Human PSF binds to RAD51 and modulates its homologous-pairing and strand-exchange activities

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ABSTRACT

RAD51, a eukaryotic recombinase, catalyzes homologous-pairing and strand-exchange reactions, which are essential steps in homologous recombination and recombinational repair of double strand breaks. On the other hand, human PSF was originally identified as a component of spliceosomes, and its multiple functions in RNA processing, transcription and DNA recombination were subsequently revealed. In the present study, we found that PSF directly interacted with RAD51. PSF significantly enhanced RAD51-mediated homologous pairing and strand exchange at low RAD51 concentrations; however, in contrast, it inhibited these RAD51-mediated recombination reactions at the optimal RAD51 concentration. Deletion analyses revealed that the N-terminal region of PSF possessed the RAD51- and DNA-binding activities, but the central region containing the RNA-recognition motifs bound neither RAD51 nor DNA. These results suggest that PSF may have dual functions in homologous recombination and RNA processing through its N-terminal and central regions, respectively.

INTRODUCTION

Homologous recombination functions in accurate chromosome segregation during meiotic cell division I (1,2). Defects in meiotic homologous recombination in mice cause infertility, indicating its required function in meiosis (3–5). Homologous recombination also has essential functions in the repair of DNA double strand breaks (DSBs) and the rescue of stalled replication forks during mitosis (6–8). The homologous-recombination defects in mitosis cause instability of the genomic DNA (8,9), leading to chromosomal aberrations and tumorigenesis. Therefore, homologous recombination is essential for both meiotic and mitotic homologous recombination (10).

meiotic and mitotic homologous recombination (10). During the homologous-recombination process, RAD51 promotes the key recombination reactions, homologous pairing and strand exchange, in an ATP-dependent manner (11–15). To promote homologous pairing, RAD51 first binds to single-stranded DNA (ssDNA), which is produced at the DSB site, and forms helical nucleoprotein filaments. The RAD51–ssDNA filaments then bind to intact double-stranded DNA (dsDNA). The homologous sequences are aligned between ssDNA and dsDNA, and new Watson–Crick base pairs (heteroduplex) are formed between the ssDNA and the complementary strand of the dsDNA within the RAD51–ssDNA–dsDNA filament. This step is called homologous pairing. The heteroduplex region formed by homologous pairing is further expanded by the RAD51-mediated strand exchange reaction. RAD51 requires cofactors for efficient promotion of the homologous-pairing and strand-exchange reactions. In humans, RAD52, RAD54, RAD54B, RAD51B–RAD51C, RAD51AP1 and BRCA2 have been reported as such cofactors for the RAD51-mediated homologous pairing and/or strand exchange reactions in vitro (7–10,16–18).

Polypyrrimidine tract-binding protein-associated splicing factor (PSF) was first identified as a component of the spliceosome (19). A tandem repeat of RNA-recognition motifs was found in the central region of PSF, suggesting that PSF functions in RNA processing through its RNA-binding activity. Subsequently, the DNA-binding activity of PSF and its regulatory function for transcription were reported (20–24). PSF was also identified in a complex form with its paralog, p54(nrb). The PSF–p54(nrb) complex reportedly binds to mRNAs, and functions in pre-mRNA processing (25,26). A biochemical study revealed that the PSF–p54(nrb) complex also stimulates the DNA end joining reaction by Ku70/80, DNA ligase IV and XRCC4 (27). These facts suggest that PSF may be a multifunctional protein, which functions in RNA processing, transcription regulation and DNA recombination.

Interestingly, PSF alone reportedly promotes homologous pairing between ssDNA and dsDNA in vitro (28),

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suggesting that PSF may also be involved in homologous recombination. To evaluate the functions of PSF in homologous recombination, in the present study, we tested whether PSF affects the recombination reactions promoted by RAD51, which is an essential recombination enzyme in both meiotic and mitotic homologous recombination. We found that purified human PSF directly binds to RAD51. Biochemical analyses revealed that PSF synergistically stimulates RAD51-mediated homologous pairing and strand exchange under low RAD51 conditions. In contrast, the RAD51-mediated recombination reactions were significantly inhibited by PSF when the RAD51 concentrations were optimal for the homologous-pairing and strand-exchange reactions without PSF. Domain mapping analyses showed that the N-terminal region (amino acid residues 1–266) of PSF and the central ATPase domain (amino acid residues 82–339) of RAD51 are involved in the PSF–RAD51 binding. The PSF central region (amino acid residues 267–468), containing a tandem repeat of RNA-recognition motifs, did not bind to DNA, while in contrast, the N-terminal 1–266 fragment of PSF bound to ssDNA and dsDNA. Therefore, the N-terminal region of PSF may be the functional domain for binding RAD51 and regulating the RAD51-mediated recombination reactions.

MATERIALS AND METHODS

Protein purification

The human PSF gene was isolated from a human cDNA pool by polymerase chain reaction, and was cloned into the pET-15b vector (Novagen, Darmstadt, Germany). In this construct, the His6 tag sequence was fused at the N-terminal end of the gene. The DNA fragments encoding PSF(1–266) and PSF(267–468) were cloned into the pET-15b vector. The His6-tagged PSF protein, the His6-tagged PSF(1–266) mutant and the His6-tagged PSF(267–468) mutant were each expressed in the Escherichia coli BL21(DE3) strain, which also carried an expression vector for the minor tRNAs (Codon(+)RP, Stratagene, La Jolla, CA, USA). The cells producing the proteins were resuspended in buffer A [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM 2-mercaptoethanol and 10% glycerol], and were disrupted by sonication. The cell debris was removed by centrifugation for 20 min at 27 700×g, and the supernatant was mixed gently with 3 ml of Ni–NTA agarose beads (Qiagen, Hilden, Germany) at 4°C for 1 h. The protein-bound beads were then packed into an Econo-column (Bio-Rad Laboratories, Hercules, CA, USA), and were washed with 120 ml of buffer A, containing 20 mM imidazole. The proteins were eluted by a 60 ml linear gradient of imidazole from 20 to 300 mM. The peak fractions were collected, and 2 units of thrombin protease (GE Healthcare Biosciences, Uppsala, Sweden) per mg of protein were added to remove the His6 tag. The samples were immediately dialyzed against buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.25 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol. After removal of the His6 tag, PSF and PSF(267–468) were loaded onto a 3 ml hydroxyapatite column (Bio-Rad Laboratories, Hercules, CA, USA), which was immediately eluted with 40 ml of buffer containing 20 mM sodium phosphate (pH 8.0), 200 mM NaCl, 0.25 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol. On the other hand, PSF(1–266) was loaded onto a 3 ml hydroxyapatite column, and the flow through fractions were collected. PSF was further purified by chromatography on a 1.5 ml SP-Sepharose column (GE Healthcare Biosciences, Uppsala, Sweden). The column was washed with 15 ml of buffer B [20 mM Tris-HCl (pH 8.5), 0.25 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol] containing 250 mM KCl, and PSF was eluted with a 30 ml linear gradient of KCl from 250 to 800 mM. PSF(1–266) was dialyzed against buffer B containing 100 mM KCl, and was further purified by chromatography on a MonoQ column (GE Healthcare Biosciences, Uppsala, Sweden). The column was washed with 10 ml of buffer B, and PSF(267–468) was eluted with a 10 ml linear gradient of KCl from 0 to 800 mM. PSF, PSF(1–266) and PSF(267–468) were dialyzed against buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol, and were stored at −80°C.

Human RAD51 and human RPA were expressed in E. coli cells (29,30), and were purified as described previously (30–32). The RAD51 used in the present study was the K313-type isoform, which is highly conserved among eukaryotes (33).

The DNA fragments encoding the N-terminal domain (1–114 amino acid residues) and the ATPase domain (82–339 amino acid residues) of RAD51 were cloned into the pET-15b vector (Novagen, Darmstadt, Germany). The His6-tagged N-terminal and ATPase domains of RAD51 were each expressed in the E. coli JM109(DE3) strain, which also carried an expression vector for the minor tRNAs (Codon(+)RIL, Stratagene, La Jolla, CA, USA). The cells producing the proteins were resuspended in buffer A [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM 2-mercaptoethanol and 10% glycerol], and were disrupted by sonication. The cell debris was removed by centrifugation for 20 min at 27 700×g, and the supernatant was mixed gently with 1.5 ml of Ni–NTA agarose beads (Qiagen, Hilden, Germany) at 4°C for 1 h. The protein-bound beads were then packed into an Econo-column (Bio-Rad Laboratories, Hercules, CA, USA), and were washed with 120 ml of buffer A, containing 20 mM imidazole. The proteins were eluted by a 60 ml linear gradient of imidazole from 20 to 300 mM. The peak fractions were collected, and 2 units of thrombin protease (GE Healthcare Biosciences, Uppsala, Sweden) per mg of protein were added to remove the His6 tag. The samples were immediately dialyzed against buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.25 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol. After removal of the His6 tag, PSF and PSF(267–468) were loaded onto a 3 ml hydroxyapatite column (Bio-Rad Laboratories, Hercules, CA, USA), which was immediately eluted with 40 ml of buffer containing 20 mM sodium phosphate (pH 8.0), 200 mM NaCl, 0.25 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol. On the other hand, PSF(1–266) was loaded onto a 3 ml hydroxyapatite column, and the flow through fractions were collected. PSF was further purified by chromatography on a 1.5 ml SP-Sepharose column (GE Healthcare Biosciences, Uppsala, Sweden). The column was washed with 15 ml of buffer B [20 mM Tris-HCl (pH 8.5), 0.25 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol] containing 250 mM KCl, and PSF was eluted with a 30 ml linear gradient of KCl from 250 to 800 mM. PSF(1–266) was dialyzed against buffer B containing 100 mM KCl, and was further purified by chromatography on a MonoQ column (GE Healthcare Biosciences, Uppsala, Sweden). The column was washed with 10 ml of buffer B, and PSF(267–468) was eluted with a 10 ml linear gradient of KCl from 0 to 800 mM. PSF, PSF(1–266) and PSF(267–468) were dialyzed against buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol, and were stored at −80°C.

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0.1 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol.

DNA and RNA substrates

In the D-loop formation assay, superhelical dsDNA (pB5Sarray DNA) was prepared by a method avoiding alkaline treatment of the cells harboring the plasmid DNA, to prevent the dsDNA substrates from undergoing irreversible denaturation. Instead, the cells were gently lysed using sarkosyl, as described previously (31). The pB5Sarray DNA contained 11 repeats of a sea urchin 5S rRNA gene (207-bp fragment) within the pBlueScript II SK(+) vector. For the ssDNA substrate used in the D-loop assay, the following high-performance liquid chromatography (HPLC)-purified oligonucleotide was purchased from Nihon Gene Research Laboratory: 50-mer, 5'-GGAGTCGGATTTATCCAGGCAGTCCATCCAAGTACACTAACGAGCTCCTATCAAAGA-3', 50-mer RNA, 5'-UCCUUUGUAAAGAGGUCCUUGGCGAAG-3'. The 5' ends of the oligonucleotide were labeled with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA) in the presence of [γ-32P]ATP at 37°C for 30 min.

For the strand exchange assay with oligonucleotides, the following HPLC-purified oligonucleotide was purchased from Nihon Gene Research Laboratory and Gene Design: 63-mer 5'-GGAATTCGGTATCCAGGGTCCTCATCCAGTACTAACGAGGCTCCTATTTGAGGATTTGAGCTATTGGCGATGG-3'. The 5' ends of the oligonucleotide were labeled with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA). The linear dsDNA was prepared by a method avoiding digestion. For the strand exchange assay with oligonucleotides, the following HPLC-purified oligonucleotide was purchased from Nihon Gene Research Laboratory and Gene Design: 63-mer 5'-GGAATTCGGTATCCAGGGTCCTCATCCAGTACTAACGAGGCTCCTATTTGAGGATTTGAGCTATTGGCGATGG-3'. The 5' ends of the oligonucleotide were labeled with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA). The linear dsDNA was prepared by a method avoiding digestion. For the strand exchange assay with oligonucleotides, the following HPLC-purified oligonucleotide was purchased from Nihon Gene Research Laboratory and Gene Design: 63-mer 5'-GGAATTCGGTATCCAGGGTCCTCATCCAGTACTAACGAGGCTCCTATTTGAGGATTTGAGCTATTGGCGATGG-3'. The 5' ends of the oligonucleotide were labeled with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA). The linear dsDNA was prepared by a method avoiding digestion. For the strand exchange assay with oligonucleotides, the following HPLC-purified oligonucleotide was purchased from Nihon Gene Research Laboratory and Gene Design: 63-mer 5'-GGAATTCGGTATCCAGGGTCCTCATCCAGTACTAACGAGGCTCCTATTTGAGGATTTGAGCTATTGGCGATGG-3'. The 5' ends of the oligonucleotide were labeled with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA).

The pull-down assay with Ni–NTA beads

Purified His6-tagged PSF, His6-tagged PSF(1–266), or His6-tagged PSF(267–468) (3.8 μg) was mixed with RAD51 (7.4 μg) in 100 μl of binding buffer, containing 15 mM sodium phosphate (pH 7.5), 95 mM NaCl, 5 mM imidazole, 0.21 mM EDTA, 2 mM 2-mercaptoethanol, 0.025% Triton X-100 and 9% glycerol, and Ni–NTA agarose beads (3 μl, 50% slurry) were then added. Purified His6-tagged PSF was also tested with RPA (10 μg) in 100 μl of binding buffer, containing 15 mM sodium phosphate (pH 7.5), 80 mM NaCl, 5 mM KCl, 5 mM imidazole, 0.2 mM EDTA, 2 mM 2-mercaptoethanol, 0.025% Triton X-100 and 9% glycerol, and then Ni–NTA agarose beads (3 μl, 50% slurry) were added. After an incubation at room temperature for 1 h, the beads were washed with 1 ml of wash buffer, containing 20 mM sodium phosphate (pH 7.5), 100 mM NaCl, 0.25 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol. The proteins bound to the beads and those in the supernatant were fractionated by 12% SDS–PAGE, and the bands were visualized by Coomassie Brilliant Blue staining.

The pull-down assay with the ssDNA beads

PSF (1 μM) and RAD51 (5 μM) were incubated with magnetic streptavidin beads conjugated with the biotinylated ssDNA 80-mer (16 μM) at 37°C for 20 min, in 10 μl of reaction buffer, containing 28 mM Tris-HCl (pH 8.0), 4 mM HEPES-NaOH (pH 7.5), 110 mM NaCl, 0.06 mM EDTA, 0.4 mM 2-mercaptoethanol, 6% glycerol, 1 mM MgCl2, 1.4 mM DTT and 1 mM ATP. After an incubation, the beads were washed with 10 μl of reaction buffer. The proteins bound to the beads were fractionated by 12% SDS–PAGE, and the bands were visualized by Coomassie Brilliant Blue staining.

The D-loop formation assay

PSF and RAD51 were incubated with the 32P-labeled 50-mer oligonucleotide (1 μM) at 37°C for 20 min, in 7 μl of reaction buffer, containing 24 mM Tris-HCl (pH 8.0), 2 mM HEPES-NaOH (pH 7.5), 55 mM NaCl, 0.03 mM EDTA, 0.2 mM 2-mercaptoethanol, 3% glycerol, 1 mM MgCl2, 1.2 mM DTT, 2 mM AMPPPNP and 0.1 mg/ml BSA. The reactions were then initiated by the addition of the pB5Sarray superhelical dsDNA (30 μM) along with 9 mM MgCl2, and were continued at 37°C for 30 min. The reactions were stopped by the addition of 0.2% SDS and 1.5 mg/ml proteinase K (Roche Applied Science, Basel, Switzerland), and were further incubated at 37°C for 15 min. After adding six-fold loading dye, the deproteinized reaction products were separated by 1% agarose gel electrophoresis in 1×TAE buffer at temperature for 2.5 h, the PSF beads were washed two times with 100 μl of wash buffer, containing 20 mM sodium phosphate (pH 8.0), 60 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol. The proteins bound to the PSF beads were fractionated by 15% SDS-polyacrylamide gel electrophoresis (PAGE), and the bands were visualized by Coomassie Brilliant Blue staining.
The analyzer (Fujifilm, Tokyo, Japan).

3.3 V/cm for 2.5 hr. The gels were dried, exposed to an imaging plate, and visualized using an FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan).

The strand-exchange assay

The φX174 circular ssDNA (20 μM), RAD51 (0.5–6.6 μM) and RPA (1.3 μM) were incubated with or without PSF (1.0 μM) at 37°C for 10 min, in the reaction buffer, containing 30 mM Tris-HCl (pH 8.0), 95 mM NaCl, 5 mM KCl, 0.05 mM EDTA, 0.2 mM 2-mercaptoethanol, 6% glycerol, 1 mM MgCl₂, 1 mM CaCl₂, 1.5 mM DTT, 1 mM ATP, 0.1 μg/ml BSA, 20 mM creatine phosphate and 75 μg/ml creatine kinase. The reactions were then initiated by the addition of the 32-mer linear dsDNA (20 μM), and were purified by polyacrylamide gel electrophoresis. The gels were dried, exposed to an FLA-7000 imaging analyzer.

The strand-exchange assay with oligonucleotides

RAD51 (3 μM) and the indicated amount of PSF were incubated with a 63-mer ssDNA (15 μM) at 37°C for 10 min, in 10 μl of reaction buffer, containing 28 mM Tris-HCl (pH 7.5), 110 mM NaCl, 0.06 mM EDTA, 0.4 mM 2-mercaptoethanol, 6% glycerol, 1 mM MgCl₂, 1 mM CaCl₂, 1.4 mM DTT, 1 mM ATP, 0.1 mg/ml BSA, 20 mM creatine phosphate and 75 μg/ml creatine kinase. The reactions were initiated by the addition of the 32-mer dsDNA or RNA/DNA hybrid (1.5 μM), which shared sequence homology with the 63-mer ssDNA. The 32-mer dsDNA and the RNA/DNA hybrid used in this assay were purified by polyacrylamide gel electrophoresis in 1xTAE buffer at 3.3 V/cm for 4 h. The products were visualized by SYBR Gold (Invitrogen, Carlsbad, CA, USA) staining.

The DNA-binding assay

The φX174 circular ssDNA (20 μM) or the linearized φX174 dsDNA (20 μM) was mixed with the indicated amount of PSF, PSF(1–266) or PSF(267–468) in 10 μl of a standard reaction solution, containing 30 mM Tris-HCl (pH 8.0), 1.5 mM DTT, 100 mM NaCl, 1 mM MgCl₂, 5% glycerol and 0.1 mg/ml BSA. The reaction mixtures were incubated at 37°C for 10 min, and were then separated by 0.8% agarose gel electrophoresis in 1xTAE buffer (40 mM Tris-acetate and 1 mM EDTA) at 3.3 V/cm for 2.5 h. The bands were visualized by ethidium bromide staining.

MALDI-TOF-MS analysis

A 2 μl aliquot of 5% trifluoroacetic acid (TFA) was added to 18 μl of a 0.4 mg/ml PSF solution, or 1 μl of 5% TFA was added to 9 μl of a 1.0 mg/ml PSF(1–266) or 1.3 mg/ml PSF(267–468) sample, and the mixture was bound to a ZipTip (C4) pipette tip (Millipore, Billerica, MA, USA). After the protein was bound, the tip was first rinsed with 0.1% TFA and 5% methanol, and then rinsed with 0.1% TFA. PSF, PSF(1–266), or PSF(267–468) was eluted with 0.1% TFA and 80% acetonitrile, and was deposited onto the sample plate, which was precoated with a dried layer of sinapic acid (Sigma-Aldrich, St Louis, MO, USA). A MALDI-TOF mass spectrometry analysis was performed with an AXIMA-CFR mass spectrometer (Kratos Analytical, Manchester, UK). Bovine carbonic anhydrase II and BSA were used as standard proteins for external calibration. Each single spectrum was obtained as an accumulation of 50 laser shots.

RESULTS

Human PSF binds to RAD51

To test whether PSF binds to RAD51, we performed pull-down assays with the PSF-conjugated beads. To prepare the PSF beads, human PSF was expressed in E. coli cells as a His₆-tagged protein, and was purified by chromatography on a Ni-NTA agarose column (Figure 1A, lane 4). The His₆ tag was removed by thrombin protease treatment (Figure 1B), and the protein was further purified by chromatography on a hydroxyapatite and SP-Sepharose columns (Figure 1A, lanes 5 and 6). The purified PSF was conjugated to Affi-Gel 10 beads, and RAD51 binding to the PSF beads was tested by the pull-down assay. As shown in Figure 1C (lane 5), substantial amounts of RAD51 were associated with the PSF beads, indicating that PSF directly binds to RAD51.

Structural studies revealed that RAD51 is composed of two distinct domains, the N-terminal and ATPase domains (34–38). To identify the PSF-binding domain of RAD51, we prepared two RAD51 fragments, amino acid residues 1–114 and 82–339, which contained the N-terminal and ATPase domains (34–38). To identify the PSF-binding domain of RAD51, we prepared two RAD51 fragments, amino acid residues 1–114 and 82–339, which contained the N-terminal and ATPase domains. To identify the PSF-binding domain of RAD51, we prepared two RAD51 fragments, amino acid residues 1–114 and 82–339, which contained the N-terminal and ATPase domains, respectively (Figure 1C, lanes 3 and 4). The pull-down assay revealed that the ATPase domain of RAD51 bound to PSF with the almost same efficiency as the full-length RAD51 (Figure 1C, lane 9). In contrast, the RAD51 N-terminal fragment containing amino acid residues 1–114 did not show significant binding to PSF (Figure 1C, lane 7). These results indicate that PSF directly binds to the ATPase domain of RAD51.

The PSF–RAD51 binding was also detected by the Ni-bead pull-down assay with His₆-tagged PSF (Figure 1D, lane 8). In contrast, PSF did not bind to RPA (Figure 1D, lane 5), suggesting that the PSF–RAD51 interaction is specific. The Ni-bead pull-down assay, in which all of the proteins could be visualized on the SDS-PAGE gel, allowed us to estimate the binding stoichiometry between PSF and RAD51. As shown in Figure 1E, His₆-tagged PSF copelleted RAD51 in a...
RAD51 concentration-dependent manner. Consistently, the amount of free RAD51 in the supernatant was also reduced in the presence of His$_6$-tagged PSF (Figure 1F and G). The PSF–RAD51 binding stoichiometry was about 1:1, in the presence of an excess of RAD51 (Figure 1E, lane 8).

**PSF modulates homologous pairing by RAD51**

We next tested the effect of PSF in the RAD51-mediated homologous pairing. PSF reportedly promotes the formation of D-loops (28), which are the products of the homologous pairing reaction between ssDNA fragments and superhelical dsDNA. Therefore, we performed the D-loop formation assay (Figure 2A). The superhelical dsDNA used in this assay was prepared by a method without alkali treatment, to avoid denaturation of the double helix of the dsDNA (31). Consistent with the previous observations, PSF or RAD51 alone promoted homologous pairing (Figure 2B, lanes 2–6, and 2C) (28). When PSF and a low amount of RAD51 (0.1 µM) were present...
together, the amount of D-loops increased, in a PSF-concentration-dependent manner (Figure 2B, lanes 7–10, and Figure 2C). This stimulation of D-loop formation was not observed when the reactions were conducted in the absence of an ATP analog, AMPPNP, which was required for the RAD51-mediated D-loop formation, but not for the PSF-mediated D-loop formation (Figure 2D).

Therefore, PSF may function as an activator for RAD51 in homologous pairing under low RAD51 conditions.

In contrast to the PSF-mediated homologous-pairing activation in the presence of 0.1 μM RAD51, PSF significantly inhibited the RAD51-mediated homologous pairing, when the reaction was conducted in the presence of RAD51 concentrations above 0.35 μM, which are optimal for the reaction without PSF. As shown in Figure 2E and F, PSF stimulated the RAD51-mediated homologous pairing in the presence of 0.05 and 0.1 μM RAD51 (compare lane 2 with lane 8, and lane 3 with lane 9).

In contrast, PSF inhibited the homologous pairing in the presence of 0.35 and 0.7 μM RAD51 (Figure 2E, compare lane 5 with lane 11, and lane 6 with lane 12, and Figure 2F). These results indicated that PSF stimulates homologous pairing in the presence of a suboptimal concentration of RAD51, but inhibits the reaction in the presence of an optimal concentration of RAD51.

**PSF modulates RAD51-mediated strand exchange**

We next tested whether PSF affects strand exchange by RAD51 (Figure 3A). To evaluate the activator function of PSF in the RAD51-mediated strand exchange, we first performed the strand-exchange assay with a suboptimal concentration of RAD51 (0.5 μM). Under these reaction conditions, a small amount of joint molecule (JM) product was detected in the absence of PSF in the 30, 60 and 90 min reactions (Figure 3B, lanes 5, 7 and 9, and
In contrast to the D-loop formation, PSF itself did not promote strand exchange (Figure 3B, lane 11, and Figure 3C). When PSF was added to the reaction mixture, the amount of the JM products formed by the RAD51-mediated strand exchange significantly increased (Figure 3B, lanes 4, 6, 8 and 10, and Figure 3C). These results indicated that PSF stimulates the RAD51 strand-exchange activity, as well as the homologous-pairing stimulating activity, in the presence of a suboptimal concentration of RAD51. This RAD51 activation by PSF was not observed when the strand-exchange reactions were performed without Ca\(^{2+}\), which stabilizes the RAD51–ssDNA filament by inhibiting ATP hydrolysis (39) (Figure 3D).

Under the reaction conditions with Ca\(^{2+}\), a 2–4 μM RAD51 concentration was optimal for strand exchange without PSF (Figure 3E, lanes 7 and 9 and Figure 3F). Consistent with the results from the D-loop formation assay, PSF inhibited the RAD51-mediated strand exchange in the presence of an optimal RAD51 concentration, although it stimulated the reactions under low RAD51 conditions (0.5 and 1.0 μM RAD51) (Figure 3E and F). Therefore, PSF functions as activator or inhibitor of the homologous-pairing and strand-exchange activities of RAD51, in a RAD51 concentration-dependent manner.

We then tested whether the order of the PSF addition affected the strand-exchange stimulation in the presence of the low RAD51 concentration (0.5 μM). As shown in Figure 3B and C, when PSF, RAD51, RPA and ssDNA were co-incubated together before initiating the reaction by the addition of dsDNA, the strand-exchange reaction was significantly enhanced (Figure 4A, lanes...
Effects of the PSF reaction order on RAD51-mediated strand exchange. The \( \phi X174 \) circular ssDNA (20\( \mu \)M), RAD51 (0.5\( \mu \)M), RPA (1.3\( \mu \)M) and PSF (1.0\( \mu \)M) were incubated at 37\( ^\circ \)C in various combinations. After this incubation, the reactions were then initiated by the addition of \( \phi X174 \) linear dsDNA (20\( \mu \)M), and were continued at 37\( ^\circ \)C for the indicated times. The deproteinized products were separated by 1% agarose gel electrophoresis, and were visualized by SYBR Gold staining. (A) The proteins and ssDNA were incubated in the combinations represented on the right side of panel A. Lane 1 indicates a negative control experiment without proteins. Lanes 2, 4, 6 and 8 indicate control experiments without PSF, and lanes 3, 5, 7 and 9 indicate experiments with PSF. The reaction time was 60 min. (B) The \( \phi X174 \) circular ssDNA was incubated with PSF at 37\( ^\circ \)C for 10 min. After this incubation, RAD51 was added to the reaction mixture, which was incubated at 37\( ^\circ \)C for 5 min. RPA was then added, and the reactions were initiated by the addition of \( \phi X174 \) linear dsDNA. Reactions were continued for the indicated times. (C) Graphic representation of the strand-exchange experiments shown in panel B. The band intensities of the JM products were quantified. Closed and open circles represent the experiments with and without PSF, respectively. (D) The \( \phi X174 \) circular ssDNA was incubated with RAD51 at 37\( ^\circ \)C for 10 min. After this incubation, PSF was added to the reaction mixture, which was incubated at 37\( ^\circ \)C for 5 min. RPA was then added, and the reactions were initiated by the addition of \( \phi X174 \) linear dsDNA. Reactions were continued for the indicated times. (E) Graphic representation of the strand-exchange experiments shown in panel D. The band intensities of the JM products were quantified. Closed and open circles represent the experiments with and without PSF, respectively. (F) The \( \phi X174 \) circular ssDNA was incubated with RAD51 at 37\( ^\circ \)C for 10 min. After this incubation, RPA was added to the reaction mixture, which was incubated at 37\( ^\circ \)C for 5 min. PSF was then added, and the reactions were initiated by the addition of \( \phi X174 \) linear dsDNA. Reactions were continued for the indicated times. (G) Graphic representation of the strand-exchange experiments shown in panel F. The band intensities of the JM products were quantified. Closed and open circles represent the experiments with and without PSF, respectively.

PSF competes with RAD51 for ssDNA binding

Under the high RAD51 conditions, PSF inhibited homologous pairing and strand exchange by RAD51, suggesting that PSF may compete with RAD51 for ssDNA binding. To test this possibility, we prepared streptavidin beads conjugated with a biotinylated ssDNA 80-mer, and performed a pull-down assay with these ssDNA beads (Figure 5A). As shown in Figure 5B (lanes 4 and 6) and 5C, RAD51 or PSF bound to the ssDNA beads. However, the amount of RAD51 bound to the ssDNA beads was significantly decreased, when both RAD51 and PSF were coincubated with the ssDNA beads (Figure 5B, lane 5, and Figure 5C). This inhibition of the RAD51–ssDNA binding by PSF was also observed when PSF was added to the ssDNA beads before and after RAD51 (Figure 5B, lanes 7–12, and Figure 5C). Therefore, we concluded that the PSF-dependent inhibition of homologous pairing and strand exchange, in the presence of an optimal amount of RAD51, may be due to RAD51 disassembly from the ssDNA by PSF. PSF may not disassemble RAD51 from ssDNA, when a RAD51-free ssDNA region is available for PSF–ssDNA binding, under the low RAD51 conditions.

PSF promotes strand exchange between ssDNA and an RNA/DNA hybrid

PSF contains RNA-binding motifs, and is known to be involved in transcription (20–24), suggesting that PSF may be involved in a transcription-associated
recombination event. Therefore, we tested whether PSF affects strand exchange between ssDNA and an RNA/DNA hybrid, which may be formed when transcription stalls. To do so, we performed a strand exchange assay with short oligonucleotides (Figure 6A). In this assay, the 32-mer dsDNA and RNA/DNA hybrid were purified by polyacrylamide gel electrophoresis. Consistent with the results from the D-loop formation assay and the strand-exchange assay with long DNA substrates, PSF stimulated the RAD51-mediated strand exchange with oligonucleotides under the suboptimal RAD51 conditions. We found that RAD51 promoted strand exchange between ssDNA and an RNA/DNA hybrid (Figure 6B and C). Intriguingly, PSF also stimulated this reaction (Figure 6B and C). Therefore, PSF has the potential to function in transcription-associated recombination.

**Domain analysis of PSF**

To identify the functional domains of PSF, we constructed three PSF deletion mutants, PSF(1–266), PSF(267–468) and PSF(469–707), which were composed of amino acid residues 1–266, 267–468 and 469–707, respectively (Figure 7A). These PSF deletion mutants were expressed in E. coli cells, and PSF(1–266) and PSF(267–468) were successfully obtained in the soluble fractions. However, PSF(469–707) was only found in the insoluble fraction, suggesting that it did not fold properly. Therefore, we purified PSF(1–266) and PSF(267–468) as recombinant proteins (Figure 7B). The purified PSF deletion mutants did not contain the His6 tag. The predicted molecular masses of PSF, PSF(1–266), and PSF(267–468) were 76, 26 and 23 kDa, respectively. However, PSF, PSF(1–266) and PSF(267–468) migrated on an SDS-denaturing polyacrylamide gel with distances corresponding to about 100, 42 and 25 kDa, respectively (Figure 7B).

A mass spectrometric analysis revealed that the molecular masses of purified PSF, PSF(1–266), and PSF(267–468) were 76, 26 and 23 kDa, respectively. Therefore, the abnormal migrations of PSF and PSF(1–266) on the SDS-denaturing polyacrylamide gel may be a consequence of their basic pI values, 9.45 and 12.01, respectively.

As previously reported by Akhmedov and Lopez (28), PSF bound to ssDNA and dsDNA (Figure 8A and B, lanes 2–4). PSF(1–266) also bound to both ssDNA and dsDNA (Figure 8A and B, lanes 5–7). In contrast, PSF(267–468), which contains RNA recognition motifs, did not bind either ssDNA or dsDNA under these reaction conditions (Figure 8A and B, lanes 8–10). Therefore, the DNA- and RNA-binding domains are separately located in the N-terminal and central domains of PSF, respectively.

**DISCUSSION**

Previous studies have suggested that PSF is a multifunctional nuclear protein, which functions in RNA synthesis, RNA processing, RNA transport, and transcription (40). In addition to these functions, interestingly, PSF was
purified from a HeLa cell extract as a protein that promotes homologous pairing between ssDNA and dsDNA in vitro (28). This finding suggested that PSF may also function in homologous recombination, and prompted us to test whether PSF affects the recombination activity of RAD51, which is a central protein for homologous recombination in eukaryotes.

In the present study, we purified human PSF as a recombinant protein, and found that PSF directly binds to RAD51. Interestingly, PSF stimulates RAD51-mediated homologous pairing and strand exchange at low RAD51 concentrations, but inhibits these reactions at high RAD51 concentrations, which are optimal for the reactions without PSF. These findings suggest that PSF may be a novel recombination factor, which has both activator and suppressor functions for the RAD51 recombinase activity. During the initial period of homologous recombination in cells, PSF may function as activator, perhaps because the RAD51 accumulation at the recombination sites is not yet sufficient. The RAD51 concentration at the recombination sites is supposed to increase during the homologous recombination processes. Excess RAD51 accumulation on chromosomes at later stages of homologous recombination may induce undesired recombination, which may cause the chromosomal aberrations frequently found in tumor cells. PSF may then function as an inhibitor of RAD51, to suppress such inappropriate recombination reactions by removing RAD51 from ssDNA during homologous recombination. We found that PSF competes with RAD51 for ssDNA binding, and removes RAD51 from ssDNA. This fact indicated that PSF does not form a three-component complex with RAD51 and ssDNA, although the PSF–RAD51 complex is formed in the absence of ssDNA. Therefore, PSF may be recruited to the recombination sites as a complex form with RAD51, and may be released from RAD51 to free the ssDNA region when RAD51 is assembled on the recombination site.

We and others found that PSF alone (i) binds to ssDNA and dsDNA, (ii) promotes D-loop formation between ssDNA and dsDNA, and (iii) anneals complementary ssDNAs (28). In this study, we also found that (iv) the N-terminal domain of PSF directly binds to the central ATPase domain of RAD51, and (v) PSF modulates the RAD51-mediated homologous pairing and strand exchange reactions. These biochemical characteristics of PSF partially overlap with those of RAD52, which is an important activator for RAD51 (7–10). In the yeast Saccharomyces cerevisiae, the rad52 mutation reportedly exhibits significant sensitivity to DSB inducing agents, such as ionizing radiation (41,42). However, the
RAD52-knockout chicken DT40 cells did not show significant defects in DSB repair (43). This discrepancy may explain the functional redundancy of RAD52 with other factors in higher eukaryotes (44). PSF may share a common function with RAD52 in vertebrates. PSF was also purified from a nuclear extract of HeLa cells, as a complex form with p54(nrb). Intriguingly, the PSF–p54(nrb) complex stimulated a non-homologous DNA end joining (NHEJ) reaction by Ku70/80, DNA ligase IV and XRCC4 (27). NHEJ is a rapid, error-prone DSB repair pathway. On the other hand, homologous-recombinational repair (HRR) is an accurate, error-free DSB repair pathway. The present study has provided new evidence that PSF physically and functionally interacts with RAD51, which is the central enzyme for the homologous recombination reactions. Therefore, PSF may be involved in both DSB repair pathways, NHEJ and HRR, with different partners, p54(nrb) and RAD51, respectively.

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