Visualization of recombination intermediates produced by RAD52-mediated single-strand annealing

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Double-strand breaks (DSBs) occur frequently during DNA replication. They are also caused by ionizing radiation, chemical damage or as part of the series of programmed events that occur during meiosis. In yeast, DSB repair requires RAD52, a protein that plays a critical role in homologous recombination. Here we describe the actions of human RAD52 protein in a model system for single-strand annealing (SSA) using tailed (i.e. exonuclease resected) duplex DNA molecules. Purified human RAD52 protein binds resected DSBs and promotes associations between complementary DNA termini. Heteroduplex intermediates of these recombination reactions have been visualized by electron microscopy, revealing the specific binding of multiple rings of RAD52 to the resected termini and the formation of large protein complexes at heteroduplex joints formed by RAD52-mediated annealing.

INTRODUCTION

In meiotic cells, programmed double-strand breaks (DSBs) are introduced into DNA to initiate meiotic recombination. DSBs are also formed in somatic cells during S phase due to the collapse of replication forks, or can be caused by DNA damaging agents. To minimize the genomic instability associated with DSB formation, eukaryotic cells have evolved two major routes for their efficient repair: homologous recombination and non-homologous end joining (Kanaar *et al.*, 1998).

In *Saccharomyces cerevisiae*, most DSBs are repaired by homologous recombination, a process that is mediated by the products of the RAD52 epistasis group of genes comprising RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54, MRE11 and XRS2. Two pathways of repair have been described, RAD51-dependent strand invasion or RAD51-

independent single-strand annealing (SSA). Both processes require the actions of the RAD52 gene product, and extreme recombination and repair defects are observed in *rad52* mutants where the introduction of a single DSB can cause cell death (Paques and Haber, 1999).

RAD52-mediated SSA is thought to involve nucleolytic resection of the DSB to produce 3'-extended single-stranded tails that, when complementary, anneal to form heteroduplex DNA. It was recently shown that the human and yeast RAD52 proteins bind specifically to DSBs and partially resected DSBs to protect them from nuclease attack and to facilitate end-to-end interactions (Van Dyck *et al.*, 1999; Kim *et al.*, 2000; Parsons *et al.*, 2000). Moreover, RAD52 protein has been shown to promote DNA annealing reactions *in vitro* (Mortensen *et al.*, 1996; Reddy *et al.*, 1997; Shinohara *et al.*, 1998; Sugiyama *et al.*, 1998).

In the work presented here, we have extended these studies to visualize annealing reactions mediated by human RAD52 in order to define the molecular mechanism of SSA. We show that RAD52 rings bind resected DNA termini and facilitate the annealing of complementary DNA sequences at these termini. Visualization of the intermediates and products of the SSA reaction reveals that RAD52 directly promotes the annealing of complementary sequences and remains bound to the heteroduplex DNA intermediate.

RESULTS AND DISCUSSION

To develop an *in vitro* system for RAD52-mediated SSA, DNA molecules containing complementary ssDNA tails were constructed. As shown in Figure 1, these were constructed by exonuclease treatment and restriction digestion of linearized

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Fig. 1. Scheme depicting the construction of the substrates DNA-1 and DNA-2 for RAD52-mediated SSA. The 497 bp *HindIII*-*EcoRI* region of pJYM3.45 (hatched) was chosen to produce linear DNA molecules with complementary single-stranded tails as indicated. The two resulting substrates contain a 3' single-stranded tail at one extremity and a short restriction site overhang at the other. This diagram is not drawn to scale.

Fig. 2. Annealing of complementary ssDNA tails by RAD52. **(A)** Schematic diagram illustrating the DNA substrates and products of RAD52-mediated SSA. DNA-1 and DNA-2, which contain duplex regions ~1.55 and 1.9 kbp in length, respectively, anneal to form a product that is ~4 kbp in length. **(B)** Annealing reactions contained 5'-³²P-labelled DNA-1 and DNA-2, and the indicated amounts of RAD52. Products were analysed by agarose gel electrophoresis following deproteinization. Lanes 1–6, RAD52 was added to a mixture of DNA-1 and DNA-2, and products were analysed after 10 min incubation at 37°C. Lanes 7–12, reactions were initiated by pre-incubation of RAD52 with DNA-2 (10 min) followed by the addition of DNA-1 as described in Methods. Lane M, 1 kbp ladder marker. **(C and D)** Time course of RAD52-mediated SSA. A reaction mixture (60 µl) containing ³²P-labelled DNA-1 and DNA-2 was supplemented with RAD52 (80 nM) and aliquots (10 µl) were taken at the indicated times. DNA products were analysed by agarose gel electrophoresis after deproteinization (lanes 1–6), or after glutaraldehyde fixation (lane 7). The graph indicates the percentage of ³²P-labelled product formed as a function of time in the reactions shown in (C).

plasmid DNA (3.45 kbp in length). The resultant duplex products, DNA-1 and DNA-2, contain complementary resected single-strand tails 500 and 700 nucleotides long, respectively.

When added to a mixture of ³²P-labelled DNA-1 and DNA-2, human RAD52 protein catalysed the formation of a 4 kbp product (Figure 2B, lanes 2–6), consistent with the annealing of the two resected DNA substrates. RAD52-mediated annealing was dependent upon protein concentration, with the reaction being most efficient at ~80 nM RAD52 (Figure 2B, lane 4). Higher concentrations of RAD52 were inhibitory (Figure 2B, lane 6). In the absence of RAD52 very little spontaneous annealing occurred under these reaction conditions (Figure 2B, lane 1). Annealing also occurred when RAD52 was incubated with DNA-2 prior to the addition of DNA-1 (Figure 2B, lanes 8–12). In contrast, however, incubation of RAD52 with each DNA individually, followed by their mixing, decreased the efficiency of annealing ~2.5-fold (data not shown).

Time course reactions indicated that the RAD52-mediated SSA reaction was rapid: indeed, immediately after addition of RAD52 almost 13% of the radiolabelled DNA had already been converted into annealed products (Figure 2C, lane 2, zero time point, sample taken ~15 s after RAD52 addition). Moreover, 50% of the DNA was annealed within 5 min, and product formation reached a plateau (73%) at ~20 min (Figure 2D). In the absence of RAD52, <2% of the DNA annealed spontaneously during the 20 min time course. Interestingly, when a sample taken from the RAD52-mediated SSA reaction was fixed with glutaraldehyde prior to electrophoresis, most of the radiolabel was found in the wells of the gel (Figure 2C, lane 7). This result indicates (i) that SSA promoted by RAD52 involves the formation of large protein–DNA networks, and (ii) that the products of annealing remain bound by RAD52 protein.

To analyse the mechanism by which RAD52 catalyses SSA, the intermediates and products of reactions similar to those described above were negatively stained with uranyl acetate and visualized by electron microscopy. DNA molecules with only their unique single-stranded tail bound by one or several RAD52 molecules could be seen (Figure 3A). These molecules were

Fig. 3. Electron microscopic visualization of RAD52-mediated SSA. Reactions contained DNA-1 and DNA-2 and were started by addition of RAD52 (80 nM). **(A)** Several RAD52 rings can be seen bound to the single-stranded tail of one DNA molecule. **(B)** Two DNA molecules are held together by a RAD52 'bridge'. The bars represent 100 nm.

characteristic of the most abundant binding complex, since they were observed on 48% of the 89 DNA molecules examined. In each case, the number of individual RAD52 rings on the single-stranded tail varied between one and six. DNA molecules with both ends bound by RAD52 were less frequent (11%), consistent with previous observations (Van Dyck *et al.*, 1999; Parsons *et al.*, 2000). In addition to the end-bound RAD52, some molecules exhibited binding to internal regions of the DNA. Indeed, of the 89 molecules examined, 31% showed binding to one end together with some internal binding, whereas 6% showed binding to both ends as well as some internal binding. Confirming the specificity of RAD52 for the single-stranded tails, <2% of the molecules showed only internally bound RAD52.

In addition to the binding of tailed duplex DNA by RAD52, complexes were observed in which two DNA molecules were connected by RAD52 (Figure 3B). The results obtained by electron microscopy were consistent with the pairing of DNA-1 with DNA-2, indicating that these molecules are likely to represent heteroduplex intermediates of the SSA reaction. However, the small differences in DNA length, together with the observed variance in length of adsorbed DNA, made it difficult to eliminate the possibility that the observed complexes consisted of two identical DNA molecules that had been brought together and stabilized by RAD52-mediated end-to-end interactions. Indeed, similar complexes were seen when either of the tailed DNA substrates was incubated alone with RAD52 (data not

shown). Finally, more intricate intermediates in which several DNA molecules were held together by RAD52 were seen upon examination of the annealing reactions. Further analyses of these small networks revealed that only one end of each DNA molecule (presumably the single-stranded tail) was bound by RAD52, with the rest of the molecule remaining unbound (data not shown, but similar to the complex shown in Figure 4D). Taken together, these results indicate: (i) that the association of single strands by RAD52 is an important step in SSA, one that occurs without regard for sequence complementarity and may potentially allow multiple attempts at annealing; and (ii) that SSA proceeds through a reaction that involves RAD52-mediated bi- and multi-molecular DNA contacts.

RAD52 protein is known to form heptameric ring structures exhibiting a diameter of ~130 Å with a large central cavity (Van Dyck *et al.*, 1998; Stasiak *et al.*, 2000). The biological relevance of these structures has until now been unclear. However, in the SSA reactions visualized by electron microscopy, we observed rings of RAD52 on both the tailed DNA substrates (Figure 3A) and also on DNA molecules that appeared to attempt annealing (Figure 3B). Finally, rings are also visible in larger protein–DNA aggregates involving several DNA molecules. These results lead us to suggest that these rings are the active structures of RAD52 during SSA.

In vivo, SSA often takes place between DNA repeats that are separated by heterologous sequences. We therefore determined whether RAD52 could promote annealing reactions using DNA in which complementary single-stranded sequences were flanked by 170-nucleotide-long heterologous 3'-termini (DNA-3 and DNA-4; Figure 4A). Incubation of DNA-3 and DNA-4 with RAD52, followed by deproteinization and gel electrophoresis, revealed the formation of an annealed product with the expected 2.7 kbp length (Figure 4B). The identity of this product was confirmed by restriction enzyme analysis, using enzymes that cut within the annealed heteroduplex region.

When the annealed products were visualized by electron microscopy, we observed bi-molecular complexes composed of two DNA molecules connected by RAD52 (Figure 4C). In this case, due to the differences in length of the two DNA substrates, it was possible to use contour length measurements to show that the majority of these complexes were formed by the annealing of one molecule of DNA-3 with DNA-4. In addition, protein–DNA networks were also observed in which several DNA molecules were held together by RAD52 (Figure 4D). Taken together, these observations indicate that RAD52 protein promotes SSA reactions with DNA molecules containing complementary single-strand termini, even when the complementary sequences are separated from the 3'-termini by heterologous DNA sequences.

The results presented in this paper show that: (i) RAD52 rings are likely to be the active catalytic species in SSA; (ii) SSA involves the association of single strands by RAD52, irrespective of their complementarity, thus allowing the annealing of complementary sequences; and (iii) annealing can take place between resected molecules that contain complementary regions flanked by homologous or heterologous termini. Our results also suggest that RAD52 remains bound to the annealed heteroduplex regions, and that the ssDNA is not easily released from non-productive (i.e. protein-mediated joints containing identical strands) complexes to permit further attempts at annealing. It is therefore possible that other proteins, possibly

Fig. 4. RAD52-mediated SSA of complementary single strands flanked by terminal heterologies. (A) Scheme depicting the annealing of DNA-3 with DNA-4. Complementary regions of ssDNA are indicated. (B) Reactions contained 3'-³²P-labelled DNA-3 and DNA-4 (3 and 1.7 μ M, respectively), and the indicated amounts of RAD52. After 15 min incubation at 37°C, DNA products were analysed by gel electrophoresis following deproteinization. Lane M, 1 kbp ladder marker. (C and D) Electron microscopic visualization of RAD52-mediated SSA with DNA-3 and DNA-4. Reactions contained DNA-3 and DNA-4 (1.5 and 0.85 μ M, respectively) and were initiated by addition of RAD52 (80 nM). (C) DNA-3 (black arrows) and DNA-4 (white arrows) are either brought into close contact (left image), or are held together (right image) by RAD52. (D) Network in which the ssDNA tails of three DNA-3 molecules (black arrows) and two DNA-4 molecules (white arrows) are held together by RAD52. The bars represent 100 nm.

recruited by RAD52, are required *in vivo* to mediate the release of RAD52 from DNAs that have attempted or undergone annealing.

Human RAD52 protein binds single-stranded oligonucleotides to form a protein–DNA complex in which the DNA is unusually sensitive to chemical probes (Parsons *et al.*, 2000). To account for this extreme chemical reactivity, it was proposed that the ssDNA lies in an exposed channel on the surface of the RAD52 ring, a structure that would facilitate its interaction with complementary DNA sequences. Our visualization of RAD52 ring structures in SSA reactions provides further support for this proposal and leads us to strengthen our argument that SSA is driven by RAD52-directed DNA–DNA contacts in which ssDNA lies exposed on the surface of the protein. In this regard, it is likely that the end-binding complex illustrated in Figure 3A represents an early intermediate of the annealing reaction in which the DNA is exposed at the surface of the rings, thus facilitating efficient interaction with a complementary single strand. That such a complex is proficient in annealing is consistent with our biochemical results showing that pre-binding of RAD52 to DNA-2 prior to addition of DNA-1 results in efficient annealing. The present data, however, indicate that it is unlikely that SSA is mediated by a single terminally bound RAD52 ring, since multiple rings were visualized on the resected duplexes and on

the heteroduplex products of annealing. Moreover, since DNA molecules containing terminal heterologies were annealed efficiently by RAD52 protein there appears to be no requirement for these terminal sequences to be complementary. Indeed, it is possible that the terminal RAD52–DNA complexes simply facilitate interactions with naked ssDNA, and that sequence complementarity permits annealing within the complex. Alternatively, SSA of substrates possessing terminal heterologies may be mediated by internally bound RAD52 rings.

Although no homologues of RAD52 have been identified in prokaryotes, RAD52 shares some structural and functional similarities with a class of recombination proteins found in bacteria and phages. Among the hallmarks of this class of proteins, whose members include *Escherichia coli* RecT and β protein from bacteriophage λ , is their ability to form ring structures that interact with ssDNA, as well as their ability to promote SSA (Passy *et al.*, 1999). The three-dimensional reconstruction of the heptameric RAD52 ring (Stasiak *et al.*, 2000), and the study of its DNA binding mode (Van Dyck *et al.*, 1998; Parsons *et al.*, 2000) and mechanism of annealing (this work), indicate that the path of the DNA on the surface of the RAD52 ring may be similar to that proposed for rings of β protein (Passy *et al.*, 1999).

METHODS

Proteins. Recombinant human RAD52 was prepared from baculovirus-infected Sf9 cells (Van Dyck *et al.*, 1998). λ exonuclease (Pharmacia), S1 nuclease (Gibco-BRL), terminal transferase (New England Biolabs), T4 polynucleotide kinase (USB) and restriction enzymes (New England Biolabs) were used as recommended by the supplier.

DNA substrates. Plasmid pJYM3.45, a gift from Dr Jean-Yves Masson, is a 3468 bp derivative of pGEM-7Z (+) (Promega) and contains unique *HindIII* and *EcoRI* sites separated by 497 bp. To produce DNA-1 and DNA-2, pJYM3.45 (20 μ g) was cut with *EcoRI* and *HindIII*, respectively, and treated with a pre-determined saturating amount of λ exonuclease (10 U/ μ g DNA) for 2 min at 25°C, in a 400 μ l reaction containing 50 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 20 mM KCl and 5 mM β -mercaptoethanol. Exonuclease treatment produced 3'-tails with average lengths of 500 n (DNA-1) or 700 n (DNA-2), as determined by agarose gel electrophoresis following S1 nuclease treatment. Subsequent digestion with *PstI* or *AflwNI*, followed by gel purification, allowed the recovery of DNA-1 and DNA-2, respectively. Each substrate therefore contains a 3' single-stranded tail at one extremity and a short restriction site overhang at the other. To permit DNA detection by autoradiography, a fraction of the DNA (<10%) was 3'-³²P-end labelled with terminal transferase or 5'-³²P-end labelled with T4 polynucleotide kinase. Amounts of DNA are expressed in moles of nucleotides.

Substrates DNA-3 and DNA-4 were produced as follows: a 300 bp PCR fragment from the *tet^R* gene of pBR322 was cloned in the unique *XbaI* site of pBEND2(Sy1) (Hanakahi and Maizels, 2000). The resulting plasmid contained an *EcoRI-HindIII* fragment comprising two direct repeats of 109 bp separated by a stretch of 340 bp with a unique *Sall* site in its middle. This *EcoRI-HindIII* fragment was then sub-cloned into the high-copy vector pUC19 to create pUC-SSA1. Uniformly ³²P-labelled pUC-SSA1 DNA (3.2 kbp) was prepared from cells grown in [³²P]orthophosphate (Van Dyck *et al.*, 1998), and 3' single-stranded tails were generated by resection of the *Sall*-cut plasmid with a predetermined saturating amount of λ exonuclease. The extent of digestion was determined to be in the order of 250 nucleotides on average based on the release of acid-soluble ³²P counts (Van Dyck *et al.*, 1999). In the products, complementary sequences (~80 nucleotides in length) were separated from the 3'-termini by 170 nucleotides of heterologous sequences. Subsequent cleavage with *ScaI* generated DNA-3 and DNA-4, containing duplex regions of ~1.7 and 0.9 kbp, respectively, which were then purified by agarose gel electrophoresis. When required, both DNA substrates were 3'-³²P-end labelled using terminal transferase and [³²P]ddATP.

Annealing assays. Reactions (10 μ l final volume) were carried out with DNA-1 (1.5 μ M) and DNA-2 (1.5 μ M) in 20 mM tri-ethanolamine-HCl pH 7.5. After 5 min at 37°C, RAD52 or enzyme diluent (1 μ l) was added and incubation was continued for a further 10 min. Alternatively, the reaction was started by addition of RAD52 to DNA-2 alone, and after 10 min at 37°C DNA-1 was added and incubation continued for a further 10 min. Reactions were terminated by addition of 2 μ l of stop buffer (0.1 M Tris-HCl pH 7.5, 3% SDS and 10 mg/ml proteinase K), followed by 15 min incubation at 37°C. DNA products were

analysed by 0.8% agarose gel electrophoresis using TAE buffer, followed by autoradiography. Annealing reactions with DNA-3 and DNA-4 were carried out as described above using a mixture of the two DNAs.

Electron microscopy. Protein-DNA complexes were prepared as described above and processed directly by dilution and washing in 5 mM Mg(OAc)₂ followed by uranyl acetate staining (Van Dyck *et al.*, 1998), or after fixation with glutaraldehyde (0.2% final concentration). Complexes were visualized at magnifications of 20 500 $\times$ or 25 500 $\times$ using a Philips CM100 electron microscope.

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REFERENCES

- Hanakahi, L.A. and Maizels, N. (2000) Transcriptional activation by LR1 at the E μ enhancer and switch region sites. *Nucleic Acids Res.*, **28**, 2651–2657.
- Kanaar, R., Hoeijmakers, J.H.J. and van Gent, D.C. (1998) Molecular mechanisms of DNA double-strand break repair. *Trends Cell Biol.*, **8**, 483–489.
- Kim, W.J., Lee, S., Park, M.S., Jang, Y.K., Kim, J.B. and Park, S.D. (2000) Rad22 protein, a Rad52 homologue in *Schizosaccharomyces pombe*, binds to DNA double-strand breaks. *J. Biol. Chem.*, **275**, 35607–35611.
- Mortensen, U.H., Bendixen, C., Sunjevaric, I. and Rothstein, R. (1996) DNA strand annealing is promoted by the yeast Rad52 protein. *Proc. Natl Acad. Sci. USA*, **93**, 10729–10734.
- Paques, F. and Haber, J.E. (1999) Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.*, **63**, 349–404.
- Parsons, C.A., Baumann, P., Van Dyck, E. and West, S.C. (2000) Precise binding to single-stranded DNA termini by RAD52 protein. *EMBO J.*, **19**, 4175–4181.
- Passy, S.I., Yu, X., Li, Z., Radding, C.M. and Egelman, E.H. (1999) Rings and filaments of β protein from bacteriophage λ suggest a superfamily of recombination proteins. *Proc. Natl Acad. Sci. USA*, **96**, 4279–4284.
- Reddy, G., Golub, E.I. and Radding, C.M. (1997) Human Rad52 protein promotes single-strand DNA annealing followed by branch migration. *Mutat. Res.*, **377**, 53–59.
- Shinohara, A., Shinohara, M., Ohta, T., Matsuda, S. and Ogawa, T. (1998) Rad52 forms ring structures and cooperates with RPA in single-strand DNA annealing. *Genes Cells*, **3**, 145–156.
- Stasiak, A.Z., Larquet, E., Stasiak, A., Müller, S., Engel, A., Van Dyck, E., West, S.C. and Egelman, E.H. (2000) The human Rad52 protein exists as a heptameric ring. *Curr. Biol.*, **10**, 337–340.
- Sugiyama, T., New, J.H. and Kowalczykowski, S.C. (1998) DNA annealing by Rad52 protein is stimulated by specific interaction with the complex of replication protein A and single-stranded DNA. *Proc. Natl Acad. Sci. USA*, **95**, 6049–6054.
- Van Dyck, E., Hajibagheri, N.M.A., Stasiak, A. and West, S.C. (1998) Visualisation of human Rad52 protein and its complexes with hRad51 and DNA. *J. Mol. Biol.*, **284**, 1027–1038.
- Van Dyck, E., Stasiak, A.Z., Stasiak, A. and West, S.C. (1999) Binding of double-strand breaks in DNA by human Rad52 protein. *Nature*, **398**, 728–731.

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