DNA double-strand breaks can be introduced by exogenous agents or during normal cellular processes. Genes belonging to the RAD52 epistasis group are known to repair these breaks in budding yeast. Among these genes, RAD52 plays a central role in homologous recombination and DNA double-strand break repair. Despite its importance, its mechanism of action is not yet clear. It is known, however, that the human homologue of Rad52 is capable of binding to DNA ends in vitro. Herein, we show that Rad22 protein, a Rad52 homologue in the fission yeast Schizosaccharomyces pombe, can similarly bind to DNA ends at double-strand breaks. This end-binding ability was demonstrated in vitro by electron microscopy and by protection from exonuclease attack. We also showed that Rad22 specifically binds near double-strand break associated with mating type switching in vivo by chromatin immunoprecipitation analysis. This is the first evidence that a recombinational protein directly binds to DNA double-strand breaks in vivo.

The RAD52 epistasis group of genes including RAD50–59, MRE11, and XRS are involved in the repair of DNA double-strand break (DSB) by homologous recombination (HR) in Saccharomyces cerevisiae (1, 2). These genes are evolutionarily well conserved from yeast to human. Mutations in these genes can cause increased sensitivity to ionizing radiation and defects in recombination. Among these, rad52 mutants show the most severe phenotypes, indicating RAD52 has a central role in DSB repair by HR (3). Purified Rad52 proteins from both S. cerevisiae and human stimulate the exchange activity of Rad51, a eukaryotic homologue of RecA (4–8). Rad52 protein by itself has a single-strand annealing (SSA) activity of two complementary DNA strands, which comprises an alternative pathway to DSB repair by HR (9, 10).

The rad22 gene of Schizosaccharomyces pombe was identified based on its ability to complement the mating type switching defect of a rad22–67 mutant, which has been originally selected by radiation sensitivity (11, 12). rad22 encodes a 52-kDa protein that has a significant identity (56%) to the S. cerevisiae Rad52 protein in its N-terminal half. The rad22 deletion mutant is inviable in a homothallic background, in which DSB occurs frequently due to mating type switching. The rad22–67 mutant also frequently gives rise to deletions in the mating type region, suggesting that Rad22 protein might be involved in DSB repair (12).

Despite the importance of RAD52 in vivo, the biochemical studies have revealed only limited roles for Rad52 as an accessory protein of strand exchange reaction and as a component required in SSA. Interestingly, it has been recently reported that human RAD52 protein (hRAD52) is able to bind to the end of linear duplex DNA in vitro (13). This suggested a new role of RAD52 as a “gate-keeper” between non-homologous end-joining (NHEJ) and HR via competition with Ku, another end-binding protein, at the DSB (13, 14). In this study, we have investigated the end-binding property of Rad22 and have provided direct evidence that the Rad52 homologue in S. pombe indeed binds to DSB in vivo.

EXPERIMENTAL PROCEDURES

Purification of Rad22 Protein—The open reading frame of rad22 gene was placed under the control of T7 promoter in pET28b plasmid (Novagen) and was named pET22. For the purification, Rad22 protein was induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside in a 1.6-liter culture of BL21 (DE3) harboring pET22 for 6 h at 30 °C. The resulting 7.2 g of cells were treated with 560 μg of lysozyme in 20 ml of lysis buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 mM NaCl) for 30 min on ice and sonicated. The cell lysate was clarified by ultracentrifugation at 40,000 rpm for 30 min and precipitated with 35% saturation of ammonium sulfate. The precipitates were resuspended in 50 ml of buffer K (20 mM KH2PO4, pH 7.4, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) containing 50 mM KCl and passed through 20 ml of Q Sepharose (Amersham Pharmacia Biotech). The flow-through was applied to 20 ml of SP Sepharose (Amersham Pharmacia Biotech), and the proteins were eluted with 100 ml of a 0.04–0.8 M linear gradient of KCl. The Rad22 fractions (around 0.2–0.3 M KCl) were applied to a 5-ml hydroxyapatite column (Bio-Rad). The proteins were eluted with 25 ml of a 0.02–0.5 M linear gradient of KH2PO4. Fractions containing Rad22, which were eluted at 0.1–0.2 M KH2PO4, were loaded onto a 1-ml denatured DNA cellulose (Amersham Pharmacia Biotech) column, and then eluted with 1 M KCl. The fractions containing Rad22 were collected and fractionated in 1 ml of Mono Q (Amersham Pharmacia Biotech) with 20 ml of a 0.04–0.5 M linear gradient of KCl. The fractions at 0.1–0.2 M were pooled, dialyzed against buffer K containing 0.2 M KCl, and stored at −70 °C. Protein concentration was determined by measuring the absorption at 280 nm using an extinction coefficient of 28,860 M−1 cm−1.

DNA and Enzymes—The Plasmid pBluescript SK(+) was prepared using the Qiagen Plasmid Midi kit. dX174 RF I DNA was purchased from New England Biolabs. A 199-base pair rad22 DNA fragment was radiolabeled by PCR in the presence of [α-32P]dCTP. The labeled DNA fragment was digested by EcoRI to generate cohesive termini. The concentration of DNA was expressed as moles of nucleotides. EcoIII and RQI DNase were purchased from Promega. HaeIII and other restriction enzymes were purchased from Boehringer.

This paper is available on line at http://www.jbc.org

Received for publication, August 4, 2000
Published, JBC Papers in Press, August 23, 2000, DOI 10.1074/jbc.M007060200

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The Journal of Biological Chemistry Vol. 275, No. 45, Issue of November 10, pp. 35607–35611, 2000
Printed in U.S.A.

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Support by Research Fellowship BK21 from the Korean Ministry of Education.

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§ The abbreviations used are: DSB, double-strand break; HR, homologous recombination; NHEJ, non-homologous end joining; SSA, single-strand annealing; PCR, polymerase chain reaction; DTT, dithiothreitol; IP, immunoprecipitation; EM, electron microscopy; bp, base pair(s).
enzymes were purchased from New England Biolabs or Takara.

Electron Microscopic Analysis—Electron microscopic analysis was performed according to Griffith et al (15). Rad22 protein was incubated with 15.4 μM PstI-digested pBR322 DNA in 20 μl of 10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM MgCl2 for 5 min at room temperature. The protein-DNA complexes were fixed with 0.25% glutaraldehyde for 5 min and filtered through 2-ml columns of Bio-Gel A5m (Bio-Rad). The fractions containing the complexes were mixed with a mounting buffer (2 mM spermidine, 2 mM MgCl2, 0.15 M NaCl, 0.1 M KCl), and directly mounted to charged carbon-coated 400-mesh copper grids. The grids were washed with series of water/ethanol rinses and were tungsten-shadowed. The protein-DNA complexes were visualized on Phillips EM 400 or Hitachi TEM.

Nuclease Protection Assay—Linearized pBluescript SK(+) DNA digested with EcoRI (15 μg) was pre-incubated with various amounts of Rad22 at 37 °C for 15 min in 20 mM Tris-Cl, pH 7.4, 1 mM DTT and subsequently treated with 0.5 unit of restriction endonuclease HaeIII or exonuclease ExoIII supplemented with appropriate buffers at 37 °C for 30 min. The reactions were deproteinized with 0.5 mg/ml proteinase K and 0.5% SDS at 37 °C for 30 min, and the products were resolved in 0.9% agarose gels. The experiment using a 199-base pair PCR product (13 nm) was performed by the same method except that 0.5 unit of RQ1 DNase was used instead of HaeIII and the reaction products were resolved on 10% polyacrylamide gels.

In Vivo Cross-linking, Immunoprecipitation, and PCR—Experiments were performed as described elsewhere (16, 17). Briefly, for cross-linking of proteins and DNA, exponentially growing cells were treated with 1% formaldehyde for 30 min at room temperature. The cells were harvested and lysed in 1.4 ml of lysis buffer (50 mM HEPES-KOH, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (v/v) sodium deoxycholate) by vortexing with glass beads. Chromosomes were sheared by sonication to generate DNA of 0.5–1 kilobase pair in length. For immunoprecipitation (IP), 0.5 mg of cell lysate in 0.5 ml of lysis buffer was incubated with immunoadfinity-purified polyclonal anti-Rad22 antiserum at 4 °C for 3 h with rotation. The anti-Rad22 sera were raised from rabbit using glutathione S-transferase-Rad22 fusion protein as antigen. Then a 10-μl slurry of protein A-Sepharose was added and incubated for another 3h. The precipitated materials were deproteinized by 100 μg of proteinase K and co-precipitated DNAs were recovered by phenol/chloroform extraction and ethanol precipitation. To detect the co-precipitated DNA by PCR, 2 μl of 1/10 diluted DNA recovered from the crude lysate and 2 μl from IP was used as template. Suitable PCR reactions were performed to amplify short fragments of mat1-M, L region, rhp511, and rad22. The resulting PCR products were analyzed by 2.5% agarose gel electrophoresis. The primers and S. pombe cell strains used in these experiments are listed in Tables I and II, respectively.

RESULTS

Rad22 Protein—Chromatographic purification of recombinant Rad22 protein is described under “Experimental Procedures.” The final step of purification yielded a 59.2-kDa protein of over 95% homogeneity, and this protein was used in all subsequent biochemical analyses (Fig. 1A). Since the observed molecular mass of Rad22 was slightly larger than the expected 52 kDa, the bacterially expressed and the endogenous Rad22 were compared by Western blot. As shown in Fig. 1B, the results showed no difference between the two proteins.

Direct Observation of Rad22-DNA Complexes by Electron Microscopy—The DNA binding property of Rad22 protein was examined by transmission electron microscopy. Rad22 formed large protein-DNA complexes at protein to nucleotide ratios higher than 1 to 2,000, which were often lost during the EM preparation processing. We found that at ratios below 1 to 2,000, single multimeric Rad22 protein complex predominantly bound on one molecule of DNA (~30%, n = 60) on each EM field. As shown in Fig. 2, Rad22 bound preferentially to the end of the duplex DNA (~70% of the Rad22-DNA complexes; Fig. 2, A and B) and sometimes induced recircularization of the linear DNA (Fig. 2C). Internal binding of Rad22 was also found to a lesser extent than end binding. In this case, Rad22-DNA complex always formed multiple loops (Fig. 2, D-F). Various sizes of Rad22 (ranging from 10 to 35 nm in diameter) were found, suggesting the protein would exist as multimeric forms. Such multimerized Rad22 mediated by self-interaction may explain the recircularization (Fig. 2C) and loop formation (Fig. 2, D–F). The data above indicate that Rad22 preferentially binds to the end of linear duplex DNA as multimers, although it could also bind internally to the DNA strand.

Rad22 Can Protect Linear Duplex DNA from Exonuclease Attack—In order to investigate preferential binding of Rad22 at the end of duplex DNA, nuclease protection experiment was performed. Linear plasmid DNA was pre-incubated with various amounts of Rad22 and then digested by the endonuclease HaeIII or the exonuclease ExoIII. The resulting protected DNA was visualized on agarose gel. As shown in Fig. 3A, HaeIII digested most of DNA, implying that Rad22 does not protect DNA from HaeIII. In contrast, DNA digestion by ExoIII was not observed at the same range (data not shown). Since Rad22 preferentially binds to the end of DNA, Rad22 could protect DNA from ExoIII digestion even at low concentration (Fig. 3B, lanes 6 and 7). Compared with the EM experiment, the protein/nucleotide ratio for DNA protection increased about 100-fold, suggesting that Rad22 would dissociate from the DNA ends over the time course of the experiment.

Due to the large size of plasmid DNA, protein/nucleotide ratio could not exceed 1 in the experiment above. To rule out the possibility that the failure of endonuclease protection experiment was caused by insufficient supply of protein, we used radiolabeled short 199-base pair DNA as substrate and increased the ratio between protein and nucleotide. Rad22 protected the short DNA from ExoIII digestion as it did with plasmid DNA (Fig. 3E). In contrast, the digestion of DNA by RQ I DNase could not be avoided even with a 670-fold molar excess of Rad22 to nucleotides (Fig. 3D, lane 7). In case of ExoIII digestion with the same small DNA fragment, more than 50% of DNA was protected by Rad22 (above 100 nm). The retarded band seen at the highest concentration of Rad22 seemed to result from the incomplete removal of DNA-bound Rad22 by proteinase K (Fig. 3E, lane 7). These results suggest that Rad22 preferentially binds to the duplex end and partially protects DNA from exonuclease digestion.

Rad22 Associates with the DNA Double-strand Break in the Mating Type Region—In order to determine whether Rad22 is able to bind to DSB in vivo, mating type locus was investigated by use of chromatin IP analysis. Mating type switching is preceded by DSB formation in the chromosomal DNA close to the right-hand border of the mat1 cassette, designated smt. About 20% of exponentially growing population of homothallic strain contains a DSB at smt (18). In vivo Rad22 binding to DSB at smt was examined by in vivo DNA-protein cross-linking followed by immunoprecipitation. Two sets of PCR primers 214 bp upstream (mat1-M, primers P1 and P2) or 125 bp downstream (L region, primers P3 and P4) of smt were designed and used as primers for PCR amplification (Fig. 4A). After DNA-protein complexes were cross-linked by formaldehyde, Rad22-DNA complexes were isolated by IP with anti-Rad22 antisera. The presence of mat1-M and L DNA fragments in the complex
precipitated by anti-Rad22 antiserum or by pre-immune serum was compared between homothallic wild type strain and \textit{smt-0} mutant background. Because the \textit{smt-0} mutant does not have a DSB due to an ~200-bp deletion around \textit{smt} (19, 20), which also includes P3 primer binding site, amplification of L fragment failed (Fig. 4A) in this strain. Both \textit{mat1-M} and L fragments were enriched in the Rad22 IP complex from the wild type strain but not in the control IP using pre-immune sera (Fig. 4B, lanes 2–4, upper panel). In contrast, the same fragments were not observed from the Rad22 IP complex from the \textit{smt-0} mutant, while the \textit{mat1-M} fragment was normally amplified from the input DNA recovered from total extract as it was in wild type strain (lanes 5–7, upper panel). Together, these results indicate that a DSB is required for Rad22 binding to \textit{smt}. In comparison, there is little enrichment of the control DNAs (\textit{rhp51} and \textit{rad22}*) from all IP complexes (Fig. 4B, middle and lower panels), showing that the enrichment of \textit{mat1-M} and L fragments by anti-Rad22 antisera is highly specific. Western blot analysis indicated that nearly equal amounts of Rad22 protein were expressed and precipitated from both strains (Fig. 4C). Quantitation analysis revealed that \textit{mat1-M} and L fragments were enriched in the IP complexes of anti-Rad22 antisera by about 7- and 23-fold respectively, when compared with the IP complex formed by pre-immune sera in wild type strain. However, \textit{mat1-M} in the \textit{smt-0} strain had no significant change (Fig. 4D). Together, the data suggest that Rad22 specifically associates with the DSB itself in vivo.

**DISCUSSION**

In the present study, we demonstrated that Rad22 directly binds to the end of linear duplex DNA and protects DNA from exonuclease digestion \textit{in vitro}. In addition, we found that Rad22 associates with a DSB at the mating type region. These \textit{in vitro} and \textit{in vivo} data suggest that Rad22 protein recognizes and directly associates with DSB in the process of DNA repair.

For appropriate DNA repair, DSB should be initially resected by the Rad50-Mre11-Xrs2 complex (21). Therefore, a very important question is whether Rad52 homologues bind to DSB before or after the resection. Several observations argue that at least prior resection may not be necessary for Rad22 and Rad52 binding to DSB \textit{in vitro}. hRad52 could bind not only to the end with long single-strand DNA tail, which mimics resected DSB, but also to the blunt end, albeit with lower affinity (13). Rad22 protected both blunt- or cohesive-ended DNA from exonuclease digestion to the same extent. These data suggested that a long single-strand tail might not be essential for Rad52 binding to DSB, and Rad52 homologues could act as an initial sensor of DSB before the resection of the ends.

Of course, it is conceivable that Rad22 binding to DSB is mating type-specific because \textit{rad22} gene was originally isolated by compensation of switching defect of \textit{rad22–67} mutant. However, it is unlikely because the \textit{rad22} deletion mutant showed phenotypes of impaired DSB repair as well as defect in mating type switching (22). It remains to be determined whether Rad22 can associate with DSBs incurred by other means such as ionizing radiation.

\[\text{References}\]

\[\text{Supplemental Data}\]
Regarding the biological significance of Rad52 homologues-DSB complex, our results support the idea that Rad52 acts as a gate-keeper between HR and NHEJ, as proposed by Van Dyck et al. (13). In this model, DSB binding of hRad52 directs DSB into HR by competing with another end-binding protein Ku, which directs DSB into NHEJ. Since no homologue of Ku has been identified in fission yeast, it is difficult to predict whether the gate-keeping role of Rad22 in recombination is the same as that of hRad52. However, a recent study measuring NHEJ activity in various S. pombe mutants provides a clue that Rad22 would compete with NHEJ components (23). Among these mutants, the rad22 deletion mutant showed more...
than 3-fold increase in NHEJ activity compared with the wild type strain, while little change in NHEJ activity was observed in rhp51 or rhp54 deletion mutants. In addition, the length of nucleotide deletion around the re-joined junction region was slightly increased in the rad22 deletion mutant. These results suggest that Rad22 might bind to the DSB, partially protect the ends from exonuclease digestion, and inhibit the NHEJ by competing with NHEJ components at DSB.

Recent biochemical studies proposed the role of Rad52 as a mediator between the single-strand binding protein RPA and the strand-exchange protein Rad51 (22, 25–27). In this model, Rad52 may bind to RPA, which is located on the single-strand tail at DSB and recruits Rad51 to replace RPA. Rad52 might also be involved in SSA. However, genetic studies of S. cerevisiae have revealed that, unlike other members of the RAD52 epistasis group, RAD52 is required for all HR events studied so far including Rad51-independent events (reviewed in Ref. 24). Therefore, a role as a mediator of Rad51-dependent HR or a component of SSA seems to be insufficient to explain the pleiotrophic phenotypes of rad52 deletion mutant. Rather, it is more likely that Rad52 and its homologues may be initial sensors of DSB and act as directors of the DSB repair system. After initial recognition of a DSB, Rad22 and other Rad52 homologues may determine which sub-pathway of HR will be addressed by recruiting proper components depending on the context of substrates.

Comparison of functional conservation among Rad52 homologues from different species would be useful to understand the in vivo function of this family. Unlike RAD52 in S. cerevisiae, its homologues in both S. pombe and vertebrates may not be essential for HR and DSB repair since their deletion mutants do not show severe defects in those processes. Both S. pombe rad22 mutant and vertebrate RAD52 knockout cells show only a small reduction of HR and moderate sensitivity to ionizing radiation (22, 25–27). This difference could be explained by the presence of redundant proteins. Recently, a rad22” homologue was found in S. pombe. The gene, named rti1” (for rad22 isogene 1), was isolated as a multicopy suppressor of temperature-sensitive cell division cycle mutant phenotype of rad22-H6. rti1” not only exhibits 65% amino acid identity in the N-terminal half to Rad22 but also suppresses UV and bleomycin sensitivity and sterility of a rad22 deletion mutant. Most intriguingly, the rad22/rti1 double mutant is inviable (28), indicating that Rad22, together with Rti1, may play the role of Rad52 in S. cerevisiae. Therefore, we suggest that the role of Rad52 in S. cerevisiae may be carried out by several proteins, such as Rad22 and Rti1, in higher eukaryotes.

Acknowledgments—We thank Drs. Onyou Hwang and Gwen Sancar for critical readings and comments on this manuscript. We are grateful for the hospitality of Dr. Jack Griffith and Dr. Steve Jett for allowing us to use EM. We thank Drs. Masayuki Yamamoto, Amikam Cohen, and Nabiheh Ayoub for S. pombe wild type and smt-0 strains.

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