (31).

UV Cross-Linking—Reactions were performed as described previously (21). In brief, 1 μM hXRC2 and 1 μM hRAD51D were incubated at 25 °C for 15 min. In 10 μl of buffer X supplemented with 1 mM [γ-³²P]ATP (60 Ci/mmol) in the presence of 6 μM εX174 ssDNA and 2 mM Mg(OAc)₂. The plate was irradiated at 254 nm in a Stratagene (Stratagene) for 10 min. Samples were resolved by 10% SDS-PAGE and proteins were visualized by Coomassie staining. After digital imaging (Epson Perfection 636), the gel was dried and radiolabel visualized with a PhosphorImager.

DNA Unwinding—DNA unwinding catalyzed by hRAD51D was examined by a modification of a method described previously (14). Relaxed DNA (form IV) was prepared in batch by incubating 9 μg of εX174 replicative form I DNA (New England Biolabs) with 30 units of calf thymus topoisomerase I (Invitrogen) in 40 μl at 37 °C for 30 min. DNA unwinding assays were then initiated by addition of 2 μl (34 μM nucleotides) of batch prepared relaxed εX174 DNA, including topoisomerase I, to the indicated amount of hRAD51D and/or XRC2 in 20 μl of buffer X supplemented with 10 mM Mg(OAc)₂ and 5 mM ATP or ADP at 37 °C for 10 min. The reactions were deproteinized by adding 2 μl of 10% SDS and 15 mg/ml protease K and incubated at 37 °C for 20 min. 2 μl of loading buffer (0.25% bromophenol blue/0.25% xylene cyanol and 0.5% glycerol) were added and 24 μl of the final sample volume subjected to electrophoresis in 0.9% agarose/TBE. DNA unwinding was measured by digestion of εX174 RFI dsDNA with ApaLI. All the reaction steps were carried out at 37 °C. The reaction was assembled by mixing 8 μM hRAD51D (16 μl) and 30 μM (nt) εX174 ssDNA or 20 μM εX174 dsDNA RFI (unless otherwise specified) and the indicated concentration of hRAD51D or hXRCC2 in 20 μl of buffer X supplemented with 10 mM Mg(OAc)₂ and 5 mM ATP or ADP at 37 °C for 10 min. The reactions were deproteinized by adding 2 μl of 10% SDS and 15 mg/ml protease K and incubated at 37 °C for 20 min. 2 μl of loading buffer (0.25% bromophenol blue/0.25% xylene cyanol and 50% glycerol) were added and 24 μl of the final sample volume subjected to electrophoresis in 0.9% agarose/TBE buffer and followed by ethidium bromide (0.5 μg/ml) staining.

Strand Exchange—Reactions were performed as described previously (19) with some modification. Linear εX174 dsDNA were prepared by digestion of εX174 RFI dsDNA with ApaLI. All the reaction steps were carried out at 37 °C. The reaction was assembled by mixing 6 μM hRAD51D (16 μl) and 30 μM (nt) circular εX174 ssDNA or 1.0 μM xHRC2 (2.6 μl) in 80 μl of final reaction volume (20 mM HEPES-NaOH, pH 7.5, 1 mM Mg(OAc)₂, 2 mM ATP, and 1 mM dithiothreitol) for Fig. 5B (otherwise in the presence of indicated amount of hXRC2 for 5C). After 5 min of incubation, (NH₄)₂SO₄ (final concentration, 100 mM) and linear duplex DNA (final concentration, 15 μM) were added to reactions in Fig. 3. At indicated times in Fig. 3, 10 μl aliquots were withdrawn, the reaction was stopped and deproteinized by adding 3 μl of 10% SDS and 15 mg/ml proteinase K, incubated further for 20 min, and subjected to electrophoresis in 0.9% agarose gels containing 0.5 μg/ml ethidium bromide in Tris-acetate/EDTA buffer.

GST-IVTT Reaction—Reactions were performed as described previously (30, 39). Full-length hRAD51 (13 μl) was subcloned into pGEX-4T-2 (Pharmacia), which allows high expression of a glutathione-S-transferase (GST) fusion protein. The fusion product was expressed in Esherichia coli, and an extract was generated as described previously (40). The cleared extract was incubated with glutathione-agarose beads (Sigma) at 4 °C for 1 h under gentle continuous agitation and subsequently centrifuged. Fractions containing 500 μl of binding buffer (20 mM Tris, pH 7.5, 10% glycerol, 150 mM NaCl, 5 mM EDTA or 10 mM MgCl₂, 1 mM DTT, 0.1% Tween 20, 0.75 mg/ml bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Under these conditions, ~20–50 ng
of protein was bound to 25 µl of beads. Lysates containing the unmodified pGEX vector were treated in the same way and used as a negative control. GST and GST-fusion protein binding to the beads was verified by denaturing gel electrophoresis (SDS-PAGE). 35S-labeled hRAD51D was synthesized using in vitro transcription/translation (IVTT) (TNT coupled reticulocyte lysate system; Promega) and added to the GST fusion protein-bound beads in binding buffer. ADP, ATP, ATP-γ-S, ATP, and NaIF4 (1 mM) were added as indicated. The samples were gently rocked at 4 °C for 1 h. The beads were then centrifuged, washed three times, as above, and the bound proteins were separated by PAGE and detected using a PhosphorImager system. No binding of the IVTT material to unmodified GST was detected under any of the conditions described previously (data not shown).

Immunoprecipitation—Protein A beads (Sigma) suspended in buffer I (25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 20 mM dithiothreitol) were exposed over night to hRAD51 polyclonal antibody. The Protein A beads were then washed with binding buffer (20 mM Tris, pH 7.5, 10% glycerol, 150 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 0.1% Tween 10, 0.75 mg/ml bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) and incubated at 4 °C for 1 h with purified hRAD51 with hXRCC2 or the heterodimer hRAD51D/hXRCC2 as indicated. ADP and NaIF4 (1 mM) were added as indicated. After three washes with binding buffer, the bound proteins were subjected to PAGE and detected by Western blot using monoclonal antibodies to hRAD51 and hXRCC2 (Novus).

IAsys Biosensor DNA-Protein Interaction—IAsys Biosensor studies were performed using an IAsys Auto+ unit (Affinity Sensors, Cambridge, UK). A model oligonucleotide (oligo-Δ50) with 5'-end biotinylated (Glen Research, Sterling, VA) was attached via streptavidin to the surface of an IAsys SPR cuvette pre-coated with biotin (Affinity Sensors). The kinetics of wild-type hRAD51 (250 nM) and K/R hRAD51 (250 nM) DNA binding was measured in 25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 5% glycerol, 2 mM MgCl2, and 1 mM dithiothreitol. Where indicated, 2.5 mM ADP, ATP, or ATP-γ-S were added to the binding mixture. Representative binding isotherms are shown.

RESULTS

Purification and Characterization of hXRCC2—To define its biochemical function, we have purified human XRCC2 (hXRCC2) to apparent homogeneity (Fig. 1A). Unlike bacterial RecA or eukaryotic RAD51 proteins (2, 9, 10, 20, 41), hXRCC2 did not seem to bind ssDNA or dsDNA (Fig. 1B). DNA binding was not observed with different length DNA substrates (oligo-Δ50 or ΔX174 and up to 30 µM ssDNA; data not shown), in the absence/presence of adenine nucleotide (AMP, ADP, ATP, ATP-γ-S; data not shown), or at hXRCC2 concentrations that were nearly 10-fold in excess of those required for complete binding by hRAD51 (Figs. 1B and 2A, lane 6; data not shown). We also evaluated the ability of hXRCC2 to bind and hydrolyze adenosine nucleotides. hXRCC2 displayed an extremely weak ATPase activity (AMP-dependent, ADP, ATP, ATP-γ-S; data not shown), and at hXRCC2 concentrations that were nearly 10-fold in excess of those required for complete binding by hRAD51 (Figs. 1B and 2A, lane 6; data not shown). We also performed analysis of hXRCC2 (250 ng) using polyclonal antisera generated to hXRCC2 described previously (21). hXRCC2 enhanced ADP binding and aggregation of hRAD51.

IAsys Biosensor studies—IAsys Biosensor binding results we performed ATP-cross-linking of hXRCC2 and hRAD51. ATPase activity of hXRCC2. 1 µM hXRCC2 was incubated at 37 °C for 30 min with 250 nM (nt) of [32P]poly(dT) or 41-mer [32P]dsDNA and analyzed by non-denaturing 4% PAGE. hXRCC2 displayed an extremely weak ATP binding activity of hXRCC2

Fig. 1. Purification and biochemical characterization of hXRCC2 protein. A, SDS-PAGE analysis of purified hRAD51 and hXRCC2. M represents molecular mass markers. Lane 1, purified hRAD51 (4.5 µg); lane 2, purified hXRCC2 (4 µg); lane 3, Western analysis of hXRCC2 (250 ng) using polyclonal antiserum generated to full-length hXRCC2. B, DNA binding activity of hXRCC2. The indicated amount of hXRCC2 was incubated at 37 °C for 30 min with 250 nm (nt) of [32P]poly(dT) or 41-mer [32P]dsDNA and analyzed by non-denaturing 4% PAGE. C, ATPase activity of hXRCC2. 1 µM hXRCC2 was incubated at 37 °C for 30 min with the indicated concentration of ATP in the absence of DNA (○); in the presence of ΔX174 ssDNA (□), or in the presence of ΔX174 dsDNA RFI (△). ATP hydrolyzed was determined as described previously (20). D, [35S]ATP-γ-S binding activity of hXRCC2 and hRAD51. hXRCC2 (0.7 µM) and/or hRAD51 (0.7 µM) was incubated at 37 °C for 15 min with the indicated amount of [35S]ATP-γ-S in the presence of ΔX174 ssDNA; □, hXRCC2 only; ○, hRAD51 only; ◯, hXRCC2 plus hRAD51. Bound [35S]ATP-γ-S was determined as described previously (21). E, [α-32P]ATP cross-linking to hXRCC2 and hRAD51. UV cross-linking of [α-32P]ATP to purified proteins (320 ng of hXRCC2 or 370 ng of hRAD51) was performed. The proteins were separated by SDS-PAGE and visualized by Coomassie stain. After digital imagery (Epson Perfection 636) of the Coomassie-stained gel, it was dried and radiolabeled visualized using a PhosphorImager (Amer sham Biosciences). The relative ratio of [α-32P]ATP cross-linking between hRAD51 and hXRCC2 was quantitated and found to be 15.4:1. F, ADP binding by hRAD51 in the presence or absence of hXRCC2. hRAD51 (1 µM) was incubated at 37 °C for 60 min in the presence or absence of hXRCC2 (1 µM) or DNA (6 µM nt or bp) with the indicated amount of ADP. Bound ADP was determined as described previously (21). □, hRAD51 alone; ○, hRAD51 plus hXRCC2; ◯, hRAD51 plus hXRCC2 with ΔX174 ssDNA; □, hRAD51 plus hXRCC2 with ΔX174 dsDNA RFI. For C, D, and F, each point represents the average and standard of deviation of at least three experiments (error bars are shown but may be obscured by symbol).
for ADP (Fig. 1F; Table I). Our previous work demonstrated that hRAD51 displayed bimodal ADP-binding: a high affinity ADP binding mode \( K_{app1} \approx 5 \mu M \) and a low affinity ADP binding mode \( K_{app2} \approx 125 \mu M \); Table I; Refs. 20 and 21). Co-incubation of hRAD51 with hXRCC2 resulted in an increase of \~{}4-fold in the high affinity equilibrium binding constant for ADP regardless of DNA cofactor (Fig. 1F; Table I). The low affinity mode has been shown to correlate with a nonspecific hRAD51-DNA aggregate (21, 22). Co-incubation of hRAD51 with hXRCC2 completely suppressed the low affinity binding of ADP by hRAD51 (Fig. 1F, inset; Table I; see Ref. 21). Taken as a whole, these results indicate that hXRCC2 influences the binding of ADP (but not ATP\( \gamma S \)) by hRAD51.

In the presence of ADP, hRAD51 binds to a model ssDNA substrate (oligo-dT\(_{50}\)) in two modes: hRAD51-DNA\(_{low}\) and hRAD51-DNA\(_{high}\) (22). The hRAD51-DNA\(_{low}\) and hRAD51-DNA\(_{high}\) modes correlate with two modes of ATP binding by hRAD51 (\( K_{app1} \approx 5 \mu M \) and \( K_{app2} \approx 125 \mu M \), respectively; 22). The structures of these complexes are unknown. We favor the notion that hRAD51-DNA\(_{low}\) represents a complex that is competent for activation because hRAD51 retains the capacity for ADP→ATP exchange (21, 22). In contrast, the hRAD51-DNA\(_{high}\) probably represents an inactive high molecular weight aggregate of hRAD51 that seems refractory to ADP→ATP exchange (21, 22). In the absence of adenosine nucleotide or in the presence of 1 mM ATP or ATP\( \gamma S \), hRAD51-DNA\(_{low}\) is the predominant form (Fig. 2A, lanes 2 and 3; 22). Quantitative conversion of hRAD51-DNA\(_{low}\) to the hRAD51-DNA\(_{high}\) form occurs in the presence of 1 mM ADP (Fig. 2A, lane 4). Consistent with ADP binding data, co-incubation of hRAD51 with hXRCC2 prevents the ADP-dependent conversion of hRAD51-DNA\(_{low}\) to hRAD51-DNA\(_{high}\) (Fig. 2A, compare lane 4 with lane 5). In addition, suppression of hRAD51-DNA\(_{high}\) is also dependent on the concentration of hXRCC2, and the IC\(_{50}\) occurred at a relative ratio of 1 hXRCC2:1 hRAD51 (Fig. 2B). Only trace amounts of hRAD51-DNA\(_{low}\) hRAD51-DNA\(_{high}\) complexes were converted to hRAD51-DNA\(_{low}\) when 4-fold excess hXRCC2 was added to preformed hRAD51-DNA\(_{high}\) complexes (Fig. 2C, lanes 2 and 3; data not shown). However, either co-incubation of hXRCC2 and

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### Table I

**Summary of ATP hydrolysis and nucleotide binding data for hXRCC2/hRAD51**

| Proteins                  | No DNA | ssDNA | dsDNA |
|---------------------------|--------|-------|-------|
| K\( \gamma S \) (\( \mu M \)) |        |       |       |
| hRAD51                    | 79 ± 48| 15 ± 1.8| 20 ± 3.4|
| hXRCC2                    | 241 ± 52| 200 ± 25| 210 ± 30|
| hRAD51+hXRCC2             | 106 ± 61| 10 ± 1.5| 15 ± 5 |
| \( k_{app1} \) (min\(^{-1}\)) | 0.069 | 0.23 | 0.11 |
| hRAD51                    | 0.039 | 0.0258 | 0.038 |
| hXRCC2                    | 0.10 | 0.32 | 0.18 |
| hRAD51+hXRCC2             | 14.0 | 255.0 | 91.6 |
| K\(_{ATP\gamma S}\) (\( \mu M \)) |        |       |       |
| hRAD51                    | 2.5 ± 0.5| 2.6 ± 0.3| 3.6 ± 0.3|
| hXRCC2                    | 5.6 ± 1.2| 6.2 ± 1.4| 5.5 ± 1.1|
| hRAD51+hXRCC2             | 15.7 | 416.0 | 155.0 |
| K\(_{app1}\) (\( \mu M \)) | 4.7 ± 0.3| 4.4 ± 0.4| 4.7 ± 0.4|
| hRAD51                    | 148 ± 85| 128 ± 85| 116 ± 52|
| hXRCC2                    | 14.0 ± 1.4| 17.7 ± 1.7| 15.7 ± 0.9|
| hRAD51+hXRCC2             | 14.0 ± 1.4| 17.7 ± 1.7| 15.7 ± 0.9|

a Hydrolysis attributable to hXRCC2 may contribute to a 10% overestimate of the \( k_{app1} \) for reactions containing hRAD51+hXRCC2.

b Unmeasurable.

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**FIG. 2.** hXRCC2 modifies ADP-dependent hRAD51-ssDNA complex formation. A, hXRCC2 suppresses hRAD51 ADP-dependent aggregation. The indicated amounts of hXRCC2 and hRAD51 were mixed with 250 nM (nt) ssDNA\((\text{53P})\text{oligo-dT}_{50}\) with or without ADP (1 mM), incubated at 37 °C for 30 min, and resolved by 4% non-denaturing PAGE. B, the influence of hXRCC2 stoichiometry on hRAD51 ADP-dependent aggregation. Reactions were performed as in A except that the indicated amount of hXRCC2 was substituted. C, the order of addition of hXRCC2 is critical for the suppression of hRAD51 ADP-dependent aggregation. In lanes 2 and 3, hRAD51, DNA, and ADP were pre-incubated at 37 °C for 15 min. hXRCC2 was subsequently added, and the reactions were incubated at 37 °C for an additional 15 min before resolution by PAGE. In lanes 4 and 5, hXRCC2, DNA, and ADP were pre-incubated at 37 °C for 15 min before resolution by PAGE. PhosphorImages are representative of at least three independent experiments.
DNA, before the addition of hRAD51 (Fig. 2C, lanes 2 and 5) or co-incubation of hXRCC2, hRAD51, and ssDNA (Fig. 2B, lanes 3-6) completely suppressed the formation of hRAD51-DNA

These data are consistent with the hypothesis that hXRCC2 can competitively bind hRAD51 DNA, reducing the affinity of hRAD51 for ADP. The order-of-addition experiments support the notion that the hRAD51-DNA complex is an inactive aggregate. In this context, hXRCC2 seems to promote the formation of a competent hRAD51-DNA complex.

hXRCC2 Enhances ATP Processing by hRAD51—The ability of hXRCC2 to reduce the affinity of hRAD51 for ADP suggested that hXRCC2 might enhance ADP release by hRAD51. We examined the ADP → ATP exchange activity directly by prebinding [3H]-ADP, and measuring the hXRCC2-dependent kinetics of ADP release by hRAD51, upon the addition of excess unlabeled ATP. A similar method has been routinely used to examine GDP → GTP exchange for G proteins (28) and ADP → ATP exchange by the human MutS homologs (26, 31, 43, 44).

In the absence of hXRCC2, little or no ADP was released by hRAD51 regardless of the addition of exogenous ssDNA or dsDNA (Fig. 3A, □, ▲, ▼). In contrast, hXRCC2 seemed to significantly stimulate ADP release (Fig. 3A, ○, △, ◇). The enhancement of ADP → ATP exchange by hXRCC2 was confined to the specific high-affinity ADP binding mode of hRAD51 and not the aggregate form of hRAD51 (Fig. 3, compare A (performed with 6 μM ADP) and B (performed with 100 μM ADP)). Little if any release of ADP by hRAD51 was observed in the absence of exogenous ATP, regardless of the ssDNA or dsDNA substrates (Fig. 3, C–E, ○). The addition of exogenous ATP only modestly increased the amount of ADP released by hRAD51 in the absence of hXRCC2 (Fig. 3, C–E, ○). These results are consistent with previous studies suggesting that ADP → ATP exchange was a rate-limiting step for the hRAD51 ATPase (20, 21).

The introduction of hXRCC2 to hRAD51 in the presence of exogenous ATP dramatically increased both the rate and total amount of ADP → ATP exchange (Fig. 3, C–E, ○, △, ◇, ▼). We note that hXRCC2 stimulated ADP release by hRAD51 seems to be modestly affected by the addition of an exogenous DNA source. A relative ratio of 1 hXRCC2 to 2 hRAD51 seemed to result in release of 50% of the ADP bound by hRAD51 (Fig. 3, C–E). In the absence of exogenous ATP, hXRCC2 is largely incapable of provoking the ADP release by hRAD51 (Fig. 3, C–E, □). Taken as a whole, these results are consistent with the conclusion that hXRCC2 enhances ADP → ATP exchange by hRAD51 (28). ADP release was found to require the addition of exogenous ATP (Fig. 3, C–E, compare ○ with ● and □ with ▼). This observation suggests that ADP release by hRAD51 must be accompanied by requisite ATP binding (ADP → ATP exchange).

The rate of ADP release by hRAD51 was increased with increasing hXRCC2 (Fig. 3F). This enhanced rate of ADP release translated to enhanced kinetics of ATP hydrolysis (Fig. 3G, compare ● with ○). Comparison of the curves shown in Fig. 3G suggests that, when hRAD51 and hXRCC2 are included together, the rate of hydrolysis is significantly greater than the sum of hRAD51 hydrolysis alone and hXRCC2 hydrolysis alone over the range of protein concentrations. We note that hydrolysis attributable to hXRCC2 might contribute to as much as a 10% overestimate of the kcat for reactions containing hRAD51+hXRCC2 (Table I). However, this contribution is largely marginalized in reactions performed in the presence of ssDNA and dsDNA, where the effect of hXRCC2 on the ATPase activity of hRAD51 seemed to be greatest (Fig. 3G; Table I; data not shown). There was little or no effect of hXRCC2 on the steady-state ATPase activity of hRAD51 in the absence of DNA, even though these conditions support hXRCC2-stimulated ADP → ATP exchange (Fig. 3F; Table I). These observations suggest that the hRAD51 ATP hydrolysis cycle contains additional rate-limiting steps that minimally include hXRCC2-enhanced ADP → ATP exchange and DNA-stimulated ATP hydrolysis.

Because hRAD51 and hXRCC2 both contain consensus Walker A/B nucleotide binding motifs, it was necessary to determine which of these proteins was responsible for the DNA-dependent stimulation of ATPase activity. We constructed a Walker A box mutation of hRAD51(K133R) and hXRCC2(K54R). We found that the hXRCC2/K54R mutant protein was insoluble in both bacteria and insect cells. The purified hRAD51(K133R) protein displayed reduced ATP binding activities (∼10% of wild-type; data not shown) and a complete absence of ATP-γ-S binding activity (data not shown). We observed a background kinetics of ATP hydrolysis by the hRAD51(K133R) (Fig. 3G, ■ and hXRCC2 proteins (Fig. 3G, △). The combination of hRAD51(K133R) with hXRCC2 did not significantly increase either the kinetics or total amount of this background ATP hydrolysis activity (Fig. 3G, □). A combination of data leads to the conclusion that hXRCC2 affects the nucleotide binding and hydrolysis activities of hRAD51, and not the reverse: 1) hXRCC2 affects ADP release by hRAD51 in an experiment in which ATP was prebound to hRAD51, and release was measured by the addition of hXRCC2 and an excess cold ATP, and 2) hXRCC2 affects the kinetics of ATP hydrolysis only in the presence of wild-type hRAD51 (i.e. background ATP hydrolysis kinetics occurs when hXRCC2 is combined with hRAD51(K133R)).

hXRCC2 Does Not Affect Kinetics or Stability of hRAD51-ssDNA Binding—One possible mechanism for hXRCC2 to alter ATP/ATP processing by hRAD51 would be to affect the kinetics and/or stability of the hRAD51 NPF. We regarded this as an unlikely scenario because hXRCC2 promoted ATP → ATP exchange by hRAD51 occurs in the absence of DNA. To address this possibility, we have used the Iasys Biosensor to examine real-time interactions between hRAD51 and a model oligo-dT 50-mer ssDNA (Fig. 4). Previous studies using the Iasys biosensor have detailed the effect of adenosine nucleotide on both bacterial RecA and hRAD51 ssDNA binding parameters (22, 45). Although bacterial RecA seems to display only cooperative ssDNA binding in the presence of ATP or ATPγ-S (consistent with an active NPF), hRAD51 seems to bind ssDNA similarly regardless of adenosine nucleotide. Yet, hRAD51 performs only homologous pairing and strand exchange in the presence of ATP as well as homologous pairing and limited stand exchange in the presence of ATPγ-S (9, 46). These results suggest that ssDNA binding by hRAD51 is not an absolute measure of an active NPF. However, the ssDNA binding behavior of hRAD51 in the presence of ATP or ATPγ-S seems to largely correlate with an active NPF.

Consistent with our gel shift data (Fig. 1B), hXRCC2 displayed no ssDNA binding activity under any conditions examined (Fig. 4, green). In addition, we observed little if any difference in hRAD51 ssDNA binding kinetics in the presence of hXRCC2 regardless of adenosine nucleotide (see Fig. 4 for representative binding isotherms). In the presence of ATP, hRAD51 displayed an equilibrium binding constant (Kd) = 140 ± 9 nM. Likewise, in the presence of ATP, the combination of hRAD51+hXRCC2 displayed a Kd value of 135 ± 22 nM. Previous studies have detailed similar equilibrium binding constants in the presence and absence of adenosine nucleotide (22, 45). These results suggest that hXRCC2 is unlikely to influence hRAD51 ADP/ATP processing by altering its DNA binding properties or kinetics. Similar ssDNA binding studies were performed with hRAD51(K133R) (Fig. 4). Although we noted...
**FIG. 3.** hXRCC2 facilitates ADP→ATP exchange and ATPase activity of hRAD51. A, effect of hXRCC2 on high affinity ADP→ATP exchange by hRAD51. Exchange was measured from hRAD51 (0.6 μM) pre-bound with ADP (6 μM) at 37 °C for 15 min. At time zero, ATP (1 mM) and hXRCC2 (1.8 μM) were introduced and the kinetics of ADP release followed (21). , reactions performed with hRAD51 and mock buffer in the absence of DNA; , hRAD51 and mock buffer with φX174 ssDNA; , hRAD51 and mock buffer with φX174 dsDNA RFI; , hRAD51 and hXRCC2 in the absence of DNA; , hRAD51 and hXRCC2 with φX174 ssDNA; and , hRAD51 and hXRCC2 with φX174 dsDNA RFI. Each point reflects the average and standard of deviation of at least three experiments. B, effect of hXRCC2 on low-affinity (100 μM ADP) ADP→ATP exchange by hRAD51. Exchange was measured from hRAD51 (0.6 μM) pre-bound with ADP (100 μM) at 37 °C for 15 min. At time zero, ATP (1 mM) and hXRCC2 (1.8 μM) were introduced and the kinetics of ADP release followed (21). , reactions performed with hRAD51 in the absence of DNA; , hRAD51 with φX174 ssDNA; , hRAD51 with φX174 dsDNA RFI; , hRAD51 and hXRCC2 in the absence of DNA; , hRAD51 and hXRCC2 with φX174 ssDNA; and , hRAD51 and hXRCC2 with φX174 dsDNA RFI. Each point reflects the average and standard of deviation of at least three experiments. Reactions were performed in the absence of DNA ( ), in the presence of φX174 ssDNA ( ), or in the presence of φX174 dsDNA RFI ( ). For , each reaction contained 0.6 μM hRAD51 and, if present, 6 μM DNA (nt φX174 ssDNA or bp φX174 dsDNA RFI) and the following amount of hXRCC2: , none; , 0.3 μM; , 0.6 μM; , 1.2 μM; , 1.8 μM. Exchange control reaction performed without the addition of exogenous ATP contained 0.6 μM hRAD51 and, if present, 6 μM DNA (nt φX174 ssDNA or bp φX174 dsDNA RFI; without hXRCC2 ) or with 1.8 μM hXRCC2 ( ). F, the rate of hXRCC2-dependent ADP release (ADP→ATP exchange) by hRAD51. The initial rate of ADP release by hRAD51 (0.6 μM) within 2 min after initiation of ADP release was measured with the indicated amounts of hXRCC2 in the presence of no DNA ( ), dsDNA (6 μM bp φX174 dsDNA; ), and ssDNA (6 μM nt φX174 ssDNA; ). G, the kinetics of hXRCC2-dependent stimulation of hRAD51 ATPase activity. ATP hydrolysis was examined in the presence of 6 μM φX174 ssDNA and 250 μM ATP. , hXRCC2 (0.7 μM) only; , wild-type hRAD51 (0.7 μM); , hRAD51(K133R) (0.7 μM); , wild-type hRAD51 (0.7 μM) plus hXRCC2 (0.7 μM); , hRAD51(K133R) (0.7 μM) plus hXRCC2 (0.7 μM). Each point in A–G reflects the average and standard of deviation of at least three experiments.
an alteration in the binding kinetics and saturation of hRAD51(K133R) compared with wild-type hRAD51 (particularly in the presence of ATP), there was no effect of exogenous hXRCC2 under any conditions. Taken together, these results are consistent with the conclusion that the hXRCC2-dependent alteration of ATP/ADP processing by hRAD51 is not a result of altered ssDNA binding properties or kinetics. It is interesting that the substantial alteration in binding kinetics of hRAD51(K133R) in the presence of ATP suggests that inappropriate ATP-binding in the absence of hydrolysis may significantly influence ssDNA binding. These results seem qualitatively similar to ATP-bound hydrolysis-defective MutS homolog mismatch repair proteins, which are largely unable to recognize mismatched nucleotides (43).

hXRCC2 Enhances DNA Unwinding by hRAD51—To address the role of hXRCC2 on the consensus recombination repair functions of hRAD51, we initially examined the hRAD51 ATP-dependent DNA unwinding activity using a topoisomerase I-coupled assay (14). ATP-dependent unwinding of parental DNA substrates is a prerequisite for homologous pairing and strand exchange catalyzed by RecA homologs (2). These activities require a minimum number of RecA monomers to be saturated with ATP and reflect the ability of NPFs to assume/ maintain an active or extended conformation (7). Consistent with previous reports (41), the addition of stoichiometric quantities of hRAD51 to the relaxed covalently closed (form IV) DNA substrate (3–5 nt DNA:1 hRAD51) resulted in DNA unwinding and the formation of a covalently closed supercoiled (form X) DNA (Fig. 5A, lane 6). The form X DNA closely co-migrated with untreated supercoiled φX174 replicative form (form I) (Fig. 5A, compare lane 1 with lanes 5–10). Substitution of prokaryotic topoisomerase I (relaxes negative supercoils only) for the eukaryotic topoisomerase I (relaxes both negative and positive supercoils) did not affect these observations, suggesting that hRAD51 binding stabilized under wound DNA (data not shown). Unwinding of form IV DNA by hRAD51 required ATP (Fig. 5A, lanes 5–7) or ATP/S (data not shown) and was not observed in the absence of nucleotide (Fig. 5A, lane 4) or in the
hXRCC2 Enhances ADP/ATP Processing by hRAD51

Fig. 6. Interaction and Function of the hXRCC2-hRAD51D with hRAD51. A, UV cross-linking of [α-32P]ATP to purified hXRCC2-hRAD51D heterodimer, hXRCC2, and hRAD51. After cross-linking, the proteins were separated by SDS-PAGE and visualized by silver stain (A, left) and PhosphorImager (Amersham Biosciences) (A, right). M, molecular mass markers; lane 1, purified hXRCC2-hRAD51D heterodimer (1.4 μg); lane 2, purified hXRCC2 (0.6 μg); lane 3, hRAD51 (0.5 μg). B, GST/IVTT precipitation analysis. A GST fusion construct of hRAD51 (GST-hRAD51) was bound to glutathione beads and exposed to 35S-labeled, in vitro-transcribed translated hRAD51D (IVTT-hRAD51D) in the presence of 1 mM ADP, ATP, ATP-γS, and/or ADP-NaAlF₄, as indicated. Precipitated proteins were separated by PAGE and visualized using a PhosphorImager system. No significant IVTT material was precipitated by GST alone (data not shown). C, immunoprecipitation of hXRCC2 and hXRCC2-hRAD51D with hRAD51. Purified hRAD51 was bound to protein A beads pre-incubated with hRAD51 antibody (α-hRAD51) and subsequently exposed to purified hXRCC2 or hXRCC2-hRAD51D in the absence of adenosine nucleotide or in the presence of 1 mM ADP or ADP-NaAlF₄, as indicated. Precipitated proteins were separated on SDS-PAGE and probed with a monoclonal antibody to hXRCC2 (α-hXRCC2; top) or the hRAD51 antibody (α-hRAD51; bottom). Lanes 1–3 contain purified proteins that had not been subjected to immunoprecipitation. D, the hXRCC2-hRAD51D heterodimer facilitates ADP→ATP exchange by hRAD51. ADP→ATP exchange assay was performed as in Fig. 3A with variable amounts of hXRCC2-hRAD51D plus 1 mM ATP. Each reaction contained 1.0 μM hRAD51, 6 μM δX174 ssDNA (nt), and the following amount of hXRCC2-hRAD51D plus 1 mM ATP: ○, none; ●, 0.25 μM; ▲, 0.5 μM; ▼, 1.0 μM; ▣, 2.0 μM.

The presence of ADP (Fig. 5A, lane 11). The addition of stoichiometric quantities of hXRCC2 to form IV DNA did not result in an unwound form X DNA product (Fig. 5A, lane 3). In the presence of less than stoichiometric quantities of hRAD51 (17 nt DNA:1 hRAD51), unwinding of the form IV DNA substrate was dramatically reduced (Fig. 5A, lane 7). The addition of hXRCC2 to this reaction significantly stimulated unwinding of the form IV DNA substrate (Fig. 5A, lanes 7–10). These results are consistent with the notion that hXRCC2 promotes the formation of an active/extended hRAD51 NPF. It is formally possible that hXRCC2 stimulates form IV DNA unwinding via the assembly of a heteropolymeric hRAD51-hXRCC2 NPF. However, attempts to detect a stable interaction between hXRCC2 and hRAD51 under a variety of conditions have been unsuccessful (8; Fig. 6). Based on its ADP→ATP exchange activity, these observations suggest that hXRCC2 promotes the ATP-bound form of the hRAD51 NPF.

hXRCC2 Stimulates hRAD51 Strand Exchange—By comparison with RecA, our results suggested that hXRCC2-dependent regulation of the nucleotide-bound form of hRAD51 within an NPF should enhance the recombinatorial strand exchange activity of hRAD51. We found a significant hXRCC2-dependent stimulation of hRAD51 homologous pairing and strand exchange activity (Fig. 5, B and C). In addition to a nearly 4-fold enhancement of total joint molecule (jm) products, the kinetics of jm-formation was significantly faster (t½ ~ 20 min for hRAD51 + hXRCC2 compared with t½ > 50 min for hRAD51 alone). The enhancement of hRAD51 strand exchange activity seemed to require less than stoichiometric quantities of hXRCC2 because it could easily be detected at a ratio of less than 1 hXRCC2 per 10 hRAD51 monomers (Fig. 5C). Because hXRCC2 does not seem to possess either DNA binding or unwinding activity, the stimulation of hRAD51 strand exchange activity is unlikely to be the result of hXRCC2-mediated DNA structure alterations.

hRAD51D Localizes hXRCC2 to hRAD51—A stable complex between hRAD51 and hXRCC2 has not been identified under a variety of physiologically relevant conditions (Ref. 8; data not shown). This observation is not entirely surprising because a long-term interaction between G proteins and GEFs usually requires the stabilization of transient reaction intermediates (47). The lack of a definitive interaction left the mechanics of hRAD51 ADP/ATP processing by hXRCC2 unresolved. In contrast, a stable complex between hXRCC2 and hRAD51D has been demonstrated in vitro and in vivo (8, 37). Whether hXRCC2 would retain ADP→ATP exchange activity toward hRAD51 in the presence of its heterodimeric partner hRAD51D was unknown.

To address these issues, we purified the hXRCC2-hRAD51D heterodimer (Fig. 6A; silver stain). Like hXRCC2, we found

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**Footnote:** C. Schmutte and R. Fishel, unpublished results.
that the hXRCC2-hRAD51D heterodimer is only weakly cross-linked with \([\alpha-^{32}P]ATP\) (Fig. 6A; PhosphorImager). The lack of ATP binding was additionally confirmed by traditional ADP or ATPγS filter-binding studies (data not shown). We conclude that both hXRCC2 and hRAD51D are incapable of significant ADP/ATP binding. We examined the ability of a glutathione S-transferase fusion protein derivative of hRAD51 (GST-hRAD51) to precipitate in vitro-transcribed/translated hRAD51D (IVTT-hRAD51D) (Fig. 6B). This method has been routinely used to identify protein interactors as well as peptide interaction regions (39, 40, 48, 49). We found that GST-hRAD51 precipitates IVTT-hRAD51D only in the presence of ADP-aluminum fluoride (Fig. 6B, lane 4). Because aluminum fluoride seems to stabilize NDP/NTP reaction intermediates of Walker A/B motif proteins, (50, 51), these results suggested that the association of hRAD51 with hRAD51D might be regulated by adenosine nucleotide.

We further examined the interaction of the purified hXRCC2-hRAD51D heterodimer with hRAD51 by immunoprecipitation analysis using an antibody specific for hRAD51 (α-hRAD51; Fig. 6C). After immunoprecipitation, separated proteins were subject to Western analysis using a specific antibody for hXRCC2 (α-hXRCC2). We found that hXRCC2 was uniquely precipitated with hRAD51 when in the hXRCC2-hRAD51D heterodimeric form and only in the presence of ADP-aluminum fluoride (Fig. 6C, lane 10). These results are consistent with the GST-IVTT precipitation studies (Fig. 6B) and with the hypothesis that the adenosine nucleotide-regulated interaction between hRAD51D with hRAD51 also co-localizes hXRCC2.

Finally, similar to hXRCC2 alone, we found that the purified hXRCC2-hRAD51D1 heterodimer retained ADP→ATP exchange factor activity for hRAD51 (Fig. 6D). Taken together, these results are consistent with the hypothesis that hRAD51D is capable of localizing hXRCC2 to the hRAD51 NPF, where it may function to enhance hRAD51 ATP processing during HRR. We note that the hXRCC2-hRAD51D1 heterodimer seemed less efficient than hXRCC2 alone in provoking ADP→ATP exchange by hRAD51. We consider two possibilities: 1) the hXRCC2-hRAD51D1 heterodimer modulates the ADP→ATP exchange activity of hXRCC2 or 2) the purified hXRCC2-hRAD51D1 heterodimer is either intrinsically less active or has been purified as less active than hXRCC2 alone. Regardless, the hXRCC2-hRAD51D1 heterodimer displays significant stimulatory activity (Fig. 6D).

**DISCUSSION**

The human RecA homolog hRAD51 displays a poorly coordinated ATPase and modest homologous pairing and strand exchange activities. This contrasts with the cooperative ATPase and robust homologous pairing and strand exchange activities of bacterial RecA. These observations present a puzzle: how does an apparently less efficient hRAD51 effectively promote homologous recombination repair within the context of the more complicated human genomes? We and others have proposed that additional proteins may support hRAD51 in its role during HRR (9, 20–22). Candidates that may fulfill such function(s) include at least five other human mitotic RecA homologs (3, 4). A number of heteromeric complexes have been identified among these mitotic RecA homologs (8). Purified complexes include hRAD51C-hXRCC3, hRAD51B-hRAD51C, hXRCC2-hRAD51D, and hRAD51B-hRAD51C-hRAD51D-hXRCC2 (11, 13, 37, 38, 52). Other than reduced or absent HRR by individual knockouts in chicken DT40 cells, the combined function of these complexes in vitro and in vivo is largely unknown.

Knockout of the RAD51 gene in mice results in highly penetrant early embryo lethality, cell inviability, and extreme radiation sensitivity (53, 54). In contrast, Xrc2−/− mice display late-stage embryo lethality and inviable neonates (55). The embryo and neonatal inviability of the Xrc2−/− mice seems related to developmental neurogenic defects and is similar to other DNA strand-break repair knockouts such as Xrc4−/− and Lgiv−/− (55). Like Rad51−/− cells, Xrc2−/− cells are inviable to long-term culture. However, Xrc2−/− cells display only a modest radiation sensitivity compared with the extreme sensitivity observed in Rad51 deficient cells (35, 36, 56, 57). The less severe genetic phenotypes associated with Xrc2−/− knockouts are consistent with an ancillary role for the XRC2 protein.

We have purified hXRCC2 in the absence of its putative binding partner(s) to distinguish its individual biochemical function(s) in the process of HRR. Our data suggest that hXRCC2 increases the ATP processing activity of hRAD51 by stimulating the rate of hRAD51-ADP→ATP exchange (Fig. 3). hXRCC2 also seems to prevent a ADP-dependent inactive hRAD51 aggregate (Fig. 2). We hypothesize that these activities ultimately result in amplified recombinational strand exchange activity (Fig. 5, B and C). A regulatory function for hXRCC2 combined with the lack of observable ATP binding and hydrolysis activities is consistent with the genetic observation that mutations in the ATPase domain does not inhibit hXRCC2-dependent DNA damage processing (42).

We note a wide range of hXRCC2 to hRAD51 ratios in the catalytic effects of hXRCC2 on hRAD51 biochemical activities. This range probably reflects the relative amount of hXRCC2-dependent ADP→ATP exchange required to affect the activity of a protein (hRAD51) that intrinsically forms a polymeric NPF. The biochemical requirements of an NPF would seem superficially different from those of small G proteins and their associated GEFs, which are generally considered to be monomeric and conformationally less complex. However, recent studies have suggested that GTP-bound Ras may function as an allosteric feedback regulator of SOS GEF activity on GDP-bound Ras (58). The concept of a multimeric feedback nucleotide-exchange complex may be comparable with similar processes that have been proposed to occur within the hRAD51 NPF and could help to explain the wide range of biochemically relevant protein ratios.

Although a stable interaction between hRAD51 and hXRCC2 has not been reported (Ref. 8),2 it is important to note that an association of GEFs and G proteins seems stabilized only when the proteins are trapped in and/or mimic a reaction intermediate (47). In contrast, we found that hRAD51D1 strongly interacts with hRAD51 in the presence of ADP-aluminum fluoride. ADP-aluminum fluoride has been found to induce the formation of an extended unwound filament of hRAD51 on ssDNA that seems largely indistinguishable from an active NPF (59). In support of this notion, ADP-aluminum fluoride promotes significant strand exchange by bacterial RecA in the absence of ATP (60). hXRCC2 and hXRCC2-hRAD51D1 do not significantly interact with hRAD51 in the presence of ADP, ATP or ATPγS (Fig. 6, B and C). These results are consistent with the observation that hRAD51-ssDNA formed in the presence of ADP, ATP or ATPγS seem to resemble an inactive filament (59). Because an extremely stable hXRCC2-hRAD51D1 heterodimer has been identified (8, 37), our results are consistent with the hypothesis that hRAD51D1 may localize hXRCC2 to an active hRAD51 NPF.

Our studies support a hypothetical model for HRR in which the multiple human RecA homologs perform discrete functions in stabilizing and/or regulating the hRAD51 NPF. Based on the historical analysis of RecA, it is likely that coordinated ATP-binding by hRAD51 at a growing three-strand junction biases the formation of a key triplex homologous pairing and strand exchange intermediate (for review, see Ref. 61). The conforma-
tional transitions associated with the formation of this triplex intermediate seem to be mimicked by RecA and hRAD51 in the presence of ADP-aluminum fluoride (59, 60). Our studies suggest that hRAD51 uniquely recognizes this reaction intermediate, perhaps localizing its heterodimeric partner hXRCC2 to an active NPF region. Once localized, hXRCRC2 may then enhance ADP → ATP exchange by hRAD51 thereby ensuring expansion of the ATP-bound triplex homologous pairing and strand exchange intermediate. This model suggests that hXRCRC2 performs a supporting role in HRR whereas hRAD51D minimally functions as an adaptor protein.

These and other studies are consistent with the notion that the human RecA homologs have evolved separate and specialized functions within the NPF that enhance ATP processing and, ultimately, the efficiency of eukaryotic HRR. Such an evolved separation-of-function would seem similar to the human MutS homologs in DNA mismatch repair and meiotic functions (62). Our results further suggest that the regulation of NTPases by NDP → NTP exchange factors is likely to be more widespread in nature than previously appreciated.

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