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PRINCIPAL INVESTIGATOR: David J. Chen, Ph.D.

CONTRACTING ORGANIZATION: Ernest Orlando Lawrence Berkeley National Laboratory
Berkeley, California 94720

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David J. Chen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Ernest Orlando Lawrence Berkeley National Laboratory
Berkeley, California 94720

E-Mail: pmgale@lbl.gov

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The BRCA2 breast cancer gene is a major contributor to a dominantly inherited predisposition to the disease. Recent evidence has indicated that the critical functions of BRCA2 associate with chromosome stability and with DNA repair medicated by homologous recombination.

Using homologous recombination assay, we have demonstrated that disruption of the BRCA2-Rad51 interaction inhibits the HRR frequency in vivo. The results indicate that the interactions between Rad51 and BRCA2 are important for regulating HRR.

The 6xHis-tagged baculoviral construct of BRC1-4, BRC5-8 and BRC1-8 fragments of bRCA2 have been made and individually expressed in insect cells. Each protein was purified using a Ni-NTA column and the identity of each protein was confirmed by Western analysis. We have used three assay systems to determine the activity of Rad51B and Rad51C proteins, including DNA binding, ATPase and DNA strand transfer. We have examined the effect of the BRC1 domain on the ATPase activity of Rad51 and found that the BRC1 domain inhibits this activity, suggesting that the BRC1 domain of BRCA2 plays a role in regulating the ATPase activity of Rad51. We will apply these three assay approaches to determine the effects of the BRC fragments (BRC1-4, BRC5-8, BRC1-8) on the activity of Rad51.

The BRC1-4, BRC5-8, and BRC1-8 fragments have been co-expressed with Rad51, Rad51B, and Rad51C in insect cells. The Ni- NTA pull-down experiments have showed that BRCA2 simultaneously interact with Rad51, Rad51B, and Rad51C in vitro. No direct interaction between BRC repeats of BRCA2 and Rad51B (or RAD51C) was observed.

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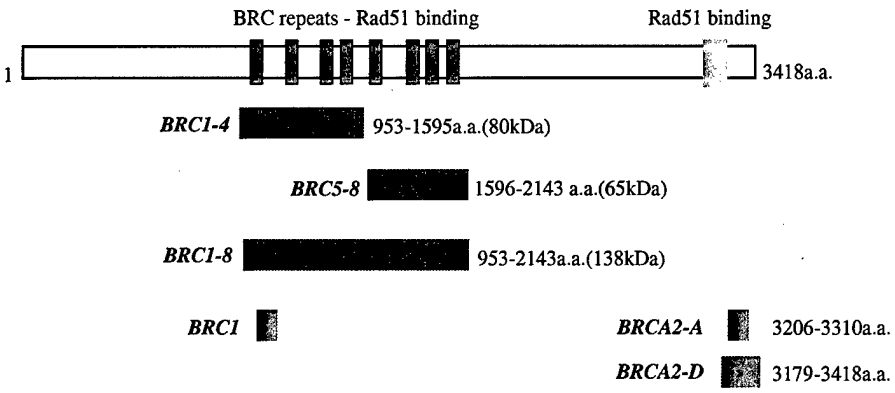
INTRODUCTION

The BRCA2 breast cancer gene is a major contributor to a dominantly inherited predisposition to the disease (reviewed in [1, 2]). Recent evidence for direct interactions between the BRCA2 protein and the homologous recombinational repair protein Rad51 [3-6] has indicated that the critical functions of BRCA2 associate with recombinational DNA repair and chromosome stability. Cells lacking BRCA2 function are hypersensitive to ionizing radiation and exhibit defective DNA repair [7-9]. In these BRCA2-deficient cells, formation of Rad51 foci is severely impaired. The evidence for the functions of BRCA2 in DNA repair has suggested a new concept for their role in predisposition to breast cancer.

This gene encodes a large protein of 3418 amino acids [10, 11]. The BRCA2 protein consists of two Rad51-binding domains, eight BRC repeats [3] and a C-terminal region [4, 5, 15]. These eight conserved BRC repeats (designated as BRC1 to BRC8) [12], located in the central portion of the protein and cover nearly a third of the protein. Since the very large size of BRCA2 might hamper attempts to obtain the full-length protein, we therefore focus our efforts on the known functional domains for Rad51-binding of BRCA2. The domain constructs used in our study are listed in Fig 1. The three specific aims and progress of this project are summarized as the following:

Fig 1. Domain constructs of human BRCA2 protein



BODY

Specific aim 1. To test the hypothesis that disruption of the BRCA2-Rad51 interaction affects homologous recombinational repair (HRR) *in vivo*. We hypothesize that the human BRCA2 protein participates directly in homologous recombinational repair (HRR) through the interaction with the DNA repair protein Rad51 and regulates this process in some critical way, such that the loss of BRCA2 function results in a reduced capacity for repair of chromosome breaks by homologous recombination. An artificial reporter locus has been developed that can detect repair of a specifically induced, chromosomal double-strand break by homologous recombination. The reporter locus has been installed in human HT1080 cells and used to measure their relative capability for homologous recombination. Using this assay system, we have found that transient expression of the BRC1 motif of BRCA2 during double-strand break induction in HT1080 cells greatly suppressed the frequency of HRR by roughly sixteen-fold, suggesting that the BRCA2-Rad51 interaction via BRC1 is important for

regulating HRR. We have further investigated the effects of two Rad51-interacting domains of BRCA2 in the C-terminal region (BRCA2 fragment "A" and "D"). We have found that expression of either one of these two C-terminal fragments of BRCA2 inhibited the HR frequency (Fig 2.), indicating that disruption of the normal interaction between BRCA2 and Rad51 in the C-terminal region of BRCA2 impairs HRR. These results support a conclusion that the BRCA2-Rad51 interaction via either BRC1 domain or a fragment in the C-terminal region of BRCA2 indeed affects homologous recombinational repair (HRR) *in vivo*.

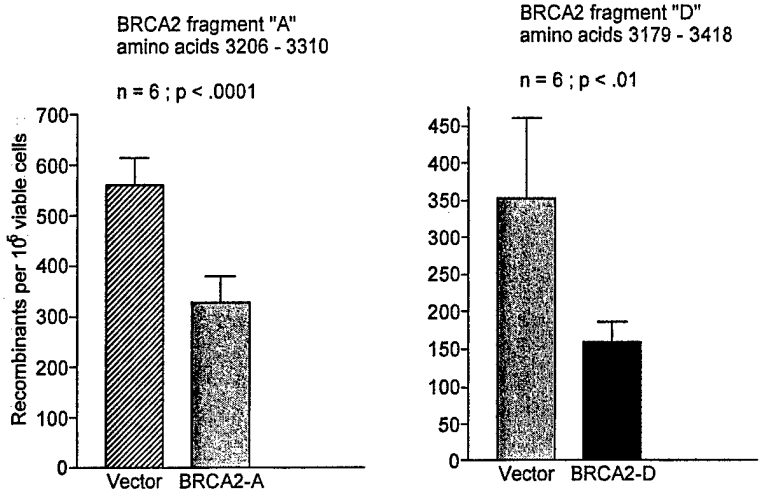


Fig 2. Expression of carboxyl-terminal fragments containing the exon 27 RAD51-binding domain of BRCA2 causes smaller but significant reductions in recombination frequency.

Although a single motif (BRC1) may suppress HRR by a dominant-negative effect, expression of various sizes of the BRC repeats might possibly show different effects. Therefore, we also examined the functions of larger BRCA2 domains for HRR. The BRC1-4, BRC5-8, and BRC1-8 domains of BRCA2 have been individually PCR amplified using our full-length BRCA2 cDNA as a template, and constructed into a modified mammalian expression vector, pIREShyg2, with a Flag tag. The individual fragments have been expressed in HT1080 cells and their effects on HRR are currently evaluated. By comparing the effects of various BRC repeats, the functions of various BRC repeats in regulating HRR can be further determined.

Specific aim 2. To test the hypothesis that the BRCA2 protein functions to mediate the biochemical activities of Rad51. It is not known how the Rad51-BRCA2 interaction regulates DNA homologous recombinational repair. We propose to investigate whether BRCA2 directly mediates the biochemical activities of Rad51 [16-18] and subsequently affects homologous recombination. We have expressed and purified the human Rad51 protein and two Rad51 paralogs, Rad51B and Rad51C, from insect cells Sf9 using the baculovirus system. Employing the baculovirus strategies, we have expressed the BRC fragments of BRCA2, including BRC1-4, BRC5-8, and BRC1-8 domains. They encode proteins of 80-kDa, 65-kDa, and 138-kDa, respectively. These three fragments have been PCR amplified using our BRCA2 full-length cDNA as a template, and individually constructed into a 6xHis-tagged baculoviral vector. Each recombinant protein has been individually expressed in Sf9 cells and the identity of the proteins was confirmed by Western Blotting using either α-BRC4 (aa1323-1346) or α-BRC5 (aa1651-1821) antibody as well as α-His antibody. The purification of the individual fragments is currently undertaking using Ni-NTA and Mono Q column.

2.1. To determine the effect of the BRC repeats on the DNA binding activity of Rad51. In a yeast two-hybrid assay, a small region near the N-terminus of mouse Rad51 has been shown to interact with the C-terminal region of the mouse BRCA2 protein, indicating the possibility of DNA binding of

Rad51 mediated by BRCA2 [3-6]. Therefore, it will be determined whether BRCA2 protein mediates the DNA binding activity of Rad51. We have established a gel shift assay to determine the DNA binding activity using [^{32}P]-labeled oligonucleotides. Using this assay, we have examined the DNA binding activity of Rad51, Rad51B and Rad51C proteins. We have shown that Rad51B and Rad51C bind both single-and double-stranded DNA (ssDNA and dsDNA), and show preference for tailed dsDNA [29]. The effects of the BRC1-4, BRC5-8 and BRC1-8 fragments on the DNA binding activity of Rad51 are currently determined using this assay.

2.2. To determine the effect of the BRC repeats on the ATPase activity of Rad51. It has been shown that the BRCA2-binding region of human Rad51 (amino acids 98-339) contains ATPase activity and is involved in oligomer formation and recombination. Therefore, whether BRCA2 functions to mediate the ATPase activity of Rad51 will be examined.

We have established an *in vitro* ATPase assay and determined the ATPase activity of the Rad51, Rad51B and Rad51C proteins [29]. We have investigated the effect of the BRC1 domain of BRCA2 on the ATPase activity of Rad51. Various ratios of BRC1 and Rad51 were tested for the ATPase activity and an inhibitory effect was observed — about 3.5 fold of inhibition was found with BRC1/Rad51 = 2. The suppression effect of the BRC1 domain on the ATPase activity of Rad51 suggests that the BRC1 domain of BRCA2 play a role in regulating the ATP binding and/or hydrolysis of Rad51.

2.3. To determine the effect of the BRC repeats on the homologous pairing and strand transfer activity of Rad51. The key biochemical activity of Rad51 in the recombinational DNA repair process is to promote strand pairing and exchange between two homologous DNA strands [16-18]. It is very likely that the interaction between BRCA2 and Rad51 influences the strand transfer activity of Rad51 and leads to effects on homologous recombination.

We have established a DNA strand transfer assay using ssDNA 63mers and [^{32}P]-labeled dsDNA 32/32mers as the substrates. The DNA strand transfer activity of Rad51 was determined as a control. The strand transfer products (3'-tailed 63/32mer) were observed and the amount of product formation is dependent on the concentration of Rad51. We also demonstrated that Rad51C displays apparent DNA strand transfer in an ATP-independent manner, while Rad51B shows no such activity [29]. The effects of BRC1-4, BRC5-8 and BRC1-8 on the DNA strand transfer activity of Rad51 are currently under investigation.

Specific aim 3. To test the hypothesis that the BRCA2 protein forms a stable complex with Rad51 and Rad51 paralogs [19-28]. Our results using a baculovirus co-expression system and Ni-NTA pull-down experiments have supported that Rad51 and two Rad51 paralogs (Rad51B and Rad51C) interact simultaneously and form a novel complex [29]. We therefore proposed to demonstrate that BRCA2 is an essential component in the Rad51-dependent recombinational complex, interacting with these three proteins (Rad51, Rad51B, Rad51C) to form a stable complex and to facilitate assembling of the complex formation. The Ni-NTA pull-down strategies have been used to reach this goal. In addition, whether BRCA2 directly interacts with Rad51B or Rad51C has also been examined.

We have co-expressed the BRC1-4 (or BRC1-8) domain with Rad51, Rad51B and Rad51C in Sf9 cells. The Ni-NTA magnetic beads were used to pull-down 6xHis-tagged BRC1-4 (or BRC1-8)

protein. We have analyzed its associated proteins in the pull-downed fractions. The results showed that a significant amount of Rad51 was pull-downed by BRC fragments as well as a little amount of Rad51C and Rad51B. The results suggested that BRCA2 interacts simultaneously with Rad51, Rad51B and Rad51C and forms a complex with these proteins. In the interaction, Rad51 binds tightly to BRCA2 and these two proteins interact weakly with Rad51C/Rad51B heterodimer through the interaction between Rad51 and Rad51C as shown in Fig 3.

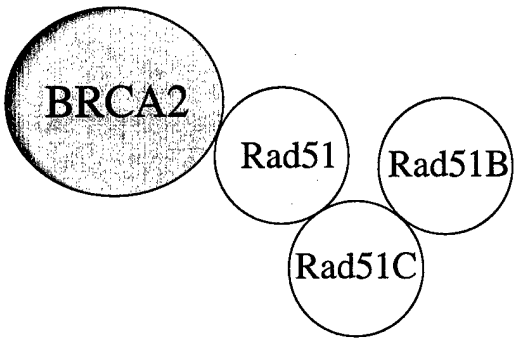


Fig 3. Complex formation between BRCA2, Rad51, Rad51B and Rad51C

In the other hand, to examine the direct interaction between the BRC repeats of BRCA2 and Rad51B (or Rad51C), we have co-transfected the 6xHis-tagged BRC1-4 (or BRC1-8) and untagged Rad51B (or Rad51C) bacmids in Sf9 cells (total four combinations). The protein co-expressions were confirmed by Western analysis using α-BRC4, α-BRC5, α-Rad51B and α-Rad51C antibody. The Ni-NTA magnetic beads were used to bind the 6xHis-tagged BRC domains and whether the untagged Rad51B (or Rad51C) can be pull-downed by the BRC domains has been determined. The results showed that either BRC1-4 or BRC1-8 protein was not able to pull-down the Rad51B or Rad51C protein, suggesting that there is no direct interactions between the BRC repeats of BRCA2 and Rad51B, or BRCA2 and Rad51C. Based on these results, we concluded that BRCA2 forms a multiprotein complex with Rad51, Rad51B and Rad51C through a strong interaction between BRCA2 and Rad51 and a weak interaction between Rad51 and Rad51C. In addition, there is no direct interaction between the BRC repeats of BRCA2 and Rad51B (or Rad51C).

KEY RESEARCH ACCOMPLISHMENTS

1. The BRC1-4 and BRC1-8 fragments of BRCA2 were expressed and purified. However, the whole fragments tend to become proteolysis during the course of purification, indicative of an unstable property of the protein.
2. The BRC1 domain of BRCA2 inhibited the ATPase activity of human Rad51, suggesting that the BRC1 domain of BRCA2 plays a role in regulating the ATP binding and/or hydrolysis of Rad51.
3. The *in vitro* complex formation of BRCA2-Rad51-Rad51C-Rad51B was demonstrated.
4. Rad51B and Rad51C were not found to individually interact with the BRCA2 protein.

REPORTABLE OUTCOMES

1. A journal article related to this project "Complex formation by the human Rad51B and Rad51C DNA repair proteins and their activities *in vitro*" was published in Journal of Biological Chemistry in January, 2003.

CONCLUSIONS

Using homologous recombination assay, we have demonstrated that the BRC1 domain and two fragments of the C-terminal region (aa3206-3310 and 3179-3418) of BRCA2 inhibit the HRR frequency. The results indicate that the interactions between Rad51 and these regions of BRCA2 are important for regulating HRR.

We have made the N-terminal 6xHis-tagged baculoviral construct of BRC1-4, BRC5-8 and BRC1-8 fragments of BRCA2. These three individual fragments have been successfully expressed in insect cells. The baculovirus titers were amplified, so that the expression level of each protein was elevated and were able to be detected by Coomassie staining. Each protein was purified using a Ni-NTA column. We observed lots of proteolysis product of each protein after Ni-NTA purification, indicating that the structure of the BRC repeats of BRCA2 protein is not stable and the proteins tend to degrade. To solve the problem, each fragment will be co-expressed with Rad51 individually. We hope to be able to co-purify a stable BRC1-4/Rad51, BRC5-8/Rad51 or BRc1-8/Rad51 complex for in vitro biochemical activity assays.

We have established three in vitro biochemical assays for Rad51 in our laboratory, including DNA binding, ATPase and DNA strand exchange. We have used these three assay systems to determine the activity of Rad51B and Rad51C proteins and the results have been published in Journal of Biological Chemistry. We have examined the effect of the BRC1 domain on the ATPase activity of Rad51 and found that the BRC1 domain inhibits this activity. The results suggest that the BRC1 domain of BRCA2 plays a role in regulating the ATPase activity of Rad51. We will apply these three assay approaches to determine the effects of the BRC fragments (BRC1-4, BRC5-8, BRC1-8) on the activity of Rad51 when we obtain the purified BRC/Rad51 complex.

The BRC1-4, BRC5-8 and BRC1-8 fragments have been co-expressed with Rad51, Rad51B and Rad51C in insect cells. The Ni- NTA pull-down experiments have showed that BRCA2 simultaneously interact with Rad51, Rad51B and Rad51C *in vitro*. No direct interaction between BRC repeats of BRCA2 and Rad51B (or Rad51C) was observed.

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APPENDICES

1. Lio, Y.-C., Mazin, A.V., Kowalczykowski, S.C., and Chen, D.J. "Complex Formation by the Human Rad51B and Rad51C DNA Repair Proteins and Their Activities *in Vitro*" (2003) *J. Biol. Chem.* **278**, 2469-2478

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Complex Formation by the Human Rad51B and Rad51C DNA Repair Proteins and Their Activities *in Vitro**

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Yi-Ching Lio‡§, Alexander V. Mazin¶‖, Stephen C. Kowalczykowski‖, and David J. Chen‡

From the ‡Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, the ¶Department of Biochemistry, Drexel University College of Medicine, Philadelphia, Pennsylvania 19102-1192, and the ‖Division of Biological Sciences, Section of Microbiology and of Molecular and Cellular Biology, Center for Genetics and Development, University of California, Davis, California 95616-8665

The human Rad51 protein is essential for DNA repair by homologous recombination. In addition to Rad51 protein, five paralogs have been identified: Rad51B/Rad51L1, Rad51C/Rad51L2, Rad51D/Rad51L3, XRCC2, and XRCC3. To further characterize a subset of these proteins, recombinant Rad51, Rad51B-$(His)_6$, and Rad51C proteins were individually expressed employing the baculovirus system, and each was purified from Sf9 insect cells. Evidence from nickel-nitrilotriacetic acid pull-down experiments demonstrates a highly stable Rad51B·Rad51C heterodimer, which interacts weakly with Rad51. Rad51B and Rad51C proteins were found to bind single- and double-stranded DNA and to preferentially bind 3′-end-tailed double-stranded DNA. The ability to bind DNA was elevated with mixed Rad51 and Rad51C, as well as with mixed Rad51B and Rad51C, compared with that of the individual protein. In addition, both Rad51B and Rad51C exhibit DNA-stimulated ATPase activity. Rad51C displays an ATP-independent apparent DNA strand exchange activity, whereas Rad51B shows no such activity; this apparent strand exchange ability results actually from a duplex DNA destabilization capability of Rad51C. By analogy to the yeast Rad55 and Rad57, our results suggest that Rad51B and Rad51C function through interactions with the human Rad51 recombinase and play a crucial role in the homologous recombinational repair pathway.

Homologous recombinational repair (HRR)[1] is an important pathway in repairing DNA double-strand breaks (DSBs) with high accuracy, which is indispensable for the maintenance of genome stability (for review see Refs. 1–4). The human Rad51 protein, a structural and functional homolog of *Escherichia coli* recombinase RecA (5, 6), plays a central role in HRR. *In vitro* studies have shown that Rad51 binds single-stranded and double-stranded DNA (ssDNA and dsDNA), exhibits DNA-dependent ATPase activity, and functions to catalyze homologous pairing and DNA strand exchange (6–8). As a prerequisite step for the action of Rad51 in HRR, Rad51 binds DNA to form highly ordered nucleoprotein filaments (9, 10). It has also been shown that these filaments form preferentially on tailed duplex DNA substrates that mimic the 3′-overhanging ssDNA tails at the sites of DSBs (11). Thus far, Rad51 is the only mitotic protein that catalyzes the key reactions of homologous pairing and strand transfer.

In yeast, the Rad51-related proteins include Rad55 and Rad57, which exist together as a tight heterodimer that interacts weakly with Rad51 (12). These two proteins appear to be Rad51 paralogs, probably derived by gene duplication followed by the evolution of new functions. The Rad55·Rad57 complex acts as a cofactor for the assembly of Rad51 onto ssDNA and functions to promote the DNA strand transfer activity of Rad51 (12). In human cells, in addition to meiotic Dmc1 protein, five Rad51 paralogs have been identified: Rad51B/Rad51L1[2] (13–15), Rad51C/Rad51L2 (16), Rad51D/Rad51L3 (15, 17, 18), XRCC2 (19–21), and XRCC3 (19, 22, 23). These Rad51 paralogs share 20–30% sequence identity with Rad51 and with each other. Interactions between these proteins have been shown by a yeast two-hybrid assay and by baculovirus co-expression experiments (24). Recent evidence from mutations in hamster and chicken DT40 cell lines (25–27), showing increased sensitivity to DNA damage and increased spontaneous chromosomal instability, suggests an important role for these paralogs in HRR.

With the aim of determining the function of these paralogs, recent efforts have been devoted to purifying the proteins and to revealing their biochemical properties. The Rad51D protein was reported to display ssDNA binding and DNA-stimulated ATPase activity, and to interact with XRCC2 *in vivo* (28). The co-purified XRCC3·Rad51C and XRCC2·Rad51D complexes bind to ssDNA and form protein·DNA networks, as visualized by electron microscopy (29–31). These heterodimers were also reported to exhibit homologous pairing activity (30, 31).

Here we report several *in vitro* activities of the human Rad51B and Rad51C proteins, including DNA binding, ATP hydrolysis, and apparent DNA strand exchange. We also compared these activities to those of Rad51. The activities of mixed proteins (Rad51 plus Rad51C, Rad51 plus Rad51B, and Rad51B plus Rad51C) were determined as well. Our results

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§ To whom correspondence should be addressed: MS74-157, Life Sciences Division, 1 Cyclotron Rd., Lawrence Berkeley National Laboratory, Berkeley, CA 94720. Tel.: 510-486-5861; Fax: 510-486-6816; E-mail: YLio@lbl.gov.

[1] The abbreviations used are: HRR, homologous recombinational repair; DSB, double-strand break; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; Ni-NTA, nickel-nitrilotriacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; RPA, replication protein A; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate.

[2] Rad51L is the recommended symbol for the Rad51-like genes/proteins (Human Gene Nomenclature Committee).

demonstrate the formation of multiprotein complexes composed of Rad51B·Rad51C and Rad51·Rad51C·Rad51B, and show that Rad51C both separates duplex DNA and facilitates the binding of Rad51 to DNA.

EXPERIMENTAL PROCEDURES

Protein Expression—The human Rad51, Rad51B, and Rad51C open reading frames were cloned into the baculoviral vector, and the recombinant proteins were expressed in Sf9 insect cells as described previously (24). The human Rad51 protein was purified by selective spermidine precipitation (32) with a modified step, an additional Q-Sepharose purification after spermidine precipitation to eliminate DNA.

The N-terminal His_6-tagged Rad51B was initially constructed and expressed in insect cells using the baculovirus system. An attempt to purify this protein was unsuccessful, because the protein did not bind to a Ni-NTA column. It is possible that the N terminus of the expressed Rad51B protein is folded into the structure and thus inaccessible to bind a Ni-NTA column. Therefore, we switched to a C-terminal-tagged construct. To do so, the C-terminal-tagged *RAD51B* gene was PCR-amplified from a human testis cDNA library with a hexahistidine-encoded sequence in the reverse primer and cloned into a pFastBac1 baculoviral vector. The sequence of *RAD51B* was confirmed to be identical to those published previously. The C-terminal His_6-tagged Rad51B protein was then expressed in Sf9 insect cells. To study the interaction between Rad51B and Rad51C, we chose to make an untagged Rad51C construct, because the Rad51B was tagged with His_6. The *RAD51C* gene was also PCR-amplified from a human testis cDNA library and cloned into a pFastBac1 vector. The sequence of *RAD51C* was confirmed and the recombinant protein was expressed in Sf9 cells.

Purification of Recombinant His_6-tagged Rad51B—All purification procedures were carried out at 4 °C. Frozen cells (from 2 liters of insect cell culture) were thawed on ice and resuspended in 120 ml of ice-cold lysis buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10% glycerol, 10 mM imidazole, and protease inhibitors. The mixture was incubated on ice for 30 min, and phenylmethylsulfonyl fluoride was added to a final concentration of 0.5 mM prior to lysis by sonication. The lysate was clarified by centrifugation at 14,000 rpm for 40 min. The resulting supernatant (soluble fraction) was then applied onto a Ni-NTA-agarose column (5 ml, Qiagen), which had been previously equilibrated with start buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10% glycerol, and 10 mM imidazole). The column was then washed with 10 column volumes of start buffer, followed by 10 column volumes of wash buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10% glycerol, and 20 mM imidazole. Finally, elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10% glycerol, and 250 mM imidazole) was added, and the eluate was collected in 2-ml fractions. The fractions containing Rad51B were dialyzed at least 4 h against T buffer consisting of 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 10% glycerol, and further loaded onto a Q-Sepharose column (10 ml, Amersham Biosciences) pre-equilibrated with the same buffer. The column was subsequently washed with 5 column volumes of T buffer and eluted with a linear gradient of 0–0.8 M KCl in T buffer. The fractions containing the large proportion of Rad51B were pooled and applied to a Mono Q column (5 ml, Amersham Biosciences) pre-equilibrated with T buffer. The column was then washed with 5 column volumes of T buffer and eluted with a linear gradient of 0–0.8 M KCl in T buffer. The Rad51B protein was eluted in a peak at ~0.3–0.4 M KCl. The pure Rad51B protein, as determined by SDS-PAGE, was finally dialyzed for 12 h against storage buffer consisting of 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol, and was stored in aliquots at −70 °C.

Purification of Recombinant Rad51C—Frozen cells (from 2 liters of insect cell culture) were thawed on ice and resuspended in 120 ml of ice-cold lysis buffer containing 100 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.1% Triton X-100, 0.5 mM DTT, 2 mM EDTA, 5% glycerol, and protease inhibitors. The mixture was incubated on ice for 30 min and was sonicated briefly to reduce viscosity. Cell debris was removed by ultracentrifugation in a Beckman 70Ti rotor at 39,000 rpm for 1 h. The clarified supernatant was dialyzed two times against 4 liters of spermidine buffer (20 mM Tris acetate, pH 7.5, 7 mM spermidine-NaOH, and 0.1 mM DTT) for a total of 10 h. The precipitate was recovered by centrifugation at 12,000 rpm for 30 min and resolubilized in 40 ml of P buffer (0.1 M KH_2PO_4, pH 6.8, 0.1 M KCl, 0.5 mM DTT, and 10% glycerol) by stirring slowly about 2 h until the pellet had largely dissolved. Insoluble material was removed by centrifugation in a Beckman 70Ti rotor at 39,000 rpm for 30 min. The clarified supernatant was loaded directly onto a hydroxyapatite column (20 ml, Bio-Rad), which had been pre-equilibrated with P buffer. After loading, the column was washed with 10 column volumes of P buffer, then eluted with a linear gradient of 0.1–0.8 M KH_2PO_4 in P buffer. The eluate was collected in 2-ml fractions. An aliquot (10 μl) of each fraction was analyzed by SDS-PAGE, and peak fractions containing Rad51C, which eluted at ~0.2–0.3 M phosphate, were pooled and dialyzed against T buffer for at least 4 h. The sample was then passed over a Q-Sepharose column (10 ml, Amersham Biosciences) pre-equilibrated with T buffer and washed with 5 column volumes of T buffer. The bound proteins were eluted with a linear gradient of 0–0.8 M KCl in T buffer and collected in 1.2-ml fractions. The fractions containing the large proportion of Rad51C were pooled and applied to a heparin affinity column (5 ml, Amersham Biosciences), which had been previously equilibrated with T buffer. The column was subsequently washed with 5 column volumes of T buffer and eluted with a linear gradient of 0–0.8 M KCl in T buffer. The major peak was eluted at 0.3 M KCl. The corresponding fractions were pooled and applied to a Mono Q HR 5/5 column (Amersham Biosciences) pre-equilibrated with T buffer. The column was then washed with 5 column volumes of T buffer, and the Rad51C protein was eluted in a sharp peak at 0.2 M KCl. The purified Rad51C was finally dialyzed against storage buffer and stored in aliquots as mentioned earlier.

Antibodies—Based on amino acid sequence analysis, a unique region on Rad51B was chosen for antibody production (CDAQLQGNLKERNKF, Zymed Laboratory). Polyclonal antiserum against human Rad51B was raised in rabbits using this specific synthetic peptide as the immunogen. The His_6-tagged bacterial recombinant protein of human Rad51C was purified and used for polyclonal antiserum production in rabbits. Both Rad51B and Rad51C antibodies were affinity-purified from the antiserum. The human Rad51 antibody was kindly provided by Dr. Akira Shinohara.

Ni-NTA Magnetic Agarose Beads Pull-down Experiments—The pull-down experiments were carried out with Rad51, Rad51C, and His_6-tagged Rad51B using the procedures as previously described (24).

DNA Substrates—The oligonucleotides used in this study were: #1, 63-mer, ACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA; #2, 63-mer, TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGCTGAATCTGGTGCTGT; #5, 32-mer, CCATCCGCAAAAATGACCTCTTATCAAAAGGA; #6, 32-mer, TCCTTTTGATAAGAGGTCATTTTTGCGGATGG; #25, 48-mer, GCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA; #26, 48-mer, TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGC; #70, 79-mer, TACAACATGTTGACCTACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA; #98, 59-mer, ATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCG. The concentrations of the oligonucleotides were determined spectrophotometrically using an extinction coefficient (ϵ_{260}) of 9833 $M^{-1}cm^{-1}$ (1 A_{260} = 33 μg/ml). All DNA concentrations are expressed in moles of nucleotides. Oligonucleotides were stored at −20 °C.

DNA Binding Assays—63-mer (#2) was 5′-end-labeled with [γ-^{32}P]ATP using standard protocols. 63-mer (#1) was annealed to 63-mer (#2) to create blunt-ended dsDNA (63/63-mer). 32-mer (#5) was annealed to 63-mer (#2) to create 3′-end-tailed dsDNA (63/32-mer). The protein (2 μl) was incubated with ^{32}P-labeled DNA (3 μM nucleotides) in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 60 mM KCl, 2 mM ATP, 1 mM DTT, and 100 μg/ml BSA in a total volume of 10 μl at 37 °C for 30 min. The reaction mixture was then mixed with a 1/10 volume of loading buffer (30% glycerol, 0.1% bromphenol blue). Samples were loaded onto a 10% polyacrylamide gel and analyzed by electrophoresis in TBE at 9 V/cm for 2 h. Gels were dried on filter paper, and ^{32}P-labeled DNA·protein complex was detected by autoradiography.

ATPase Assays—Reactions (25 μl) contained 1.25 μg of ssDNA and 3 μM protein in 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 100 μg/ml BSA, and 0.2 mM ATP (0.2 μCi of [γ-^{32}P]ATP) and were incubated at 37 °C. At the indicated time points, a 5-μl aliquot was removed, and the reaction was quenched by the addition of 2.5 μl of 0.5 M EDTA. 1 μl of the samples was spotted on a thin layer chromatography plate, and the plate was developed in 0.75 M KH_2PO_4. The percentage of [γ-^{32}P]ATP hydrolysis was quantified with a Model 425E PhosphorImager (Amersham Biosciences).

DNA Strand Exchange Assays—To form nucleoprotein complexes, Rad51C protein was incubated with ssDNA in the standard buffer containing 33 mM HEPES (pH 7.0), 2 mM DTT, 100 μg/ml BSA, 2 mM ATP, and 3 mM magnesium acetate at 37 °C for 15 min. DNA strand exchange was initiated by the addition of dsDNA to the nucleoprotein complexes and incubated at 37 °C. Aliquots were withdrawn from the reaction mixture, deproteinized by the addition of EDTA to 50 mM, SDS to 1%, and proteinase K to 700 μg/ml, followed by incubation for 15 min

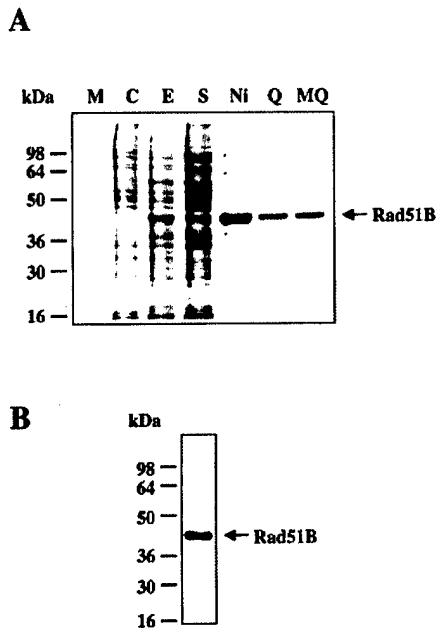

FIG. 1. **Purification of Rad51B protein.** *A*, Coomassie Blue-stained 10% SDS-polyacrylamide gel of extracts of Sf9 cells before (*C*) and after (*E*) expression of Rad51B protein. Soluble fraction (*S*) was purified by Ni-NTA-agarose (*Ni*), Q-Sepharose (*Q*), and Mono Q (*MQ*) column chromatography. Molecular weight markers were shown in *lane M*, and their sizes in kilodaltons are shown on the *left*. The Rad51B protein is indicated by an *arrow*. *B*, Western blot of the purified Rad51B protein eluted from the Mono Q column using α-Rad51B antibody.

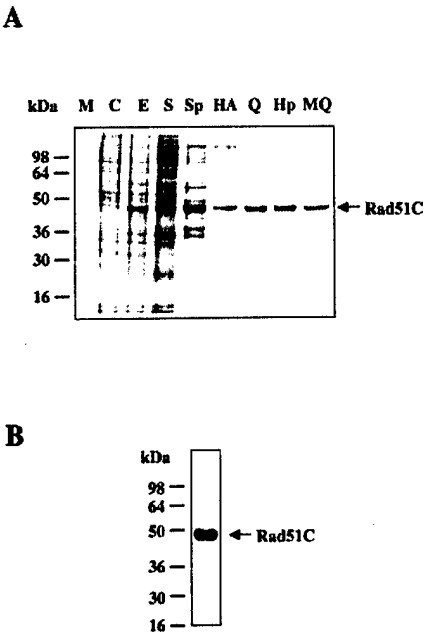

FIG. 2. **Purification of Rad51C protein.** *A*, Coomassie Blue-stained 10% SDS-polyacrylamide gel of extracts of Sf9 cells before (*C*) and after (*E*) expression of Rad51C protein. Soluble fraction (*S*) was purified by spermidine precipitation (*SP*) and column chromatography using hydroxyapatite (*HA*), Q-Sepharose (*Q*), heparin affinity (*Hp*), and Mono Q (*MQ*). Molecular weight markers were shown in *lane M*, and their sizes in kilodaltons are shown on the *left*. The Rad51C protein is indicated by an *arrow*. *B*, Western blot of the purified Rad51C protein eluted from the Mono Q column using α-Rad51C antibody.

at 37 °C, mixed with a 1/10 volume of loading buffer (30% glycerol, 0.1% bromphenol blue), and loaded onto a 10% polyacrylamide gel. Products of DNA strand exchange were quantified using a Storm 840 PhosphorImager (Amersham Biosciences).

Nuclease Assays—To control for nuclease activity, the Rad51C protein preparation was assayed at the standard conditions for DNA strand exchange. Rad51C nucleoprotein complexes were assembled on 63-mer ssDNA (#2). Duplex DNA (#1–2) was used as a substrate for DNA strand exchange. The dsDNA was ^{32}P-labeled at the 5′-end of the identical DNA strand (#2) using T4 polynucleotide kinase and [γ-^{32}P]ATP. DNA strand exchange was carried out for 30 min at 37 °C. After deproteinization, the products of this reaction were separated using a 10% polyacrylamide gel run in TBE. The DNA strand that was displaced from dsDNA was eluted from the gel, and its integrity was confirmed by electrophoresis in a 15% polyacrylamide gel containing 8 M urea.

RESULTS

Purification of Rad51B Protein—After cells were lysed, roughly 50% of the His_6-tagged Rad51B protein was found in the soluble fraction, and the protein bound to a Ni-NTA column. The Ni-NTA column provides a powerful purification step yielding 90% pure Rad51B. The eluate from the Ni-NTA column was then run through a Q-Sepharose column to remove contaminating DNA from the protein preparation. Finally, Rad51B was further purified to near homogeneity by a Mono Q column (Fig. 1*A*). About 400 μg of homogeneous Rad51B protein was obtained from two liters of insect cell culture. The identity of the protein was confirmed by Western blotting analysis with α-Rad51B antibody (Fig. 1*B*).

Purification of Rad51C Protein—The solubility of Rad51C protein was determined to be ~50%, and the soluble fraction was used for purification. In light of the selective precipitation of the *E. coli* RecA (33)[3] and human Rad51 protein by spermidine (32, 35–38), the effect of spermidine on Rad51C was examined. We found that Rad51C is indeed precipitated by spermidine in a concentration-dependent manner (data not shown). Soon after the soluble fraction of cell lysate was dialyzed against spermidine buffer, a white precipitate was observed. The amount of precipitate accumulated continuously up to 10 h with a substantial increase following the second buffer change. Approximately 70% of the Rad51C was precipitated by spermidine. The Rad51C protein was subsequently recovered from the precipitate by slowly stirring the precipitate ~2 h at 4 °C, with about 70% of the protein being dissolved into the solution. The solution was then subjected to sequential column chromatography using hydroxyapatite, Q-Sepharose, heparin, and Mono Q columns. About 500 μg of homogeneous Rad51C protein was obtained from 2 liters of insect cell culture (Fig. 2*A*). The identity of the protein was confirmed using α-Rad51C antibody (Fig. 2*B*).

Complex Formation between Rad51, Rad51B, and Rad51C—The His_6-tagged Rad51B and untagged Rad51 and Rad51C were co-expressed in Sf9 cells. Using Ni-NTA beads, we found that Rad51 could be pulled down from insect cell extracts by the His_6-tagged Rad51B through its interaction with Rad51C (Fig. 3*A*). This result demonstrates a simultaneous interaction between Rad51, Rad51B, and Rad51C, implying a heterotrimer formation between these three proteins. However, the amount of Rad51 in the complex is much less than the amount of Rad51C. This observation was found consistently in several experiments, and may indicate that the association between Rad51C and Rad51 is weak. In addition, to ensure that the observed protein associations were not caused by DNA, the Ni-NTA pull-down experiments were also carried out in the presence of an increasing amount of ethidium bromide. We found that up to 700 μg/ml ethidium bromide did not affect the pull-down (data not shown). In the presence of ethidium bromide, a consistent amount of Rad51 was detected after pull-down, suggesting that the presence of Rad51 in the complex is

[3] J. Mirshad and S. C. Kowalczykowski, submitted for publication.

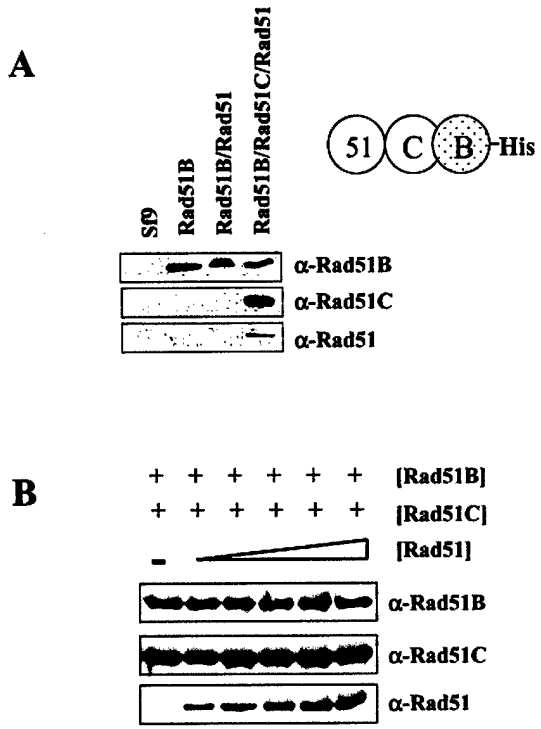

FIG. 3. **Rad51, Rad51B, and Rad51C form a heterotrimer *in vitro*.** *A*, Ni-NTA pull-down experiments using baculovirus co-expressed insect cells. *Lane 1*, uninfected Sf9 cell extract; *lane 2*, Sf9 singly infected with His_6-tagged Rad51B; *lane 3*, Sf9 doubly co-infected; and *lane 4*, Sf9 triply co-infected. Each gel was run in triplicate, and three Western blots were done, probing with three different antibodies. *B*, Ni-NTA pull-down experiments using purified Rad51, Rad51B, and Rad51C proteins. Rad51B and Rad51C were mixed with increasing amounts of Rad51 in the presence of 500 μg/ml ethidium bromide. The pull-down samples were subject to Western blot analysis with α-Rad51, α-Rad51B, and α-Rad51C.

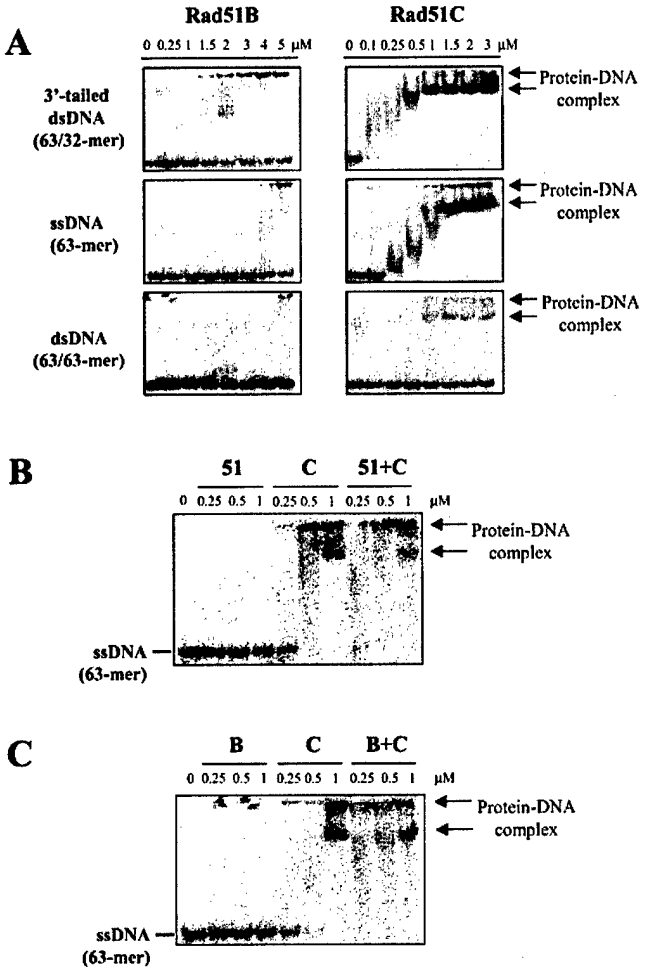

FIG. 4. **DNA binding activity of Rad51B and Rad51C proteins.** *A*, gel shift assays, using oligonucleotide substrates, were carried out in the presence of various amounts of Rad51B or Rad51C. *B*, Rad51C promotes the DNA binding activity of Rad51. Equal molar amounts of individual proteins (Rad51 and Rad51C) and the mixed proteins (Rad51 plus Rad51C) were added to separate reaction mixtures. To do so, a half-molar amount of each protein was mixed to make the final molar amount of total protein equal in the three different samples (Rad51, Rad51C, and Rad51 plus Rad51C). A gel shift assay was performed to determine their DNA binding activity. *C*, Rad51B promotes the binding activity of Rad51C. The molar amounts of each individual protein (Rad51B and Rad51C) and of the total amount of mixed proteins (Rad51B plus Rad51C) are indicated.

due to protein-protein interactions rather than to DNA binding.

To further confirm this observation, individual purified proteins were mixed and used in similar Ni-NTA pull-down experiments. Fixed amounts of purified Rad51B and Rad51C (molar ratio = 1:1) were mixed with increasing amounts of Rad51 in the absence and presence of 500 μg/ml ethidium bromide. The results showed that an increasing amount of Rad51 was pulled down in both conditions (Fig. 3*B*), confirming that the interaction is not due to DNA association. Importantly, no detectable Rad51B and Rad51C were found in the supernatant (data not shown), suggesting a strong interaction between Rad51B and Rad51C. However, the majority of Rad51 remained in the supernatant, and only ~5–10% of Rad51 was pulled down by Rad51B (data not shown), indicative of a weak interaction between Rad51B·Rad51C and Rad51.

Based on these results, we conclude that Rad51B and Rad51C form a stable complex with a strong association constant and that Rad51, Rad51C, and Rad51B form a heterotrimer involving a weak interaction between Rad51 and Rad51C. To our knowledge, this is the first *in vitro* evidence showing the simultaneous interaction between Rad51B·Rad51C and Rad51.

Rad51B and Rad51C Preferentially Bind ssDNA-tailed dsDNA—Based on sequence alignments, we have found that four of the human Rad51 paralogs, including Rad51B, Rad51C, Rad51D, and XRCC3, contain a conserved helix-hairpin-helix motif (39, 40) in their N terminus.[4] The helix-hairpin-helix motif is a domain of around 20 amino acids widely present in prokaryotic and eukaryotic proteins involved in non-sequence-specific DNA binding, *e.g. E. coli* RadC, eukaryotic ERCC1/Rad10 family, yeast SW10, and human Rad51 (41). Therefore, we examined the DNA-binding ability of Rad51B and Rad51C using an electrophoretic mobility-shift assay. Both Rad51B and Rad51C were found to bind ϕX174 single-stranded DNA (data not shown). To further determine the substrate preference of these two proteins, oligonucleotide substrates were employed: ssDNA (63-mer), dsDNA (63/63-mer), and tailed dsDNA with 3′-tailed ssDNA (63/32-mer). Both Rad51B and Rad51C preferentially bind 3′-tailed dsDNA over ssDNA, and they have the lowest affinity for dsDNA (Fig. 4*A*). Two forms of Rad51C·DNA complexes were observed: an aggregate that was trapped in the loading well, and a protein·DNA complex that entered the gel (as indicated in Fig. 4*A*). However, for Rad51 (data not shown) and Rad51B, only the aggregates were observed. It is possible that Rad51C exists as multimers of limited size, some of which form a large aggregate of protein·DNA complexes. Our gel filtration results demonstrating tetramer formation by Rad51C

[4] Y.-C. Lio, S. Mian, and D. J. Chen, unpublished data.

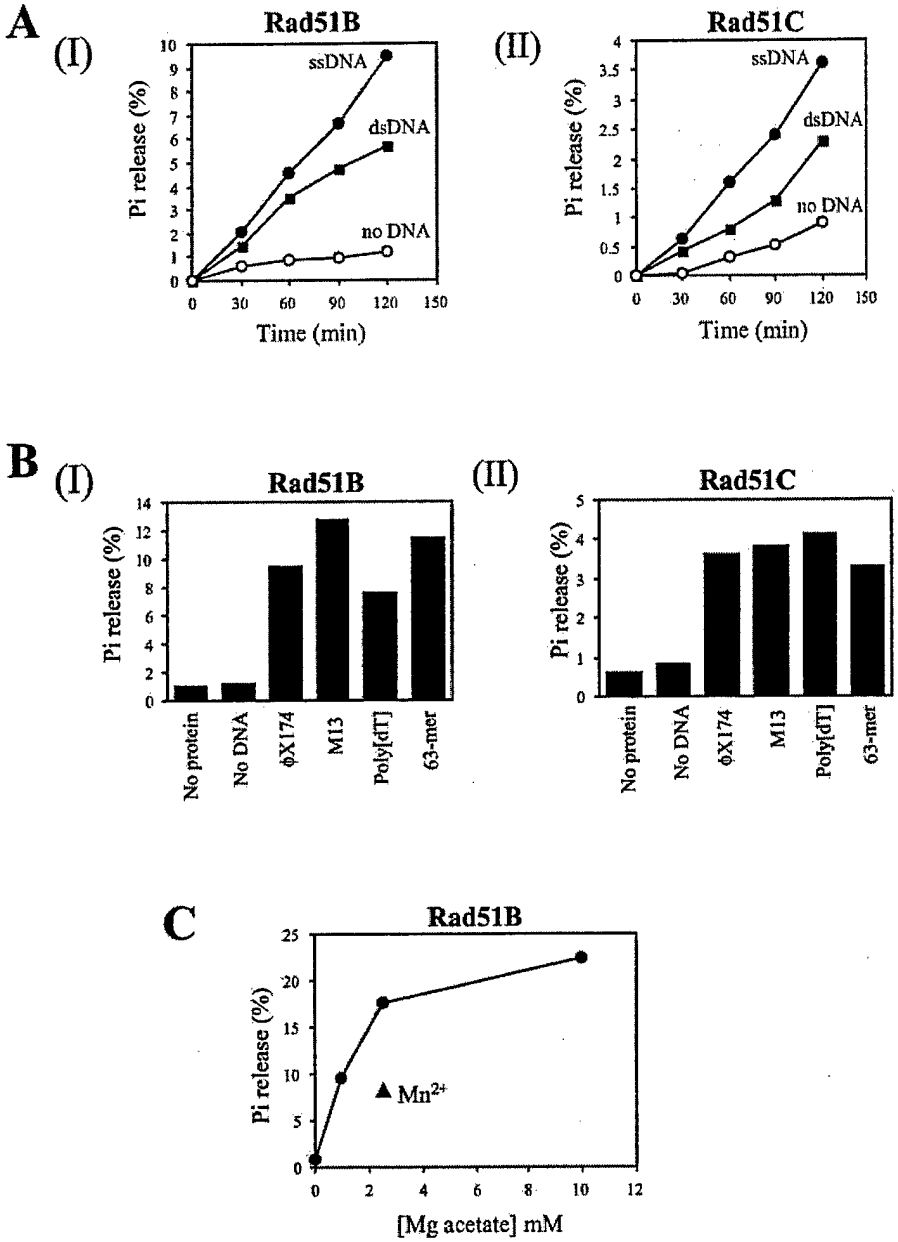

FIG. 5. **ATP hydrolysis activity of Rad51B and Rad51C proteins.** *A*, time course for ATPase activity of Rad51B (*panel I*) and Rad51C (*panel II*). Rad51B or Rad51C was incubated in the absence (○) or presence of single-stranded (●) or double-stranded (■) ϕX174 DNA as described under "Experimental Procedures." At the indicated time points, an aliquot was removed and the reaction was quenched by EDTA. *B*, the ATPase activity of Rad51B and Rad51C was stimulated by various single-stranded DNA. ATPase activity was quantified for Rad51B (*panel I*) and Rad51C (*panel II*) after 2-h incubation in the absence of DNA and in the presence of circular ϕX174 ssDNA, circular M13 ssDNA, poly[dT] DNA, and a 63-mer oligonucleotide (76 μM nucleotides). Each value represents an average from two independent determinations. *C*, the ATPase activity of Rad51B is dependent on a divalent cation. Rad51B was incubated with ϕX174 ssDNA and three different amounts of Mg^{2+} (●) or 2.5 mM of Mn^{2+} (▲) at 37 °C for 2 h, and the ATP hydrolysis activity was determined.

(data not shown) indeed support this speculation. Because the repair of DNA double-strand breaks (DSBs) by homologous recombination requires processing of the break to produce a 3′-overhanging ssDNA tail, our observations indicate that Rad51B and Rad51C proteins preferentially bind the 3′-overhanging ssDNA tail created during the repair process.

We also compared the DNA binding capabilities of Rad51B and Rad51C with Rad51 using the same three DNA substrates: 63-mer, 63/63-mer, and 63/32-mer. We found that Rad51C possesses a higher affinity for all of three DNAs than does Rad51 (Fig. 4*A* and data not shown). The relative affinity of these three proteins for DNA was found to be: Rad51C > Rad51 > Rad51B (Fig. 4*A* and data not shown).

In addition, we investigated the DNA binding capacity of various mixed proteins. For the 63-mer ssDNA substrate, we observed enhanced DNA binding with mixed Rad51 and Rad51C (Fig. 4*B*), as well as with mixed Rad51B and Rad51C (Fig. 4*C*). As is particularly evident from substrate use, comparison of the DNA binding ability of an individual protein (Rad51 or Rad51C) with the mixed proteins (Rad51 plus Rad51C) shows that the DNA binding activity of protein mixture is elevated. Using 3′-tailed dsDNA (63/32-mer) as the substrate, DNA binding by the Rad51B·Rad51C complex was also enhanced over the individual proteins (data not shown). However, no stimulatory effect was found with mixed Rad51 and Rad51B compared with individual Rad51 or Rad51B (data not shown). It is likely that the direct interaction between Rad51 and Rad51C, as well as between Rad51B and Rad51C, is responsible for their increased ability to bind DNA.

Both Rad51B and Rad51C Exhibit DNA-stimulated ATPase Activity—Amino acid sequence analysis indicates that the five human Rad51 paralogs contain the conserved ATP-binding domains (3), the Walker A and B motifs, suggesting ATP binding and hydrolysis activities for these proteins. Like human Rad51 and Rad51D (7, 28), both Rad51B and Rad51C were found to display ATPase activity, and this activity was stimulated by DNA (Fig. 5*A*). Single-stranded DNA was found to be a better stimulator of the ATP hydrolysis activity than dsDNA for both proteins (Fig. 5*A*). The effect of various ssDNA species on the activity of each protein was also determined, including ϕX174 ssDNA, M13 ssDNA, poly(dT), and a 63-mer oligonucleotide (Fig. 5*B*). Each ssDNA stimulated the activity of

Rad51B and Rad51C, indicating that the secondary structure of ssDNA has no significant effect on the stimulation. The ATPase activity of Rad51B was shown to be dependent on the presence of a divalent cation, Mg^{2+}, and the activity is Mg^{2+} concentration-dependent; the ATPase activity of Rad51B increased with higher concentration of Mg^{2+}. In addition, Mn^{2+} was able to partially substitute for Mg^{2+} (Fig. 5*C*). Similar effects by a divalent cation were also found for Rad51C (data not shown) and were reported for Rad51D (28).

The ATP hydrolysis activity of Rad51B and Rad51C in the presence of ssDNA was found to be lower than that of Rad51 (data not shown). When the ATPase activity of mixed proteins was examined (Rad51 plus Rad51B, Rad51 plus Rad51C, and Rad51B plus Rad51C), an additive effect on the ATP hydrolysis activity was observed, rather than a stimulatory effect (data not shown).

Rad51C Promotes an Apparent DNA Strand Exchange Reaction—Using oligonucleotide DNA substrates, we tested the ability of Rad51B and Rad51C proteins to promote DNA strand exchange *in vitro*. For this purpose, Rad51C nucleoprotein complexes were formed with ssDNA 63-mer (#2), and these complexes were mixed with dsDNA of three different lengths: 32, 48, and 63 bp. To detect the products of DNA strand exchange, the strand in dsDNA that was identical in sequence to the ssDNA (#2) was labeled (*i.e.* the strand that was expected to be displaced from the dsDNA). After incubation, we observed the appearance of the displaced ssDNA, the product of DNA strand exchange (Fig. 6*A*). Rad51C generated ~20–30% displaced ssDNA product after 30 min of incubation. As shown in Fig. 6*B*, the reactions work equally well with the 32-, 48-, and 63-bp dsDNA substrates. The activity of Rad51C protein only slightly depended on DNA length in the tested range of dsDNA substrate, with the activity increasing slightly with increasing dsDNA length. Consistent DNA strand exchange products were observed using three independent Rad51C protein preparations. In contrast to Rad51C, Rad51B protein did not show any activity under the conditions examined (data not shown).

For comparison, the DNA strand exchange activity of Rad51 was examined using the same DNA concentrations and assay conditions as used for Rad51C, except that the optimal concentration of magnesium acetate (20 mM) was used and the concentration of Rad51 (0.6 μM) was the optimal 1:3 stoichiometric ratio relative to ssDNA. Under these conditions, Rad51 catalyzed the formation of ~15–35% of ssDNA product after a 30-min reaction (Fig. 6*C*). However, Rad51 shows a preference for the shorter dsDNA substrates. Thus, we conclude that Rad51C displays a significant apparent DNA strand exchange activity, which is comparable to that of Rad51.

The Apparent DNA Strand Exchange Promoted by Rad51C Is Protein Concentration and Magnesium Ion-dependent but Not ATP-dependent—To determine the reaction stoichiometry between Rad51C and ssDNA, the DNA strand exchange reaction was carried out with a fixed amount of ssDNA (1.5 μM) and various amounts of Rad51C. Using 63-mer ssDNA and dsDNA substrates, the reaction yield was found to increase with increasing concentrations of Rad51C and saturated at ~5.5 μM (Fig. 7*A*). This amount greatly exceeds the classic 1:3 stoichiometry for Rad51 and DNA, and it indicates that the binding stoichiometry of Rad51C and DNA is different from that of the Rad51 protein.

Because both RecA and Rad51 proteins (7) require a nucleotide cofactor for DNA strand exchange, we tested whether this requirement applied to the Rad51C-mediated reactions. We found that, contrary to expectations based on the RecA/Rad51 family members, the reaction promoted by Rad51C protein is independent of a nucleotide cofactor (Fig. 7*B*). We also found that AMP-PNP had no stimulatory effect on the reactions (Fig. 7*B*).

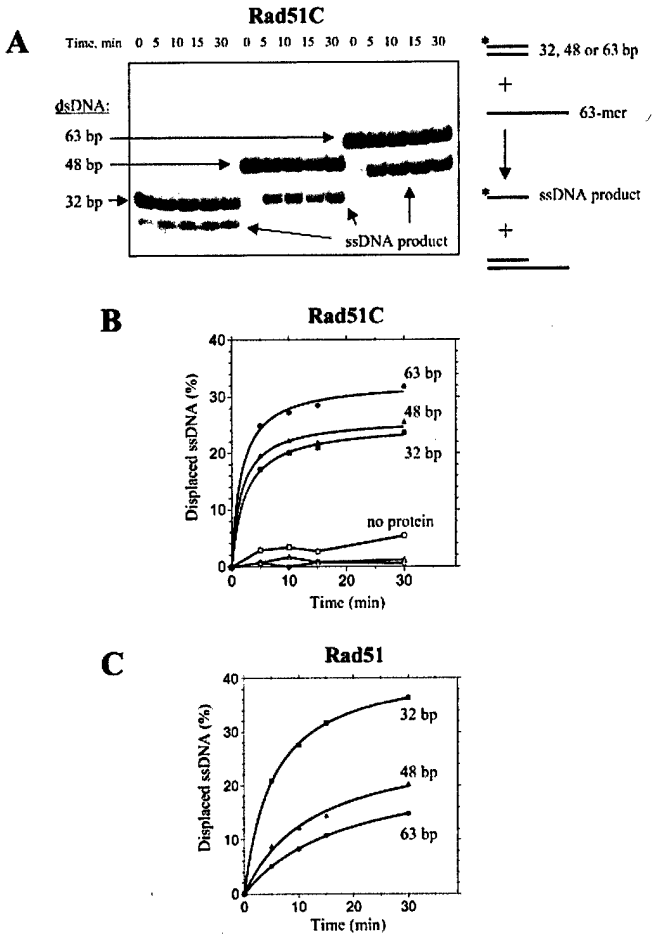

FIG. 6. **Rad51C protein displays an apparent DNA strand exchange activity.** *A*, DNA strand exchange between labeled dsDNA and homologous ssDNA, as a function of dsDNA length. Nucleoprotein complexes were formed by incubating 1.5 μM of ssDNA (63-mer, #2) with 6 μM of Rad51C protein. To initiate DNA strand exchange, these complexes were mixed with ^{32}P-labeled dsDNA and incubated for various time points at 37 °C as indicated. The nucleotide concentration of dsDNA was 1.5 μM for 32-bp (*#5–6*), 2.25 μM for 48-bp (*#25–26*), and 3.0 μM for 63-bp (*#1–2*) duplexes. The concentration of dsDNA was equimolar to ssDNA in terms of molecule concentrations. Control reactions without protein were carried out under identical conditions. The products of DNA strand exchange were determined as described under "Experimental Procedures." The products of DNA strand exchange reaction were quantified and plotted in *panel B*. The *open symbols* represent the protein-free controls. *C*, DNA strand exchange activity of Rad51. The DNA concentrations and buffer conditions were the same as used for Rad51C except that 20 mM magnesium acetate and 0.6 μM of Rad51 was used for the reactions. The products of DNA strand exchange were quantified and plotted.

Because the DNA strand exchange activity of Rad51 shows a strong dependence on magnesium ion concentration, we examined the effect of magnesium acetate concentration on the activity of Rad51C. A titration experiment (Fig. 7*C*) shows that the Rad51C-promoted reaction exhibits a dependence on magnesium ion concentration, with maximal activity at 3 mM magnesium that remains approximately constant to 20 mM. Because ammonium sulfate was found to stimulate the DNA strand exchange activity of Rad51 (42), we also tested its effect on Rad51C. However, under our conditions, 100 mM ammonium sulfate instead inhibited the reaction promoted by Rad51C (Fig. 7*C*).

The Rad51C-promoted DNA Strand Exchange Reaction Results From Its dsDNA-melting Ability—An observed exchange

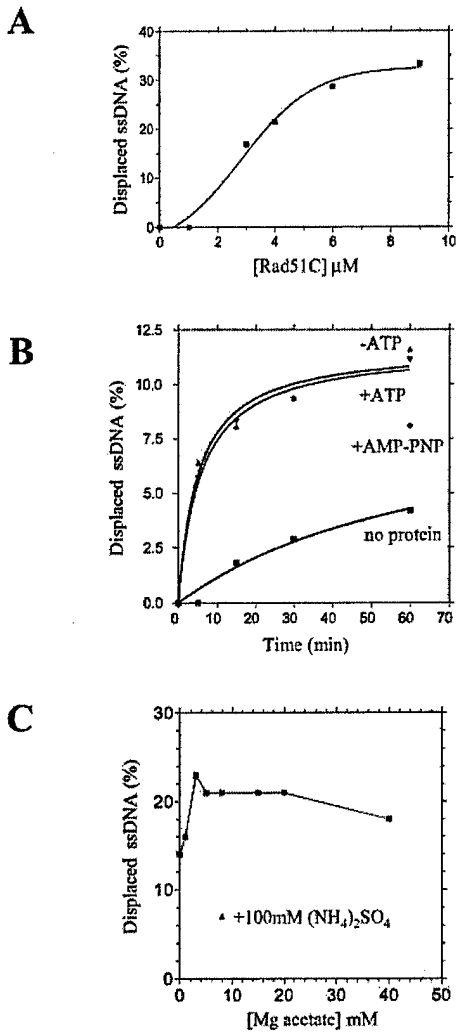

FIG. 7. **Effects of Rad51C, ATP, and magnesium acetate concentration on DNA strand exchange.** *A*, the reaction catalyzed by Rad51C is protein-concentration dependent. Nucleoprotein complexes were formed by incubating 1.5 μM ssDNA (63-mer, *#2*) with Rad51C protein at concentrations of 0, 1, 3, 4, 6, or 9 μM. To initiate DNA strand exchange, these complexes were mixed with 3 μM of ^{32}P-labeled 63-bp dsDNA (*#1–2*) and incubated for 30 min at 37 °C. The products of DNA strand exchange were determined as described under "Experimental Procedures" and plotted. *B*, the reaction catalyzed by Rad51C protein is ATP-independent. The reaction conditions of DNA strand exchange were identical to those described in *panel A*, except that 6 μM Rad51C was used and the reactions were assayed in the presence (▼) or absence (▲) of ATP, or ATP was replaced with AMP-PNP (♦), when indicated. The control reaction without protein (■) was carried out under identical conditions. The aliquots were withdrawn from the reaction mixtures at the indicated time points, deproteinized, and analyzed by electrophoresis in a 10% polyacrylamide gel run with TBE buffer. *C*, the reaction catalyzed by Rad51C protein is dependent on the magnesium ion concentration. The reaction conditions for DNA strand exchange were identical to those described in *panel A*, except that the Rad51C protein concentration was 6 μM and assayed in the presence of 100 mM ammonium sulfate or increasing concentrations of magnesium acetate.

of DNA strands can occur by several different biochemical processes. The canonical RecA-promoted reaction occurs in the absence of any nucleolytic resection of the DNA duplex and in the absence of any detectable dsDNA strand separation (*i.e.* generation of free ssDNA). To investigate whether the observed Rad51C-mediated DNA strand exchange occurs without the generation of free ssDNA from the dsDNA as an intermediate, three control experiments were performed. The first was to eliminate the possibility that DNA strand exchange was the result of partial nucleolytic resection of the DNA strand in duplex substrate that is identical to the ssDNA region, followed by annealing of this recessed dsDNA to the homologous ssDNA; thermal branch migration would lead to DNA strand exchange. To exclude this possibility, we examined the integrity of the identical strand that is displaced by DNA strand exchange. For this purpose, the ^{32}P-labeled displaced DNA strand was analyzed by electrophoresis in a 15% polyacrylamide gel containing 8 M urea. As shown in Fig. 8*A*, the length of ssDNA displaced in the Rad51C-promoted reaction (*lane 3*) was indistinguishable from the ssDNA displaced by heating (*lane 2*), and from the original ^{32}P-labeled 63-mer (*lane 1*). This result indicates that the observed DNA strand exchange was not caused by the nucleolytic resection of dsDNA followed by spontaneous branch migration of the annealed DNA molecules. A second possibility is that Rad51C is simply destabilizing and melting the dsDNA, resulting in the production of a free ssDNA strand that in a reaction does not require the homologous ssDNA as a strand exchange partner. Therefore, the homology dependence of the reaction was examined. We found that no ssDNA was produced when the DNA substrates were heterologous; in comparison, product formation was ~30% when the partner DNA was homologous (Fig. 8*B*). This result demonstrates that Rad51C protein-promoted DNA strand transfer is homology-dependent. Finally, we were concerned that Rad51C might still be melting the dsDNA but that the free ssDNA strands would quickly re-anneal after deproteinization. Hence, the reaction would appear homology-dependent, but only because the homologous ssDNA was acting to trap the strand-separated DNA duplex. To investigate this possibility, the standard homology-dependent experiment was carried out, but in addition, an excess amount of homologous ssDNA was added to the stop buffer. This added homologous ssDNA, although complementary to the displaced ^{32}P-labeled ssDNA product (#2), was of a different length (79-mer) than the original ssDNA (63-mer) present in DNA strand exchange reaction. Thus, pairing that might occur during the deproteinization step would be distinguished from DNA strand exchange with the intended partner as diagramed in Fig. 8*C*, *panel I*. We found that, in the absence of 79-mer ssDNA in the stop/quench mix, a displaced 63-mer ssDNA product was generated as observed before (Fig. 8*C*, *panel II*, *lane 1*). When the 79-mer ssDNA was present, the displaced 63-mer ssDNA quickly annealed with 79-mer ssDNA to form a 79/63-mer duplex (Fig. 8*C*, *lane 2*); this result was fully expected and showed that the 79-mer ssDNA was an effective quench for any free ssDNA produced. However, when the homologous ssDNA was replaced by heterologous ssDNA (*lane 3*), the 79/63-mer duplex was still formed, even though DNA strand exchange could not occur. The only way that the 79/63-mer duplex could form in the presence of heterologous ssDNA was if Rad51C was melting the 63 base pair duplex into separate DNA strands that were then annealing with the 79-mer ssDNA when the sample was being deproteinized. In agreement with this interpretation, omitting Rad51C results in only a background level of 79/63-mer duplex (*lane 4*). To test whether a Rad51C·ssDNA complex was required for this melting activity, the 63-mer ssDNA was omitted; the 79/63-mer duplex was nevertheless formed (*lane 5*). This reaction was also Rad51C-dependent (*lane 6*), showing that Rad51C indeed possesses the capability of melting duplex DNA, which quickly anneals with the 79-mer ssDNA after deproteinization. This interesting result indicates that the apparent DNA strand exchange activity of Rad51C results from its ability to melt and separate duplex DNA. The 79-mer quench experiment was also performed with Rad51. No DNA melting or strand separation by Rad51 was observed (data not shown), suggesting that the DNA melting/strand separation activity is unique to Rad51C.

Because Rad51C was found to associate with Rad51, we were

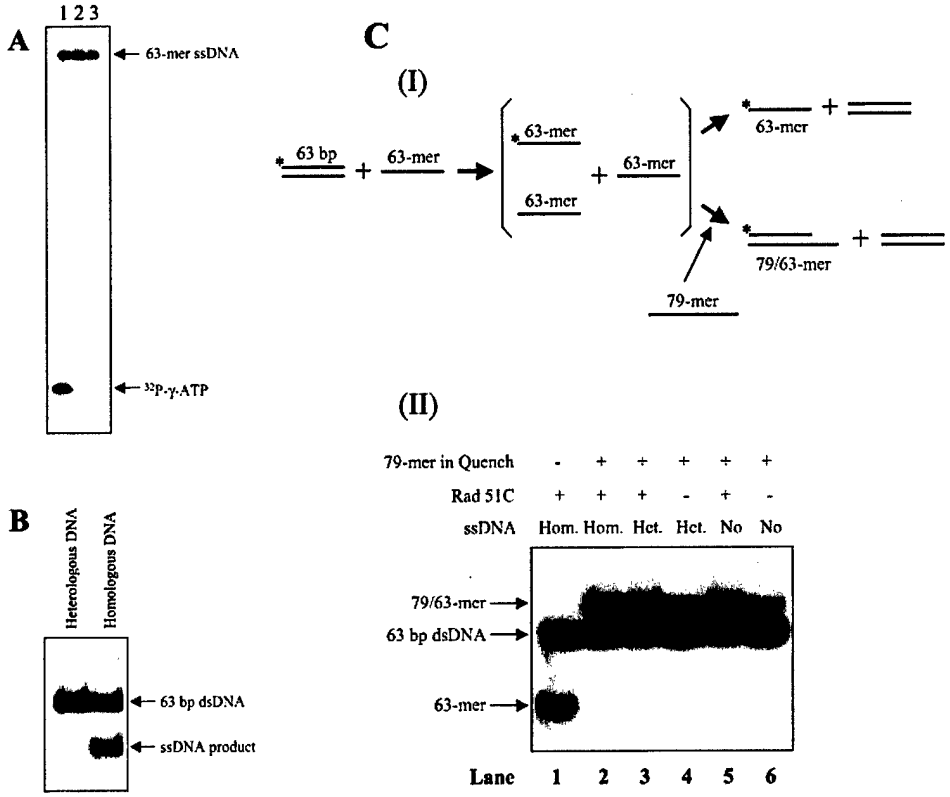

FIG. 8. **Rad51C-promoted DNA strand exchange is not due to nuclease activity but is mediated by a DNA melting activity.** *A*, the ssDNA strand displaced during strand exchange promoted by Rad51C is intact. The reaction conditions for DNA strand exchange were identical to those described above. Rad51C was mixed with 1.5 μM ssDNA (63-mer, #2). DNA strand exchange was initiated by addition of homologous 3 μM of 63-bp dsDNA (*#1–2*), in which the strand that would be displaced during DNA strand exchange (#2) was ^{32}P-labeled. The ssDNA displaced was isolated, and its integrity was analyzed by electrophoresis in a 15% polyacrylamide gel containing 8 M urea (*lane 3*) along with ssDNA-displaced strand produced in a spontaneous DNA strand exchange (*lane 2*) and with the original ^{32}P-labeled ssDNA 63-mer (*lane 1*). *B*, Rad51C-promoted DNA strand exchange requires a homologous DNA. Either 1.5 μM heterologous 59-mer (*#98*) or homologous 63-mer (#2) ssDNA was separately incubated with 6 μM Rad51C protein. To initiate DNA strand exchange, these nucleoprotein complexes were mixed with 3 μM ^{32}P-labeled 63-bp dsDNA (*#1–2*) and incubated for 10 min at 37 °C. The products of DNA strand exchange were determined as described under "Experimental Procedures." *C*, Rad51C displays DNA melting activity. The reactions are carried out in the same conditions as used in *panel B* except that 13.1 μM of a 79-mer ssDNA (*#70*) was included in the reaction stop buffer.

concerned that the observed DNA strand exchange activity might originate from contaminating Rad51 protein. To exclude the possibility that the purified Rad51C (and Rad51B) preparation contains Rad51 from insect cells, the same amount of the purified Rad51B and Rad51C preparation used in all assays was examined by probing with Rad51 antibody. The α-Rad51 recognized the insect Rad51 in control samples from Sf9 cells, but no Rad51 was detected in either preparation (data not shown), indicating that the DNA binding, ATPase, and apparent DNA strand exchange activities are intrinsic to Rad51B and Rad51C proteins, rather than due to Rad51 contamination.

DISCUSSION

To establish a purification strategy for the Rad51 paralogs, we discovered that spermidine selectively precipitates overexpressed Rad51C from cell extracts, resulting in ~70% purity of the protein. This step greatly facilitated the purification of Rad51C protein. Based on our unpublished observations, Rad51B also favorably precipitates with spermidine. Thus, in addition to purification of RecA and Rad51 as described previously (32, 33, 35–38),[3] spermidine precipitation is demonstrated here as a powerful strategy for purification of Rad51-related proteins. Our results suggest that this strategy might work for purifying other untagged Rad51 paralogs. However, because spermidine also precipitates DNA, it is important to include a Q-Sepharose column to eliminate DNA in the purification process.

In previous studies using a yeast two-hybrid assay and baculovirus co-expression with Ni-NTA pull-down analysis (24), we reported that Rad51B directly interacts with Rad51C, and that Rad51C weakly interacts with Rad51. Here we demonstrate a simultaneous interaction among these three proteins. This is the first *in vitro* evidence for Rad51·Rad51C·Rad51B complex formation. However, in the course of our study, it was reported that overexpressed His_6-tagged Rad51C did not pull down Rad51 in human cells (43, 44). Because we observed that the interaction between Rad51C and Rad51 is weak, only ~5–10% of the overexpressed Rad51 was pulled down by the Rad51B·Rad51C complex in insect cell extracts as well as by purified complex of Rad51B and Rad51C proteins, the failure to detect the Rad51C-Rad51 interaction in human cells (43, 44) is likely due to the weak interaction between the Rad51B·Rad51C complex and Rad51.

To elucidate the mechanism of homologous recombination involving Rad51 and Rad51 paralogs and to understand the functions of each protein, the biochemical activities of these proteins were determined. Using established *in vitro* assays for Rad51, including DNA binding and ATPase and DNA strand exchange assays, the biochemical properties of Rad51B and Rad51C were determined and compared with those of Rad51.

To catalyze DNA strand transfer, Rad51 must first bind to ssDNA to form a nucleoprotein filament (9, 10). Because the Rad51 paralogs play a role in HRR, a key question is whether the paralogs are involved in the DNA binding step. We found that four of the five Rad51 paralogs, including Rad51B,

Rad51C, Rad51D, and XRCC3, contain a non-sequence-specific DNA binding domain, a helix-hairpin-helix motif (39, 40). The DNA binding ability of each paralog was assessed using an electrophoretic mobility-shift assay. In addition to the previously reported DNA binding activity of Rad51D (28), we demonstrated that both Rad51B and Rad51C proteins exhibit DNA binding capacity. This finding suggests a role for the Rad51 paralogs in the DNA binding step. Because the break sites of DNA are nucleolytically processed after DSB formation to produce ssDNA with a 3′-overhanging end for binding by RPA, Rad51, and other associated proteins, we examined the substrate binding preferences of Rad51B and Rad51C. Our data, showing that both Rad51B and Rad51C proteins bind the 3′-end-tailed dsDNA substrates in preference to either ssDNA or dsDNA, support the notion that the Rad51 paralogs bind the damaged sites and play a role in the repair processes. The observation that the DNA binding activity of Rad51C is higher than that of Rad51 is somewhat interesting. It may suggest that Rad51C plays a crucial role in the DNA binding processes of the HRR-related proteins. Another significant finding is that the complex formed by Rad51 and Rad51C, as well as by Rad51B and Rad51C, displays a higher DNA binding activity than that of the individual proteins. This result suggests that the direct protein-protein interaction serves possibly to recruit Rad51, and perhaps other proteins as well, to the processed ssDNA.

Alignments of the amino acid sequences of Rad51 and five Rad51 paralogs revealed that all proteins contain the Walker A and B motifs (3), suggesting ATP binding and hydrolysis capability. The ATPase activity of each paralog was confirmed *in vitro*. We showed that purified Rad51B and Rad51C proteins display ATPase activity that is stimulated by DNA. Little activity was observed in the absence of DNA, implying that DNA is the key cofactor in activating ATP binding/hydrolysis by Rad51B and Rad51C. The role of ATP binding and hydrolysis by the Rad51 paralogs in HRR is still unclear. It was reported that the ATPase mutant of XRCC2 does not affect the formation of Rad51 foci (45), indicating that XRCC2 does not use ATP binding or hydrolysis to promote its function. Whether the ATPase mutants of Rad51B and Rad51C are altered in their DNA binding activity remains to be determined.

Thus far, Rad51 is the only protein found to catalyze the key reactions of homologous pairing and DNA strand transfer that are needed by HRR to repair DNA double-strand breaks in human mitotic cells. Current evidence supports a mediating role for Rad51 paralogs in HRR, where these proteins may function as accessory cofactors assisting the action of Rad51 (4). During the course of our research, the Rad51B·Rad51C complex was purified in the Sung laboratory (46). They reported that the Rad51B·Rad51C complex acts to facilitate the replacement of RPA from the nucleofilaments by Rad51 and to promote the DNA strand exchange activity of Rad51 (46), and they suggest a mediator role for the Rad51B·Rad51C complex in HRR. We found, using oligonucleotide DNA substrates, that Rad51C, but not Rad51B, promotes a DNA strand exchange reaction. After further investigation, we discovered that the observed DNA strand exchange products were generated by a DNA melting and strand separation activity of Rad51C. This result implies a distinct role for Rad51C in HRR and suggests that Rad51C may have a more direct function in addition to its mediator role. It is very possible that Rad51C recruits Rad51 to ssDNA and mediates the separation of duplex DNA to promote DNA strand exchange. This proposed function is based on several aspects of our work: (i) Rad51 and Rad51C (with and without Rad51B) physically interact; (ii) Rad51 and Rad51C show synergy in their DNA binding affinity; and (iii) Rad51C promotes dsDNA separation for 32-, 48-, and 63-bp substrates equally well, but Rad51 acts preferentially on the shorter substrates, suggesting that Rad51C might assist in the melting of longer duplex regions. Together, these results demonstrate that Rad51C behaves very differently from Rad51, indicating the Rad51C may serve a different role from Rad51 in the DNA strand exchange stage of homologous recombination. It is most likely that Rad51C "helps" with the strand exchange by Rad51 in several ways: Rad51C may not only directly bind DNA at DSB sites but also facilitate the DNA binding of Rad51 by complex formation and, subsequently, catalyze the separation of duplex DNA for strand exchange. It was recently reported that Rad54 promotes transient separation of the strands in duplex DNA via its ATP hydrolysis-driven DNA supercoiling function and the ability is stimulated by Rad51 (47). It will be interesting to see whether Rad51C functions with Rad54 in mediating the separation of duplex DNA for strand exchange.

Among the protein-protein interactions between the five Rad51 paralogs, Rad51C has been shown to be a central player that interacts directly with Rad51B, Rad51D, XRCC3, and weakly with Rad51 (24), and which is present in various multiprotein complexes in human cells, including Rad51B·Rad51C, Rad51C·XRCC3, and Rad51B·Rad51C·Rad51D·XRCC2 (43, 44, 46, 48, 49). The evidence of multiple protein interactions supports the notion that Rad51C may play multiple roles in the recombinational repair processes. In addition, two hamster cell lines that are mutated in Rad51C, irs3, and CL-V4B, were recently identified, and these cells show a reduction in sister chromatid exchange and genomic stability (34, 50), emphasizing a key role for Rad51C in HRR. Here we described several biochemical properties that help define the functions of Rad51C in this process, including complex formation, DNA binding, ATP hydrolysis, and DNA melting/separating activities. These findings underpin the significant biological function of Rad51C protein in the DNA strand exchange events of homologous recombination.

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