Homologous-pairing activity of the human DNA-repair proteins Xrcc3·Rad51C

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The human Xrcc3 protein is involved in the repair of damaged DNA through homologous recombination, in which homologous pairing is a key step. The Rad51 protein is believed to be the only protein factor that promotes homologous pairing in recombinational DNA repair in mitotic cells. In the brain, however, *Rad51* expression is extremely low, whereas *XRCC3*, a human homologue of *Saccharomyces cerevisiae RAD57* that activates the Rad51-dependent homologous pairing with the yeast Rad55 protein, is expressed. In this study, a two-hybrid analysis conducted with the use of a human brain cDNA library revealed that the major Xrcc3-interacting protein is a Rad51 paralog, Rad51C/Rad51L2. The purified Xrcc3·Rad51C complex, which shows apparent 1:1 stoichiometry, was found to catalyze the homologous pairing. Although the activity is reduced, the Rad51C protein alone also catalyzed homologous pairing, suggesting that Rad51C is a catalytic subunit for homologous pairing. The DNA-binding activity of Xrcc3·Rad51C was drastically decreased in the absence of Xrcc3, indicating that Xrcc3 is important for the DNA binding of Xrcc3·Rad51C. Electron microscopic observations revealed that Xrcc3·Rad51C and Rad51C formed similar filamentous structures with circular single-stranded DNA.

Double-strand breaks (DSBs) in chromosomal DNA frequently occur when cells are exposed to various DNA-damaging agents, including ionizing radiation, crosslinking reagents, and oxidative stress. In eukaryotes, two major pathways that repair lethal chromosomal DSBs have been identified: homologous recombination and nonhomologous DNA end joining (1–7). The homologous recombination pathway is important for accurate DNA repair, which is indispensable for chromosomal maintenance (8–11). Homologous pairing, in which a single-stranded DNA (ssDNA) tail derived from a DSB site invades the homologous double-stranded DNA (dsDNA) to form a heteroduplex, is a key step in the homologous recombination pathway (12–14). In meiotic cells, the human Dmc1 protein is known to catalyze homologous pairing (15). However, thus far, the human Rad51 protein (HsRad51) has been considered to be the only protein factor that catalyzes homologous pairing during the recombinational repair process in mitotic cells (16–19).

The chromosomal DNA in the brain is at particularly high risk of DNA damage, including DSBs, because of the by-products of extensive oxidative respiration (20). However, in mammals, the expression of *RAD51* mRNA is extremely low in the brain (21, 22). Therefore, the brain might have a brain-specific activator of Rad51 or an unidentified functional homologue of Rad51. In the yeast *Saccharomyces cerevisiae*, the homologous pairing catalyzed by the Rad51 protein (ScRad51) is remarkably enhanced by the heterodimer of the Rad55 and Rad57 proteins (ScRad55 and ScRad57, respectively), probably through direct binding of ScRad55 to ScRad51, in the presence of replication protein A (19, 23–25). No human homologue has been identified for ScRad55. On the other hand, the human Xrcc3 protein appears to be a homologue of ScRad57, because of the significant sequence identity of about 30% (26). A phylogenetic analysis also revealed that Xrcc3 is evolutionarily very close to the *Schizosaccharomyces pombe* rhp57 protein, a functional homologue of ScRad57 (27).

The Xrcc3 protein is the product of one of the human x-ray-repair cross-complementing (*XRCC*) genes, which complement the hamster mutant cell phenotypes of extreme sensitivity to DNA damage and severe chromosome instability (26, 28, 29). Recently it has been reported that Xrcc3 is important to the promotion of the repair of DSBs by homologous recombination and is associated with the development of melanoma skin cancer (30, 31). In mammals, the *XRCC3* mRNA is expressed in the brain, as well as in the testis and the spleen, unlike in the case of HsRad51 (21, 26). In a yeast two-hybrid analysis, it has been reported that Xrcc3 interacts with HsRad51 (26). The Xrcc3 protein might enhance the HsRad51-dependent homologous pairing to overcome the risk of unrepaired DNA due to the small amount of HsRad51 in the brain. On the other hand, it has been found, by a two-hybrid analysis, that Xrcc3 interacts with the human Rad51C protein (Rad51C), which exhibits the highest sequence identity (27%) with HsRad51 (32).

In the present study, a two-hybrid screening with a human brain cDNA library showed that the major Xrcc3-interacting protein is Rad51C, not HsRad51. The stable complex formed between Xrcc3 and Rad51C exhibited homologous-pairing activity *in vitro*.

Materials and Methods

Proteins. The *XRCC3* and *Rad51C* genes were cloned from human brain cDNA (purchased from CLONTECH) by PCR. The HsRad51 protein was purified by the method previously described, with an additional Mono-Q column chromatography step (33). The overexpression plasmid for either Xrcc3·Rad51C or Rad51C was introduced into *Escherichia coli* JM109 (DE3) cells with the plasmid containing the genes for *E. coli* tRNA Arg-3 and tRNA Arg-4, which recognize the CGG and AGA/AGG codons, respectively. The cells were grown in 10 liters of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 30°C for 10 h, and then 200 μM isopropyl β-thiogalactopyranoside was added to induce protein expression. The proteins were produced for 12 h at 30°C in the presence of isopropyl β-thiogalactopyranoside, and the cells were harvested. The Rad51C protein contains a His_6 tag, and Xrcc3

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Abbreviations: DSB, double-strand break; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; XRCC or Xrcc, x-ray-repair cross-complementing.

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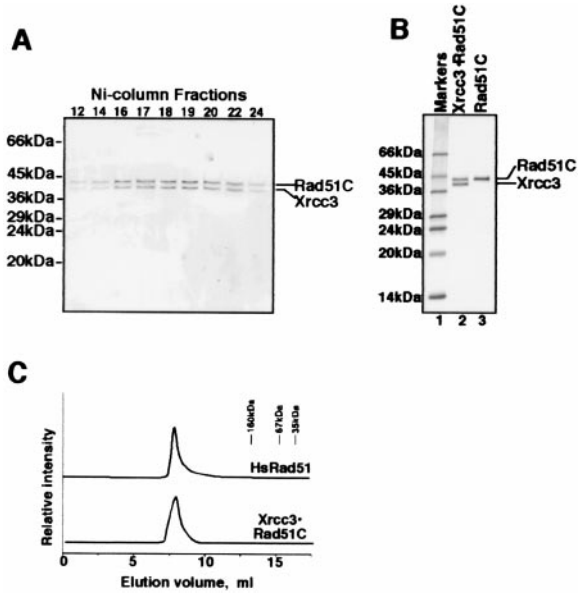

Fig. 1. The purified Xrcc3·Rad51C and Rad51C proteins. (*A*) SDS/12% PAGE of Ni column fractions of Xrcc3·Rad51C. Fractions (3 μl) containing Xrcc3·Rad51C were analyzed by SDS/12% PAGE. Fraction numbers are indicated at the top of the gel. (*B*) SDS/12% PAGE of the purified Xrcc3·Rad51C and Rad51C proteins. Lane 2 is Xrcc3·Rad51C (0.5 μg) eluted from an Affi-Gel-heparin column, and lane 3 is Rad51C (0.5 μg) eluted from Ni nitrilotriacetate agarose. Lane 1 is molecular mass markers. The proteins were visualized by Coomassie brilliant blue staining. (*C*) Elution profiles of Xrcc3·Rad51C and HsRad51 in Superdex 200 h chromatography. Xrcc3·Rad51C (20 μg) and HsRad51 (20 μg) were analyzed by Superdex 200 h column chromatography. (*Upper*) Elution profile of HsRad51. (*Lower*) Elution profile of Xrcc3·Rad51C.

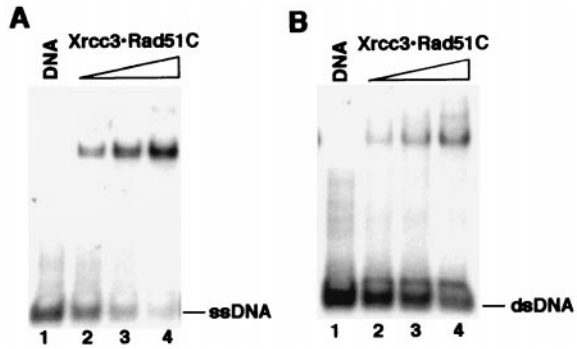

Fig. 2. DNA-binding activity of Xrcc3·Rad51C. (*A*) The ssDNA binding of Xrcc3·Rad51C. A ^{32}P-labeled single-stranded 50-mer oligonucleotide (100 nM), which does not contain intramolecular base pairing, was incubated with Xrcc3·Rad51C at 37°C for 10 min, and the reactions were analyzed by nondenaturing 8% PAGE in TBE buffer. Concentrations of Xrcc3·Rad51C were 55 nM (lane 2), 110 nM (lane 3), and 220 nM (lane 4) and were calculated as heterodimers. (*B*) The dsDNA binding of Xrcc3·Rad51C. A ^{32}P-labeled double-stranded 50-mer oligonucleotide (200 nM) was used as the substrate. Concentrations of Xrcc3·Rad51C were 55 nM (lane 2), 110 nM (lane 3), and 220 nM (lane 4) and were calculated as heterodimers.

contains a FLAG tag at the N terminus. The cells producing either Xrcc3·Rad51C or Rad51C were resuspended in 20 ml of buffer, containing 20 mM sodium phosphate (pH 7.9) and 0.5 M NaCl, and were disrupted by sonication. The samples were centrifuged for 20 min at 30,000 $\times$ *g*, and the supernatants were incubated with 4 ml of Ni nitrilotriacetate agarose (Qiagen, Chatsworth, CA) for 1 h at 4°C. Then, the resin bound to either Xrcc3·Rad51C or Rad51C was packed into a column (1.6 $\times$ 2 cm; 4 ml matrix) and was washed with 300 ml of buffer containing 20 mM Tris·HCl (pH 8.5), 30 mM imidazole, and 0.5 M NaCl. Subsequently, the resin was washed with 150 ml of buffer containing 20 mM Tris·HCl (pH 8.5) and 30 mM imidazole. The proteins were eluted by a gradient of imidazole from 30 mM to 400 mM in 20 mM Tris·HCl (pH 8.5), and Xrcc3·Rad51C (3 mg) and Rad51C (2.5 mg) were eluted at 100 mM imidazole. The samples eluted from the Ni column were applied to an Affi-Gel-heparin column (1.6 $\times$ 5 cm, 10 ml; Bio-Rad) previously equilibrated with buffer containing 20 mM Tris·HCl (pH 8.0), 2 mM DTT, and 10% glycerol. The Xrcc3·Rad51C proteins (1–2 mg) were eluted at 600 mM NaCl by a gradient of NaCl from 0 mM to 1,200 mM. The proteins were dialyzed against a buffer containing 20 mM Tris·HCl (pH 8.1), 2 mM DTT, and 10% glycerol. The proteins eluted from the Affi-Gel-heparin column were further analyzed by Superdex 200 h gel filtration chromatography (Amersham Pharmacia). The purified proteins were stored in 20 mM Tris·HCl (pH 8.1)/5 mM DTT/10% glycerol. The concentrations of the proteins were determined with a Bio-Rad protein assay kit (Bio-Rad), with BSA as the standard protein.

To confirm the functional integrity of the tagged Xrcc3 and Rad51C proteins used in this study, we expressed these tagged proteins in chicken DT40 cells lacking the corresponding genes (M.T. and S.T., unpublished observations). The two knockout cell lines, *XRCC3*$-/-$ and *Rad51C*$-/-$, showed extreme sensitivities to cisplatin, a DNA cross-linking agent (data not shown). Both tagged Xrcc3 and Rad51C proteins fully complemented the cisplatin sensitivity of the *XRCC3*$-/-$ and *Rad51C*$-/-$ cells, respectively (data not shown), as well as the tag-free proteins.

Oligonucleotides. HPLC-purified oligonucleotides (50-mer and 120-mer) for ssDNA/dsDNA-binding and D-loop-formation assays were purchased from Roche Molecular Biochemicals. The nucleotide sequences were as follows: 50-mer: 5′-ATTTC ATGCT AGACA GAAGA ATTCT CAGTA ACTTC TTTGT GCTGT GTGTA-3′; 120-mer: 5′-ATTTC TTCAT TTCAT GCTAG ACAGA AGAAT TCTCA GTAAC TTCTT TGTGC TGTGT GTATT CAACT CACAG AGTGG AACGT CCCTT TGCAC AGAGC AGATT TGAAA CACTC TTTTT GTAGT-3′.

These sequences were derived from a clone of human α-satellite DNA. The 5′ ends of the oligonucleotides were labeled with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP. A 198-bp fragment of the human α-satellite sequence was cloned into the pGEM-T Easy vector (Promega).

Preparation of Closed Circular dsDNA. To avoid irreversible denaturation of the dsDNA, we prepared plasmid DNA without any treatment that would potentially cause denaturation, such as alkaline treatment. The plasmid DNA (pGsat4; 3,216 bp) containing the human α-satellite sequence was introduced into the *E. coli* DH5α strain, and the cells were cultured for 12–16 h at 37°C. The cells were harvested, mildly disrupted with 0.5 mg/ml lysozyme and 0.1% Sarkosyl, and centrifuged at 28,000 rpm in a Hitachi RP42 rotor for 1 h. The supernatant containing the pGsat4 plasmid DNA was extracted with phenol/chloroform three times, and the pGsat4 DNA in the aqueous phase was precipitated by ethanol. The pellet was dissolved in 1 ml of TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) and was treated with 0.15 mg/ml RNase A at 37°C for 30 min. The pGsat4 DNA was purified by 5–20% sucrose gradient centrifugation at 24,000 rpm in a Hitachi RPS40T rotor for 18 h (34). DNA concentrations are expressed in moles of nucleotides.

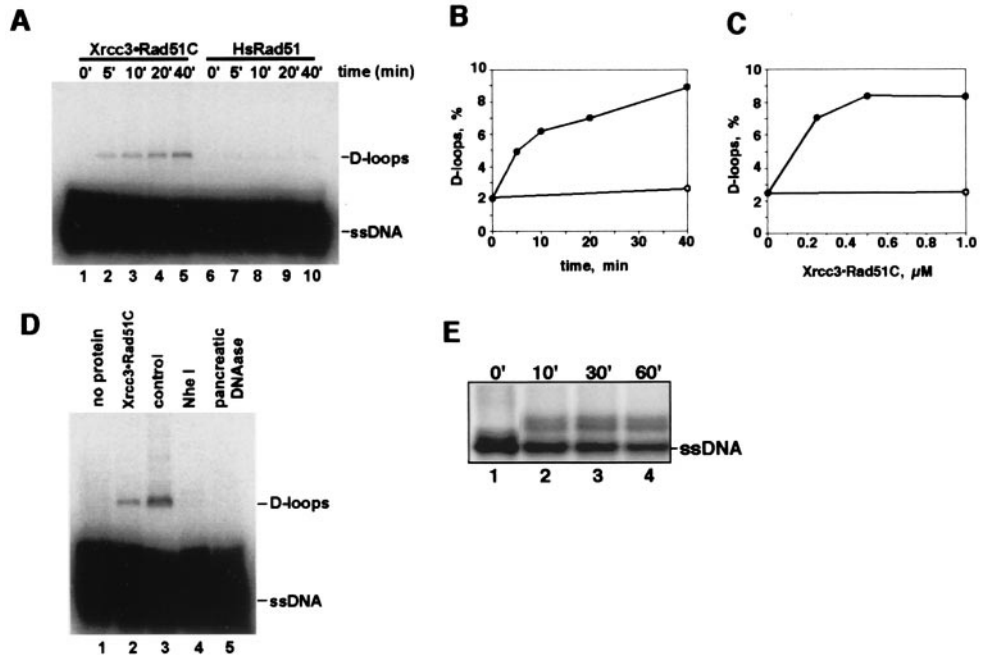

Fig. 3. Homologous-pairing activity of Xrcc3·Rad51C. A ^{32}P-labeled single-stranded 120-mer oligonucleotide (1.6 μM) was incubated with Xrcc3·Rad51C in the standard reaction mixture at 37°C for 5 min, and the reactions were initiated by the addition of 12 mM $MgCl_2$ and pGsat4 form I DNA (13 μM). (*A*) Time course experiments. The concentrations of Xrcc3·Rad51C (as heterodimers) and HsRad51 were 0.5 μM. The reaction times were 0 min (lanes 1 and 6), 5 min (lanes 2 and 7), 10 min (lanes 3 and 8), 20 min (lanes 4 and 9), and 40 min (lanes 5 and 10). (*B*) Graphic representation of time course experiments. The reactions were conducted with a 6.7-fold excess amount of ssDNA (molecule); the percentage of D-loop formation was calculated relative to the limiting amount of dsDNA. ●, Experiments with 0.5 μM Xrcc3·Rad51C; ○, control experiment without Xrcc3·Rad51C. (*C*) Graphic representation of protein titration experiments. The reactions were continued for 40 min. The reactions were conducted with a 6.7-fold excess amount of ssDNA (molecule); the percentage of D-loop formation was calculated relative to the limiting amount of dsDNA. ●, Experiments with homologous ssDNA and dsDNA; ○, control experiment with heterologous ssDNA and dsDNA. (*D*) Dissociation of D-loops by spontaneous branch migration. After a 40-min incubation with 0.5 μM Xrcc3·Rad51C, the D-loops formed by the reactions were treated with *Nhe*I (5 units) for 30 min at 37°C (lane 4) or pancreatic DNase (15 ng/ml) for 30 s at room temperature (lane 5). Lane 1 is a negative control without protein, and lane 2 is a control without DNase treatment. Lane 3 is a D-loop formed by the nonenzymatic method (36). (*E*) Homologous-pairing activity of Xrcc3·Rad51C between double-stranded and single-stranded oligonucleotides. A ^{32}P-labeled single-stranded 50-mer oligonucleotide (600 nM) and a double-stranded 50-mer oligonucleotide (1.2 μM) were used as substrates. The concentration of Xrcc3·Rad51C was 200 nM. Reactions were continued for 0 min (lane 1), 10 min (lane 2), 30 min (lane 3), and 60 min (lane 4). Products were deproteinized and were analyzed by 12% PAGE.

ssDNA-Binding Assay. A ^{32}P-labeled single-stranded 50-mer oligonucleotide (100 nM) or single-stranded pGsat4 DNA (30 μM) was mixed with Xrcc3·Rad51C (55 nM, 110 nM, and 220 nM) in 10 μl of standard reaction buffer, containing 20 mM Tris·HCl (pH 8.1), 2 mM ATP, 20 mM creatine phosphate, 1 mM DTT, 100 μg/ml BSA, 12 units/ml creatine kinase, 15 mM $MgCl_2$, and 3% glycerol. The reaction mixtures were incubated at 37°C for 10 min and were analyzed by nondenaturing 8% polyacrylamide (single-stranded 50-mer) or by 0.8% agarose gel (single-stranded pGsat4) electrophoresis in TBE buffer (90 mM Tris/64.6 mM boric acid/2 mM EDTA, pH 8.0) (for polyacrylamide gel), or 0.5× TBE buffer (for agarose gel).

dsDNA-Binding Assay. The HPLC-purified oligonucleotide (50-mer) was annealed to its complementary strand (50-mer) to create a blunt-ended, double-stranded 50-mer. The double-stranded 50-mer was purified by nondenaturing 8% PAGE. Superhelical pGsat4 DNA (18 μM; 3,216 bp) or ^{32}P-labeled double-stranded 50-mer oligonucleotide (100 nM) was mixed with Xrcc3·Rad51C (55 nM, 110 nM, and 220 nM) in 10 μl of the standard reaction buffer, containing 20 mM Tris·HCl (pH 8.1), 2 mM ATP, 20 mM creatine phosphate, 1 mM DTT, 100 μg/ml BSA, 12 units/ml creatine kinase, 15 mM $MgCl_2$, and 6% glycerol. The reaction mixtures were incubated at 37°C for 10 min and were analyzed by 0.8% agarose gel (pGsat4 DNA) or 8% nondenaturing polyacrylamide gel (double-stranded 50-mer oligonucleotide) electrophoresis. In the case of the agarose gel assay, the samples were electrophoresed for 4 h at 3 V/cm in 0.5× TBE buffer, and the bands were visualized by ethidium bromide staining.

Assay for Homologous Pairing of ssDNA and dsDNA. A ^{32}P-labeled single-stranded 120-mer oligonucleotide (1.6 μM) was mixed with Xrcc3·Rad51C in 10 μl of standard reaction buffer, containing 50 mM Tris·HCl (pH 8.0), 2 mM ATP, 20 mM creatine phosphate, 1 mM DTT, 100 μg/ml BSA, 12 units/ml creatine kinase, and 1 mM $MgCl_2$. The mixtures were incubated at 37°C for 5 min, and the reaction was started by the addition of 12 mM $MgCl_2$ and pGsat4 form I DNA (13 μM). After an incubation at 37°C for the indicated time, the reaction was terminated by the addition of 0.5% sodium lauryl sulfate (SDS), and the proteins were removed from the DNA by treatment with proteinase K (700 μg/ml), followed by an incubation at 37°C for 15 min. The products of homologous pairing (D-loops) were separated from the unreacted DNA substrates by 0.8% agarose gel electrophoresis in 0.5× TBE buffer. The amounts of labeled ssDNA fragments incorporated into the D-loops were quantified with a Fuji BAS2500 image analyzer.

In the experiments with *Nhe*I (5 units) or pancreatic DNase (15 ng/ml), concentrations of these nucleases were just enough to convert form I DNA into form III or form II.

Results and Discussion

Interaction Between Xrcc3 and Rad51C in the Human Brain cDNA Library. We searched for Xrcc3-interacting factors from the human brain cDNA library by means of a two-hybrid analysis, because

XRCC3 mRNA is expressed in the brain. No HsRad51 clone was detected among those positively interacting with Xrcc3, probably because of the very low level of *RAD51* expression (21, 22). On the other hand, half of the positive clones (9 of 18) encoded Rad51C. The other nine clones were all different (data not shown).

The Xrcc3 and Rad51C Complex Formation *in Vitro*. We coexpressed Xrcc3 and Rad51C in the *E. coli* recA-JM109(DE3) strain as FLAG-tagged and His_6-tagged proteins, respectively. Both Xrcc3 and Rad51C were detected in the soluble fraction and were coeluted from Ni nitrilotriacetate agarose (Fig. 1*A*) or Anti-FLAG M2 affinity gel (Kodak). The Xrcc3 and Rad51C proteins were further copurified by Affi-Gel-heparin (Bio-Rad) column chromatography (Fig. 1*B*, lane 2) and were eluted in the column void volume from Superdex 200 by gel filtration chromatography (Amersham Pharmacia), which was similar to the process used to purify HsRad51 (Fig. 1*C*). These results indicate that Xrcc3 and Rad51C form a stable complex. When the recombinant Xrcc3 and Rad51C proteins were independently overexpressed, Rad51C was detected in the soluble fraction, but Xrcc3 was not. Therefore, we purified Rad51C alone by Ni nitrilotriacetate agarose chromatography (Fig. 1*B*, lane 3). Endonuclease and exonuclease activities were not detected in the Xrcc3·Rad51C and Rad51C fractions (data not shown). The purified Xrcc3·Rad51C complex showed apparent 1:1 stoichiometry on an SDS/12% polyacrylamide gel stained with Coomassie brilliant blue (Fig. 1*B*, lane 2) and exhibited both ssDNA-binding and dsDNA-binding activities (Fig. 2).

Homologous-Pairing Activity of the Xrcc3·Rad51C Complex. It has been reported that the ScRad57·ScRad55 complex interacts with ScRad51 and enhances the ScRad51-dependent homologous pairing (19, 25). Assuming an analogy to the ScRad57·ScRad55 complex, we tested the Xrcc3·Rad51C complex for its effect on HsRad51-dependent homologous pairing. In fact, Xrcc3·Rad51C did not enhance the homologous-pairing activity of HsRad51 (data not shown). To test for the homologous-pairing activity, we used the D-loop formation assay, which is a standard assay for homologous pairing by the *E. coli* RecA protein (12). The superhelical dsDNA used in this assay was prepared by a method without alkali treatment to avoid denaturation of the double helix of the dsDNA. In this assay, the D-loop formed between homologous ssDNA fragments and superhelical dsDNA is the product of homologous pairing. As shown in Fig. 3, the homologous pairing promoted by HsRad51 was very low when a 120-mer oligonucleotide (38% GC content) was used as a substrate (Fig. 3*A*, lanes 7–10). It has been reported that in the case of HsRad51, DNA having a GC content of 37% showed decreased homologous pairing (2-fold) and strand exchange (4-fold) relative to a 16% GC sequence (18). In addition, Mazin *et al.* (35) reported that HsRad51 formed little D-loops when a short oligonucleotide (90-mer) was used as the substrate. These previously reported results are consistent with the results presented in Fig. 3*A*. On the other hand, we found that the Xrcc3·Rad51C complex itself can catalyze homologous pairing between a ssDNA fragment and superhelical dsDNA without HsRad51 (Fig. 3 *A–C*). The homologous pairing by Xrcc3·Rad51C was detected only with a homologous combination of ssDNA and dsDNA, but not with a heterologous combination (Fig. 3*C*). The migration distance of the D-loop formed by Xrcc3·Rad51C was exactly the same as that of the control D-loop formed by the nonenzymatic method of Holloman *et al.* (36) (Fig. 3*D*, lanes 2 and 3). Furthermore, the D-loops formed by Xrcc3·Rad51C were dissociated by spontaneous branch migration, when the superhelical tension of the D-loops was released by cutting at a nonhomologous site with *Nhe*I (Fig. 3*D*, lane 4) or by nicking with pancreatic DNase (37) (Fig. 3*D*, lane 5). The Xrcc3·Rad51C complex also promotes homologous pairing between single-stranded and double-stranded oligonucleotides (Fig. 3*E*), indicating that superhelicity of the dsDNA is not required for the activity. These data clearly show that the reaction products were authentic D-loops, and thus we conclude that Xrcc3·Rad51C promotes homologous pairing, that is, the invasion by ssDNA of a double helix at the homologous region followed by the formation of a heteroduplex with its complementary sequence in the dsDNA.

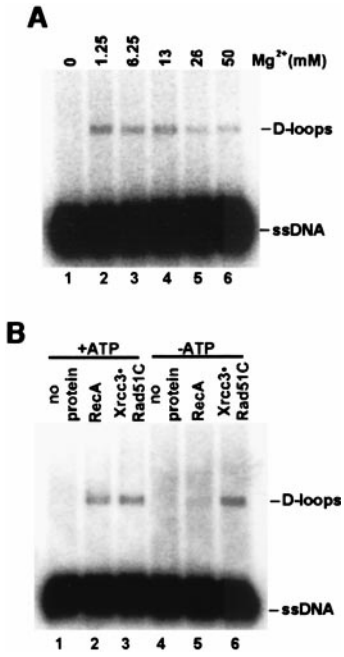

Fig. 4. Mg^{2+} and ATP requirements for homologous pairing by Xrcc3·Rad51C. (*A*) Mg^{2+} dependence of homologous pairing by Xrcc3·Rad51C. A ^{32}P-labeled single-stranded 120-mer oligonucleotide (1.6 μM) was incubated with Xrcc3·Rad51C (0.5 μM) at 37°C for 5 min in the presence of various amounts of $MgCl_2$. The reactions were initiated by the addition of pGsat4 form I DNA (13 μM) and were continued for 10 min. $MgCl_2$ concentrations are 0 mM (lane 1), 1.25 mM (lane 2), 6.25 mM (lane 3), 13 mM (lane 4), 26 mM (lane 5), and 50 mM (lane 6). (*B*) ATP dependence of homologous pairing by Xrcc3·Rad51C. A ^{32}P-labeled single-stranded 120-mer oligonucleotide (1.6 μM) was incubated with RecA (0.1 μM) or Xrcc3·Rad51C (0.5 μM) at 37°C for 5 min in the presence or absence of ATP, and the reactions were initiated by the addition of 12 mM $MgCl_2$ and pGsat4 form I DNA (13 μM). Lanes 1–3 are reactions with ATP, and lanes 4–6 are reactions without ATP. Lanes 1 and 4 are negative controls without protein. Lanes 2 and 5 are the reactions with RecA, and lanes 3 and 6 are the reactions with Xrcc3·Rad51C.

The Xrcc3·Rad51C complex required Mg^{2+} for homologous pairing. There are little differences in the efficiency of D-loop formation in the range of 1.25–13 mM Mg^{2+}, and the products were detected even in the presence of 50 mM Mg^{2+} (Fig. 4*A*). ATP does not appear to be required for the homologous pairing by Xrcc3·Rad51C, although it is required for the reaction by RecA (Fig. 4*B*). This reaction may be an ATP-independent homologous pairing, like those catalyzed by the *E. coli* RecT protein (38) and the *S. cerevisiae* and human Rad52 proteins (39, 40), but we cannot exclude the possibility that the recombinant Xrcc3·Rad51C sample contained tightly bound ATP (41, 42).

The Roles of Xrcc3 and Rad51C in the Complex. As shown in Fig. 5, Xrcc3·Rad51C binds superhelical dsDNA and circular ssDNA (Fig. 5 *A* and *B*, lanes 2–4). Interestingly, we found that Rad51C alone still bound dsDNA and ssDNA, but its abilities were drastically lower than those of the Xrcc3·Rad51C complex (Fig. 5 *A* and *B*). These results indicate that Xrcc3 is an important subunit for DNA binding by the Xrcc3·Rad51C complex. Elec-

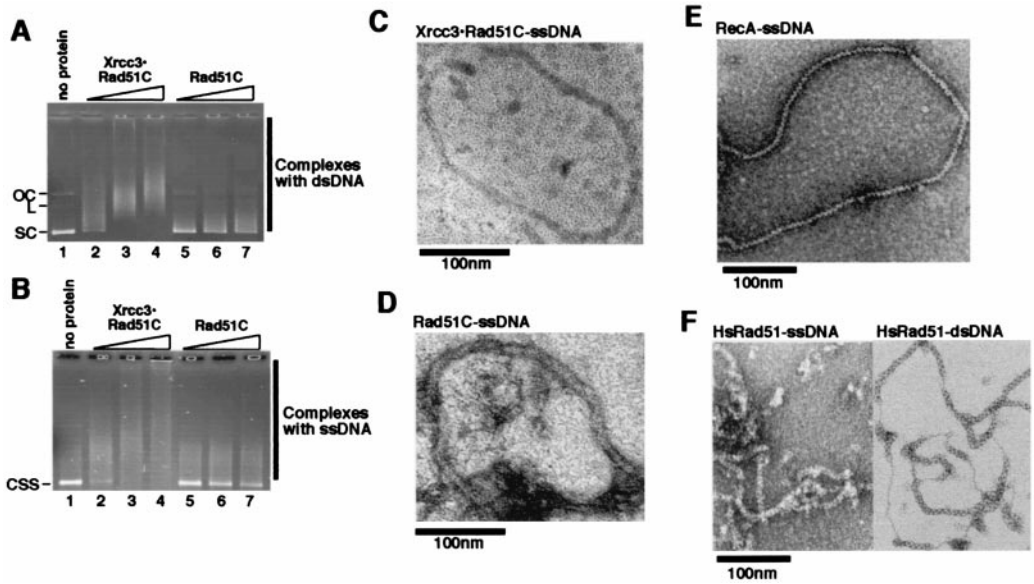

Fig. 5. DNA binding and electron microscopic visualizations of Xrcc3·Rad51C and Rad51C. (*A*) Superhelical dsDNA binding by Xrcc3·Rad51C and Rad51C. Superhelical dsDNA (10 μM) was incubated with Xrcc3·Rad51C or Rad51C at 37°C for 10 min, and the reactions were analyzed by 0.8% agarose gel electrophoresis in 0.5× TBE buffer. The concentrations of Xrcc3·Rad51C and Rad51C used in the DNA-binding experiments were 0.4 μM, 0.7 μM, and 1.0 μM. The concentration of Xrcc3·Rad51C was calculated as heterodimers. (*B*) Circular ssDNA binding by Xrcc3·Rad51C and Rad51C. Superhelical dsDNA (30 μM) was incubated with Xrcc3·Rad51C at 37°C for 10 min, and the reactions were analyzed by 0.8% agarose gel electrophoresis in 0.5× TBE buffer. The concentrations of Xrcc3·Rad51C and Rad51C used in the DNA-binding experiments were 0.4 μM, 0.7 μM, and 1.0 μM. The concentration of Xrcc3·Rad51C was calculated as heterodimers. (*C*) Electron microscopic visualization of Xrcc3·Rad51C complexed with ssDNA. The complexes were visualized by negative staining with uranyl acetate. (The magnification bar represents 100 nm.) (*D*) Electron microscopic visualization of Rad51C complexed with ssDNA. (*E*) Electron microscopic visualization of RecA complexed with ssDNA. (*F*) Electron microscopic visualization of HsRad51 complexed with ssDNA and dsDNA. (*Left*) HsRad51 complexed with ssDNA. (*Right*) HsRad51 complexed with dsDNA.

tron microscopic observations revealed that Xrcc3·Rad51C bound circular ssDNA and formed a filamentous structure with ssDNA (Fig. 5*C*). It is known that the proteins that promote homologous pairing, for example, RecA and HsRad51, also form filamentous structures on ssDNA (Fig. 5 *E* and *F*). The formation of the filamentous structure on ssDNA, by which the secondary structure of the ssDNA is unfolded, may be a common key step for the homologous-pairing proteins. Surprisingly, Rad51C alone, which bound circular ssDNA, also formed a filamentous structure similar to that of Xrcc3·Rad51C (Fig. 5*D*). This result suggests that Rad51C may be a catalytic subunit for homologous pairing of Xrcc3·Rad51C. Indeed, Rad51C alone promoted homologous pairing, although at a reduced rate as compared with that of Xrcc3·Rad51C (Fig. 6).

Conclusions and Perspectives. Our results presented here show that Xrcc3·Rad51C has homologous-pairing activity. The Xrcc3-deficient cells, irs1SF, are defective in chromosome maintenance through homology-dependent recombinational repair (28, 30), indicating that the homologous-pairing activity of the Rad51 protein is not sufficient to maintain the chromosomal integrity. These results suggest that these homologous-pairing proteins, Xrcc3·Rad51C and HsRad51, have specific functions during recombinational repair. It has been reported that sister chromatid exchanges, which occur after DNA replication, are mediated by Rad51-dependent homologous recombination (43). On the other hand, neuron cells require homologous chromosomes, rather than the sister chromatids, as templates for homologous recombinational repair, because the cells are arrested before DNA synthesis (the G_0 phase). In the brain, *XRCC3* is expressed, as mentioned above, and *Rad51C* expression has also been observed (32). The Xrcc3·Rad51C complex may be important for the catalysis of homologous pairing between homologous chromosomes during recombination repair in the brain, where the Rad51 protein is very low. In tissues other than the brain, the Xrcc3·Rad51C complex may be required for recombinational repair before DNA replication during the cell cycle (G_1 phase), in which the sister chromatids are not available. In this context, it should be noted here that the yeast Dmc1 protein, a meiosis-specific homologue of Rad51, is known to have a specific function in recombination between homologous chromosomes (44). Further analyses will be required to establish the role of the Xrcc3·Rad51C-dependent homologous pairing between homologous chromosomes *in vivo*. It has been reported that Xrcc3 is necessary for HsRad51 assembly on damaged chro-

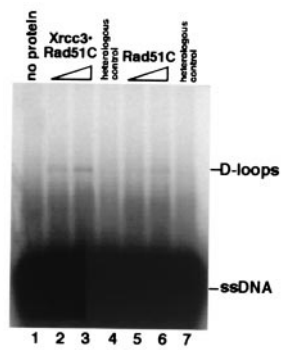

Fig. 6. Homologous-pairing activity of Rad51C. A ^{32}P-labeled single-stranded 50-mer oligonucleotide (1 μM) was incubated with Xrcc3·Rad51C in the standard reaction mixture at 37°C for 5 min, and the reactions were initiated by the addition of 12 mM $MgCl_2$ and pGsat4 form I DNA (13 μM). The reactions were continued for 20 min. Lane 1 is the negative control without protein. Lanes 2–4 are the experiments with Xrcc3·Rad51C, and lanes 5–7 are the experiments with Rad51C alone. The concentrations of Xrcc3·Rad51C and Rad51C used in the DNA-binding experiments were 0.2 μM (lanes 2 and 5) and 0.6 μM (lanes 3, 4, 6, and 7). Lanes 4 and 7 are the negative controls with heterologous ssDNA and dsDNA. The concentration of Xrcc3·Rad51C was calculated as heterodimers.

mosomes *in vivo* (45). The assembly of both Xrcc3·Rad51C and HsRad51 on the damaged sites of chromosomes might be important for efficient homologous recombinational repair in dividing cells.

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1. Moore, J. K. & Haber, J. E. (1996) *Mol. Cell. Biol.* **16,** 2164–2173.
2. Teo, S. H. & Jackson, S. P. (1997) *EMBO J.* **16,** 4788–4795.
3. Wilson, T. E., Grawunder, U. & Lieber, M. R. (1997) *Nature (London)* **388,** 495–498.
4. Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A. & Takeda, S. (1998) *EMBO J.* **17,** 5497–5508.
5. Kanaar, R., Hoeijmakers, J. H. & van Gent, D. C. (1998) *Trends Cell Biol.* **8,** 483–489.
6. Paques, F. & Haber J.E. (1999) *Microbiol. Mol. Biol. Rev.* **63,** 349–404.
7. Jasin, M. (2000) *Cancer Invest.* **18,** 78–86.
8. Sonoda, E., Sasaki, M. S., Buerstedde, J-M., Bezzubova, O., Shinohara, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y. & Takeda, S. (1998) *EMBO J.* **17,** 598–608.
9. Baumann, P. & West, S. C. (1998) *Trends Biochem. Sci.* **23,** 247–251.
10. Thacker, J. (1999) *Biochimie* **81,** 77–85.
11. Thompson, L. H. & Schild, D. (1999) *Biochimie* **81,** 87–105.
12. Shibata, T., DasGupta, C., Cunningham, R. P. & Radding, C. M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1638–1642.
13. McEntee, K., Weinstock, G. M. & Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2615–2619.
14. Sung, P. (1994) *Science* **265,** 1241–1243.
15. Li, Z., Golub, E. I., Gupta, R. & Radding, C. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11221–11226.
16. Baumann, P., Benson, F. E. & West, S. C. (1996) *Cell* **87,** 757–766.
17. Gupta, R. C., Bazemore, L. R., Golub, E. I. & Radding, C. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 463–468.
18. Gupta, R. C., Folta-Stogniew, E., O'Malley, S., Takahashi, M. & Radding, C. M. (1999) *Mol. Cell* **4,** 705–714.
19. Mazin, A. V., Zaitseva, E., Sung, P. & Kowalczykowski, S. C. (2000) *EMBO J.* **19,** 1148–1156.
20. Jellinger, K. A. (1999) *Drugs Aging* **14,** 115–140.
21. Shinohara, A., Ogawa, H., Matsuda, Y., Ushio, N., Ikeo, K. & Ogawa, T. (1993) *Nat. Genet.* **4,** 239–243.
22. Habu, T., Taki, T., West, A., Nishimune, Y. & Morita, T. (1996) *Nucleic Acids Res.* **24,** 470–477.
23. Hays, S. L., Firmenich, A. A. & Berg, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6925–6929.
24. Johnson, R. D. & Symington, L. S. (1995) *Mol. Cell. Biol.* **15,** 4843–4850.
25. Sung, P. (1997) *Genes Dev.* **11,** 1111–1121.
26. Liu, N., Lamerdin, J. E., Tebbs, R. S., Schild, D., Tucker, J., Shen, M. R., Brookman, K. W., Siciliano, M. J., Walter, C. A., Fan, W., *et al.* (1998) *Mol. Cell* **1,** 783–793.
27. Tsutsui, Y., Morishita, T., Iwasaki, H., Toh, H. & Shinagawa, H. (2000) *Genetics* **154,** 1451–1461.
28. Tebbs, R. S., Zhao, Y., Tucker, J. D., Scheerer, J. B., Siciliano, M. J., Hwang, M., Liu, N., Legerski, R. J. & Thompson, L. H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6354–6358.
29. Cui, X., Brenneman, M., Meyne, J., Oshimura, M., Goodwin, E. H. & Chen, D. J. (1999) *Mutat. Res.* **434,** 75–88.
30. Pierce, A. J., Johnson, R. D., Thompson, L. H. & Jasin, M. (1999) *Genes Dev.* **13,** 2633–2638.
31. Winsey, S. L., Haldar, N. A., Marsh, H. P., Bunce, M., Marshall, S. E., Harris, A. L., Wojnarowska, F. & Welsh, K. I. (2000) *Cancer Res.* **60,** 5612–5616.
32. Dosanjh, M. K., Collins, D. W., Wufang, F., Lennon, G. G., Albala, J. S., Shen, Z. & Schild, D. (1998) *Nucleic Acids Res.* **26,** 1179–1184.
33. Aihara, H., Ito, Y., Kurumizaka, H., Yokoyama, S. & Shibata, T. (1999) *J. Mol. Biol.* **290,** 495–504.
34. Shibata, T., Cunningham, R. P. & Radding, C. M. (1981) *J. Biol. Chem.* **256,** 7557–7564.
35. Mazin, A. V., Bornarth, C. J., Solinger, J. A., Heyer, W. D. & Kowalczykowski, S. C. (2000) *Mol. Cell* **6,** 583–592.
36. Holloman, W. K., Wiegand, R., Hoessli, C. & Radding, C. M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 2394–2398.
37. Radding, C. M., Beattie, K. L., Holloman, W. K. & Wiegand, R. C. (1977) *J. Mol. Biol.* **116,** 825–839.
38. Noirot, P. & Kolodner, R. D. (1998) *J. Biol. Chem.* **273,** 12274–12280.
39. Ogawa, T., Shinohara, A., Nabetani, A., Ikeya, T., Yu, X., Egelman, E. H. & Ogawa, H. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **58,** 567–576.
40. Kurumizaka, H., Aihara, H., Kagawa, W., Shibata, T. & Yokoyama, S. (1999) *J. Mol. Biol.* **291,** 537–548.
41. Söderhäll, S. & Lindahl, T. (1973) *J. Biol. Chem.* **248,** 672–675.
42. Zarembinski, T. I., Hung, L. W., Mueller-Dieckmann, H. J., Kim, K. K., Yokota, H., Kim, R. & Kim, S. H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 15189–15193.
43. Sonoda, E., Sasaki M.S., Morrison, C., Yamaguchi-Iwai, Y., Takata, M. & Takeda, S. (1999) *Mol. Cell. Biol.* **19,** 5166–5169.
44. Schwacha, A. & Kleckner, N. (1997) *Cell* **90,** 1123–1135.
45. Bishop, D. K., Ear, U., Bhattacharyya, A., Calderone, C., Beckett, M., Weichselbaum, R. R. & Shinohara, A. (1998) *J. Biol. Chem.* **273,** 21482–21488.