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13. ABSTRACT ***(Maximum 200 Words)***

A large fraction of familial breast cancers arise from mutations in two suppressors of breast tumors, BRCA1 and BRCA2, but no molecular information is as yet available to pinpoint the underlying basis for the breast tumor suppressor functions of these two factors. Importantly, recent studies have indicated that BRCA1 and BRCA2 are associated with proteins indispensable for homologous recombination and the recombinational repair of DNA double-strand breaks (DSBs), thus implicating these two tumor suppressors in modulating the activities of the recombination machinery. For dissecting the biochemical roles of BRCA1 and BRCA2 in recombination and repair, it will be important to first define the mechanistic underpinnings of the recombination machinery. For this purpose, we have been carrying out detailed biochemical analyzes of the Rad50/Mre11/NBS1 complex known to be required for the processing of DNA ends during recombination and DSB repair, and have established in vitro systems for examining the homologous DNA pairing and strand exchange activities of Rad51 recombinase germane for the formation of DNA joints during recombination processes. Further studies will examine the functional and physical interactions among BRCA1, BRCA2, the Rad50/Mre11/NBS1 complex, and the Rad51 recombinase.

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FOREWORD

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ANNUAL SUMMARY

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INTRODUCTION

Homologous recombination and the recombinational repair of DNA double-strand breaks are mediated by genes of the *RAD52* epistasis group (reviewed by Paques and Haber, 1999 and Sung et al, 2000). Recent studies have strongly suggested an involvement of the recombination machinery in the suppression of breast and other forms of tumors. Specifically, BRCA1 associates with and appears to influence the activities of the protein complex consisting of three members of the *RAD52* group - Rad50, Mre11, and NBS1 (Zhong et al, 1999; Wang et al, 2000), required for the processing of DNA double-strand breaks (Paull and Gellert, 1998; Trujillo et al, 1998). Consistent with this observation, BRCA1 is necessary for normal levels of homologous recombination (Moynahan et al, 1999) and resistance to genotoxic agents (Gowen et al, 1998; Zhong et al, 1999). Rad51, another key member of the *RAD52* group, binds to the BRC repeats and the carboxyl-terminal domain of BRCA2 (Chen et al, 1998; Wong et al, 1998; Sharan et al, 1997). Mouse embryos harboring a BRCA2 truncation are hypersensitive to ionizing radiation (Sharan et al, 1997). In addition, the BRCA2 deficient human cell line Capan-1 is impaired for the ability to assemble Rad51 nuclear foci upon DNA damaging treatment (Yuan et al, 1999). Taken together, it seems probable that BRCA2 modulates the efficiency of the recombination machinery *via* its specific association with Rad51.

BODY

The hRad51 protein shows a high degree of structural similarity to the *E. coli* recombination protein RecA (Shinohara and Ogawa, 1995). RecA promotes the **homologous DNA pairing and strand exchange** between recombining DNA molecules to form heteroduplex DNA joints (Kowalczykowski et al, 1994; Roca and Cox, 1997), an enzymatic activity central to recombination and DNA repair processes. However, published studies have suggested that hRad51 has only a very limited ability to make heteroduplex DNA joints (Baumann et al, 1996; Baumann and West, 1997 and 1999; Gupta et al, 1997 and 1999). Given the central role of hRad51 in recombination processes and the fact that the activities of hRad51 are apparently subject to modulation by tumor suppressor proteins such as BRCA2, establishing an efficient hRad51-mediated homologous DNA pairing and strand exchange system is important for dissecting the functional interactions among hRad51, other human recombination factors, and tumor suppressors. We have therefore explored a variety of reaction conditions that may influence the recombinase activities of hRad51 and have found that under a new set of conditions, hRad51 in fact makes DNA joints avidly and can promote highly efficient DNA strand exchange over at least 5.4 kilo base pairs, as documented below.

Systems for homologous DNA pairing and strand exchange - Figure 1 shows the two most commonly used systems for studying the homologous DNA pairing and strand exchange reaction. In the **presynaptic phase** of the reaction, Rad51 polymerizes onto ssDNA to form a right handed nucleoprotein filament (Benson et al, 1994; Ogawa et al, 1993; Sung and Robberson, 1995), called the presynaptic filament, in which the DNA is held in an extended conformation (reviewed in Bianco et al, 1999 and Sung et al, 2000). Biochemical studies have indicated that the search for DNA homology in the incoming duplex and formation of DNA joints with the duplex occur within the confines of the Rad51-ssDNA nucleoprotein filament. System A was used in the current study.

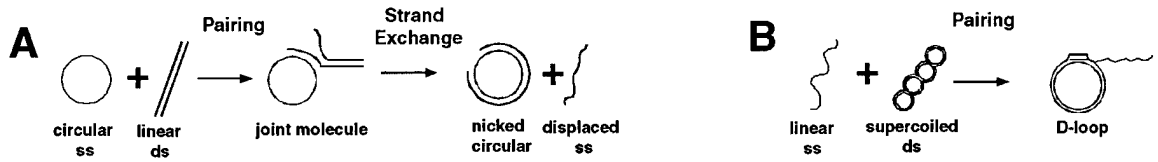

Figure 2. Two systems used to study homologous DNA pairing and strand exchange. **A** Commonly used system involving viral (+) strand and linear duplex from phage DNA to study homologous pairing and strand exchange. **B.** System measuring formation of a D-loop, the first DNA intermediate formed *in vivo*, by using linear viral (+) strand and supercoiled form of phage DNA.

Protein Purification **(i)** ***hRad51*** - The cDNA coding for hRad51 was cloned from a B cell library, sequenced, and then placed under the control of the T7 promoter. The resulting plasmid phRad51.1 (kind gift of Wen Hwa Lee and Phang Lang Chen of our Institute) was introduced into *E.coli* BLR (DE3) containing pLysS. The hRad51 protein was induced with IPTG and purified to near homogeneity (≥ 98%) using a six-step chromatographic procedure. We routinely obtain 2 to 3 mg of purified hRad51 per liter of bacterial culture. **(ii)** ***hRPA*** - For the purification of hRPA, extract was made from *E. coli* BL21 (DE3) harboring the plasmid p11d-tRPA (Henricksen et al, 1994) and subject to the purification procedure we have used for yeast RPA (Sung, 1997a). Five mg of highly purified hRPA (≥ 98%) was routinely obtained from 10 liters of bacterial culture.

Human Rad51 mediates extensive homologous DNA pairing and strand exchange - The schematic of the reaction is shown in Figure 1 (System A) and the order of addition of reaction components is summarized in Figure 2A. Specifically, the reaction (Figure 2B; 50 µl final volume) was assembled by mixing hRad51 (7.5 µM) added in 2 µl of storage buffer and ϕX174 viral (+) strand (30 µM nucleotides) added in 2 µl TE (10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA) in 40 µl of buffer R (40 mM Tris-HCl, pH 7.8, 2 mM ATP, 1 mM $MgCl_2$, 1 mM DTT, and an ATP regenerating system consisting of 8 mM creatine phosphate and 28 µg/ml creatine kinase). After a 5 min incubation, hRPA (2 µM) in 2 µl of storage buffer was added, followed by a 5 min incubation. To complete the reaction, linear ϕX174 replicative form I DNA (30 µM nucleotides) in 4 µl and 4 µl of 50 mM spermidine (4 mM) were incorporated. At the indicated times, 4.5 µl portions were deproteinized (Sung, 1994) before electrophoresis in 0.9 % agarose gels in TAE buffer (40 mM Tris-HCl, pH 7.4, 20 mM NaOAC, 0.5 mM EDTA). The gels were stained in ethidium bromide (2 µg/ml in H_2O), destained in a large volume of water, and then subject to image analysis in a NucleoTech gel documentation station equipped with a CCD camera. For comparison, another reaction was carried out following the procedure precisely as described by Baumann and West (Figure 2C; Baumann and West, 1997 and 1999).

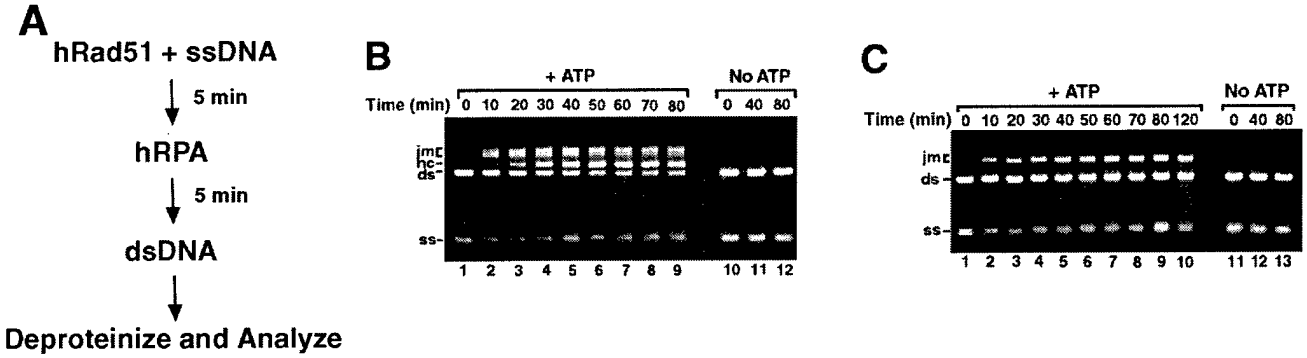

Figure 2. hRad51 promotes efficient DNA pairing and strand exchange. **A.** The order of addition of reaction components. **B** and **C.** Comparison of strand exchange in the presence and absence of ATP using our reaction conditions (**B**) and previously published conditions (**C**)(Baumann and West; 1997 and 1999). Symbols: jm-joint molecule, nc-nicked circular duplex, ds-linear duplex, ss-single stranded circular and linear molecules.

A three to four fold increase in total homologous DNA pairing and strand exchange products (joint molecules and nicked circular duplex) were seen under our reaction conditions compared to the published conditions of Baumann and West (1997 and 1999) (compare B and C in Figure 2). More importantly, while the published conditions (Baumann and West, 1997 and 1999) gave little if any full DNA strand exchange product, the nicked circular duplex (Figure 2C), highly robust strand exchange activity was seen under the new reaction conditions, as indicated by the conversion of > 60% of the input substrates into nicked circular duplex after 60 min of reaction (Figure 2B).

Effect of hRPA on hRad51-mediated DNA pairing and strand exchange - Under the newly devised reaction conditions, the efficiency of homologous DNA pairing and strand exchange is strictly dependent on hRPA. With 7.5 µM hRad51 and 30 µM ssDNA (in nucleotides), the optimal level of hRPA was found to be 1.5 to 2.0 µM, although addition of as little as 0.5 µM of hRPA

resulted in a highly notable stimulation of the reaction. The requirement for hRPA to ensure DNA strand exchange efficiency was also observed over a wide range of hRad51 concentrations below and above that used in Figure 2 (data not shown). These findings are highly significant, as in published studies, a stimulatory effect of hRPA is seen only when the hRad51 concentration is below what is required to fully saturate the ssDNA substrate (Baumann and West, 1997 and 1999).

SUMMARY

The findings documented here demonstrate for the first time the catalytic potential of hRad51 in DNA joint formation and DNA strand exchange. Importantly, using our protein purification protocols and newly devised reaction conditions, the efficiency of the hRad51-mediated DNA strand exchange reaction is strongly dependent on hRPA over a wide range of Rad51 concentrations tested. In future studies, a variety of biochemical experiments will be carried out to fully characterize the effects of different protein components and reaction parameters on the hRad51-mediated homologous DNA pairing and strand exchange reaction. In particular, the synergistic interactions of hRPA and recombination factors including BRCA2 in promoting the assembly of the functional hRad51-ssDNA presynaptic complex will be characterized. The proposed studies should shed light on how various human recombination factors functionally cooperate in mediating the homologous DNA pairing and strand exchange reaction central to all recombination processes including DNA double-strand break repair. The biochemical systems we have now established should prove to be useful for defining the functions of various tumor suppressors including BRCA1 and BRCA2 in DNA recombination and repair.

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APPENDED INFORMATION

(1) Key Research Accomplishments to date:

- Established procedure for the purification of the human Rad50/Mre11/NBS1 complex from nuclear extracts, and extensive biochemical characterization of this complex for activities germane for homologous recombination and DNA repair.

- Purification of human Rad51 and RPA and demonstration of functional cooperation between these two factors in the homologous DNA pairing and DNA strand exchange reaction central for the formation of heteroduplex DNA joints during homologous recombination and DNA repair.

- Cloning, expression, and purification of other human recombination factors including XRCC2 and Rad54.

(2) Reportable Outcomes

Presentations:

- Invited Speaker, EMBO workshop on mechanisms of genetic recombination. Seillac, France. Title of presentation: "Rad51 and Rad54 Constitute a DNA Supercoiling Motor Indispensable for DNA Joint Formation in Recombination and Repair".

- Invited Speaker, Department of Biochemistry, University of Delaware. Title of presentation: "Mechanisms of Heteroduplex DNA Formation in Recombination Process".

- Invited Speaker, Department of Biochemistry, University of Wisconsin at Madison. Title of presentation: "Action Mechanism of Recombination Factors of *Saccharomyces cerevisiae*.".

- Invited Speaker, National Cancer Institute - Frederick Cancer Research and Development Center. Title of Presentation "Role of Rad52 Group Recombination Proteins in Heteroduplex DNA Formation".

(3) Publications

Sung, P., Van Komen, S., and Trujillo, K. (2000) Recombination factors of *Saccharomyces cerevisiae. Mutation Res. In the press.*

(4) Funding applied for and approved, based on work supported by this grant:

"Crosstalk Between Recombination and Cell Cycle Checkpoints" Human Frontier Science Program for funding considerations. I am the leader of team 3 on this project. Total direct costs requested for my unit is $210,000. Project period: 04/01/00 -03/31/03.