Identification of the RecR Toprim Domain as the Binding Site for both RecF and RecO: A Role of RecR in RecFOR assembly at dsDNA-ssDNA junctions*

Masayoshi Honda¹ ², Jin Inoue¹ ², Masatoshi Yoshimasu¹ ³, Yutaka Ito¹ ³ ⁴, Takehiko Shibata¹ ² ³ ⁵, and Tsutomu Mikawa¹ ² ³ ⁵

From the¹ RIKEN Discovery Research Institute, 2-1, Hirosawa, Wako, Saitama 351-0198, Japan, the² Graduate School of Integrated Science, Yokohama City University, 1-7-29, Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan, the³ CREST/Japan Science and Technology Corporation (JST), the⁴ Department of Chemistry, Tokyo Metropolitan University, 1-1, Minami-osawa, Hachioji-shi, Tokyo 192-0397, Japan and the⁵ RIKEN Harima Institute/Spring-8, Mikazuki cho, Hyogo 679-5148, Japan

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Address correspondence to: Tsutomu Mikawa, Bio-supramolecular Structure-Function Group, RIKEN Discovery Research Institute, 1-7-29, Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan, Tel. 81-45-508-7224; Fax. 81-405-508-7364; E-mail: mikawa@riken.jp

The RecR protein forms complexes with RecF or RecO that direct the specific loading of RecA onto gapped DNA. However, the binding sites of RecF and RecO on RecR have yet to be identified. In this study, a Thermus thermophilus RecR dimer model was constructed by NMR analysis and homology modeling. NMR titration analysis suggested that the hairpin region of the helix-hairpin-helix motif in the cavity of the RecR dimer is a binding site for double-stranded DNA (dsDNA) and that the acidic cluster region of the Toprim domain is a RecO binding site. Mutations of E84, D88 and E144 residues comprising that acidic cluster were generated. The E144A and E84A mutations decreased the binding affinity for RecO, but the D88A did not. Interestingly, the binding ability to RecF was abolished by E144A, suggesting that the region surrounding the RecR E144 residue could be a binding site for not only RecO but also RecF. Furthermore, RecR and RecF formed a 4:2 heterohexamer in solution that was unaffected by adding RecO, indicating a preference for RecR over RecO. The RecFR complex is considered to be involved in the recognition of the dsDNA-ssDNA junction, while RecO binds single-stranded DNA (ssDNA) and ssDNA-binding protein. Thus, the RecR Toprim domain may contribute to the RecO interaction with RecFR complexes at the dsDNA-ssDNA junction site during recombinational DNA repair mediated by the RecFOR.

Maintaining genomic integrity by repairing damaged DNA is crucial for all organisms. DNA damage can arise during normal DNA metabolism such as the introduction of mismatches during replication and the deamination of bases, or it can be caused by exposure to exogenous factors such as ultraviolet radiation, γ-radiation and chemical mutagens. Such DNA damage is mostly repaired by base excision repair, nucleotide excision repair and mismatch repair pathways, based on information provided by the complementary strand in a damaged duplex. However, double-stranded DNA (dsDNA)¹ break, and base lesions in single-stranded DNA (ssDNA) gap regions having no complementary strands can also arise, mainly during replication. To cope with this category of DNA damage, organisms have developed recombinational repair pathways, which minimize the loss of genetic information by using homologous DNA as a template for repair.

RecFOR and/or RecBCD pathways are required to initiate homologous DNA recombination in bacteria (1). The Escherichia coli RecFOR pathway is mainly used for ssDNA gap repair, while the RecBCD pathway is responsible for dsDNA break repair. However, the RecFOR pathway can also repair dsDNA breaks, as has been demonstrated in recBC sbcB mutants, which are deficient in the RecBCD
pathway (2,3). In the RecFOR pathway, dsDNA breaks are unwound by the RecQ helicase and processed by the RecJ 5’ to 3’ exonuclease, and the resulting 3’-tailed ssDNA is coated with the ssDNA-binding protein (SSB). The RecF, RecO and RecR proteins then mediate the loading of RecA protein onto the SSB-coated ssDNA, specifically at junctions with dsDNA (4). Afterwards, RecA forms a nucleoprotein filament on ssDNA that interacts with free homologous dsDNA to promote heteroduplex formation between the ssDNA and the complementary strand of the dsDNA (5-7). It was shown recently that the function of RecF, RecO and RecR proteins is conserved in higher eukaryotes. The human tumor suppressor protein BRCA2 homologue Brh2 directs homologous recombination specifically to the dsDNA-ssDNA junction, suggesting a functional similarity between the RecFOR and BRCA2 proteins (8,9). Moreover, Rad52 proteins in human and yeast, which are functional homologues of RecO, facilitate the binding of the eukaryotic RecA homologue Rad51 to ssDNA coated with the eukaryotic SSB homologue RPA (10-12).

E. coli RecF, RecO, and RecR proteins are well studied. The RecR protein plays a critical role in recombinational DNA repair by forming complexes with RecO or RecF (13-15). The RecOR complex facilitates RecA filament formation on SSB-coated ssDNA and prevents the dissociation of RecA from ssDNA ends (16). The RecFR complex binds dsDNA and attenuates the extension of RecA filaments beyond the region of ssDNA in the gapped DNA (14). In addition, the RecFR complex is required for reassembly of the replication holoenzyme after recombinational DNA repair at replication forks (17,18). The RecF protein itself binds DNA and has a weak ATPase activity (19), both of which are enhanced by RecR binding (20,21). Furthermore, specific loading of the RecA protein onto the dsDNA-ssDNA junction is mediated by the RecF, RecO and RecR proteins acting in concert, with no interaction between RecF and RecO detected (4). Therefore, RecR is thought to be the key mediator of RecF and RecO association at dsDNA-ssDNA junctions.

The crystal structure of Deinococcus radiodurans RecR (drRecR) was recently determined (22). As predicted from sequence analysis, drRecR consists of a helix-hairpin-helix (HhH) motif, a zinc finger motif, a Toprim domain and a Walker B motif. drRecR forms a ring-like tetramer, and it has been suggested that the HhH motifs in the central hole are important for DNA binding. However, the RecR binding sites for the RecO and RecF have not been identified. In addition, E. coli RecR has been reported to form a dimer in solution (15,20), and its structural conformation has not been determined. Previously, we reported the backbone NMR assignments of Thermus thermophilus RecR (ttRecR) and suggested that it forms a symmetric homodimer in solution (23).

Here, we have constructed a dimer model of ttRecR and have analyzed its specific interactions with DNA, RecO and RecF. We have also discussed the role of RecR in recombinational DNA repair based on these protein-protein and protein-DNA interactions.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification** – Unlabeled and uniformly $^2$H/$^15$N- or $^{15}$N-labeled T. thermophilus HB8 RecR and RecA (ttRecA) proteins were prepared as previously described (23,24); and RecO (ttRecO), RecF (ttRecF) and SSB (ttSSB) proteins were prepared as described below. Each gene was amplified by PCR and cloned in an expression vector with a T7 promoter. Then, sequences were confirmed and proteins overexpressed in E. coli BL21(DE3)/pLysS cells. Proteins were purified from cell extracts by heat treatment and column chromatography as follows: ttRecO: SP Sepharose Fast Flow (Amersham Biosciences), TOYOPEARL Phenyl-650S (TOSOH), Superdex 75 10/300 GL (Amersham Biosciences) and RESOURCE S 6 ml (Amersham Biosciences); ttRecF: SP Sepharose Fast Flow and Superdex 200 10/300 GL (Amersham Biosciences); and ttSSB: Heparin Sepharose Fast Flow (Amersham Biosciences), TOYOPEARL Butyl-650S (TOSOH) and RESOURCE Q 6 ml. Purified proteins were confirmed by N-terminal sequence analysis and mass spectrometry.

**Preparation of ttRecR Mutants** – The gene encoding the deletion mutants ttRecR$_{75-194}$ and ttRecR$_{1-173}$ were amplified by PCR and cloned into the vector pET-28a (Novagen) after sequence confirmation. The N-terminal His-tagged ttRecR$_{75-194}$ and ttRecR$_{1-173}$ were overexpressed in
E. coli BL21(DE3) and purified from cell extracts with the Magtration System 6GC (PSS Bio Instruments, Inc.) and Superdex 75 10/300 GL. To prepare the ttRecR E84A, D88A and E144A mutants, point mutations were incorporated into the ttRecR expression plasmid using the QuickChange site-directed mutagenesis kit (Stratagene) and were confirmed by sequencing. The ttRecR mutants were expressed and purified using the same methods as for wild-type ttRecR.

**Ultracentrifugation Analysis** – Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A equipped with a four-hole rotor (An60Ti) with six channel standard cells at 15,000 rpm for ttRecR and 7,000 rpm for the ttRecFR complex for 13h at 20°C. The ttRecR and ttRecFR complex were dissolved to 1, 2 or 4 mg/ml in buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl). For molecular mass analysis, the data obtained were tailored to an ideal, single-component model using a solution density of 1.05 g/cm³ and partial specific volume of 0.71 cm³/g for ttRecR and 0.68 cm³/g for the ttRecFR complex.

**Size-exclusion Chromatography** – T. thermophilus RecF (30 µM), RecR (100 µM), RecO (50 µM), RecF (50 µM)+ RecR (100 µM), or RecF (30 µM) + RecR (60 µM) + RecO (30 µM) in buffer (25 mM HEPES-KOH pH 7.2, 150 mM NaCl) were applied to a Superdex-200 HR 10/300 column (Pharmacia) and eluted at a flow rate of 0.5 ml/min in the same buffer.

**NMR Spectroscopy** – NMR experiments were performed at 313K in a triple-resonance cryoprobe fitted with a Z-axis-pulsed field gradient coil using a 600 MHz Bruker DRX spectrometer. Data were processed on a Linux PC with a combination of customized macro-programs in the OpenGL-version of ANSIG v3.3 software (25). For processing three-dimensional (3D) NMR data, a two-dimensional (2D) maximum entropy algorithm was applied for indirect dimensions (26). Peak assignments for ttRecR spectra were obtained by transfer from previously described data (23).

**H/D Exchange and 3D NOESY NMR Experiments** – ¹H/¹⁵N-labelled ttRecR (0.4 mM) in 20 mM Tris-HCl (pH 7.5), 150 mM KCl and 1 mM EDTA was lyophilized and dissolved in D₂O (99.96%). Immediately after the addition of D₂O, protection of amide protons against deuterium was identified by analyzing a series of 2D ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra. The distance constraints around ttRecR residues 170-194 were determined by analysis of the Nuclear Overhauser Effect (NOE) from a 3D ¹⁵N-separated Nuclear Overhauser Effect spectroscopy (NOESY)-HSQC spectrum (27).

**Titration Analysis** – NMR samples of ¹⁵N-labelled ttRecR were prepared at 0.4 mM in 20 mM Tris-HCl (pH 7.2), 150 mM KCl and 1 mM EDTA. Unlabeled protein and DNA were directly added into the sample from a concentrated stock to prevent changes in concentration and pH. For DNA titration experiments, chemical shift mapping was performed by recording the chemical shift of each peak before and after adding DNA to 1.6 mM. The chemical shift change (Δν) was calculated and normalized using the following formula: [(Δ¹H)² + (Δ¹⁵N)²]¹/², where Δ¹H and Δ¹⁵N are the chemical shift differences (Hz) along the ¹H and ¹⁵N axes, respectively (28). For titration experiments with ttRecO, 0.05, 0.1 or 0.2 mM ttRecO were added into 0.2 mM ¹⁵N-labelled ttRecR. The relative intensity of HSQC signals in the presence of 0.2 mM ttRecO [64 scans per free induction decay (FID)] or in its absence [16 scans per FID] were plotted against the number of amino acids and mapped onto the ttRecR dimer structure.

**Structure Modeling** – A structural model of ttRecR dimer was generated with MODELLER version 7.7, a comparative homology modeling software (29). For homology modeling calculations, the drRecR structure was obtained from the Protein Data Bank, 1VDD (22). The integrity and quality of the models were assessed using the program PROCHECK, which generates Ramachandran plot of model structure. The molecular diagrams were depicted using PyMOL.

**Native PAGE Analysis** – Wild-type or mutant ttRecR (10 µM) and wild-type ttRecO (0, 2 and 10 µM) or ttRecF (0, 2 and 10 µM) were incubated in 30 µl of 20 mM Tris-HCl (pH 7.5), 150 mM KCl and 1 mM EDTA. After 15 min of incubation at 37°C, samples were analyzed by polyacrylamide gel electrophoresis (PAGE) in...
12.5% gels under non-denaturing conditions at 4 °C.

**ATP Hydrolysis Assays** – The ssDNA-dependent ATP hydrolysis activity of tTRcR in the presence of tTRcF pathway proteins was measured as described by Bork *et al.* (16) with minor modifications. The ssDNA was first incubated with SSB in 190 µl of the following buffer: 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.3 mM phosphoenolpyruvate, 8 unit/ml pyruvate kinase, 13 units/ml lactate dehydrogenase, 1 mM ATP, and 250 µM NADH at 37°C for 5 min. 10 µl of the tTRcO and tTRcR mixture were added to the SSB-ssDNA solution and incubated for 10 min before tTRcA was added to initiate the ssDNA-dependent ATPase reaction. The standard reaction included 20 µM (with respect to nucleotides) ssDNA, 1 µM tTRcA, 1 µM tTRsB, 1 µM tTRcR and 1 µM tTRcO. The kinetics of ATP hydrolysis was followed by measuring the absorption of NADH at 340 nm using an Ultrospec 4300 pro spectrometer (Amersham Pharmacia).

**Agarose Gel Retardation Assay** – The DNA-tTRcFR complex formation was detected using an agarose gel assay according to the protocol described by Webb *et al.* (20). Each substrate DNA was incubated with wild-type or mutant tTRcR (5 µM) and tTRcF (10 µM) in 20 µl of reaction mixture containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl₂ and 1 mM EDTA at 37°C for 10 min. Samples were analyzed by electrophoresis in a 3% agarose gel in TAE buffer. DNA and DNA-protein complexes were visualized by Gel Star (Cambrex Bio Science).

**CD spectroscopy** – Circular dichroism (CD) measurements were carried out on a Jasco spectropolarimeter, model J720-W.

### RESULTS

**Prediction of tTRcR Dimer Structure by Homology Modeling** – To clarify the biophysical and structural properties of tTRcR, we initially examined its oligomeric state using analytical ultracentrifugation (Fig. 1A). At a tTRcR concentration of 1 mg/ml (47 µM), the molecular mass was calculated to be 48,448 Da, which corresponds to the mass of the tTRcR dimer. Using size-exclusion chromatography, we confirmed that tTRcR exists as a dimer from 1 µM to 1 mM of the protein concentrations. Since the tTRcR concentration (1 µM) was diluted (below 50 nM) during chromatography, these results suggest that tTRcR exists as a dimer at a broad range of concentrations (data not shown). In contrast, the crystallized drRecR formed a ring-like tetramer, in which N-terminal HhH motifs (1-53 residues) contacted with each other, and C-terminal regions (175-198 residues) swapped as described in Figure 1B (22).

In order to investigate the dimerization interface of the tTRcR dimer, we used NMR to perform a proton/deuterium (H/D) exchange experiment, which can identify residues exposed on the protein surface by detecting the exchange of observable amide protons for non-observable deuterium (30,31). The panels in Figure 1C show the ¹H-¹⁵N HSQC spectra of the tTRcR dimer at 15 min, 4 hr and 24 hr after the sample was lyophilized and dissolved in D₂O. Each peak in the panels corresponds to an amide proton from a tTRcR amino acid residue. Amide proton signals were lost due to H/D exchange in a time-dependent manner (Fig. 1C). However, even after 4 hr exposure to D₂O, approximately one-third of the amide protons were protected from deuterium exchange, suggesting that these protons are not on the tTRcR dimer surface. Since tTRcR and drRecR amino acid sequences share 57% identity and 74% homology, and since the secondary structure of the RecR dimer that was previously determined based on our NMR assignments was identical to that of the drRecR crystal structure (Supplemental Fig. 1), the monomeric structures of tTRcR and drRecR are thought to be quite similar despite the differences in their oligomeric states. Therefore, we next sought to explore the interface of the tTRcR dimer by mapping non-exchange tTRcR amide protons to corresponding drRecR regions. When these amide protons were mapped onto the drRecR monomer structure (red spheres in Fig. 1D, left panel), seven residues with protected amide protons were observed at the protein surface: L17, G21, E41, H55, I59, G83 and G89. With the exception of G83 and I59, these residues are located near the HhH motif, making it plausible that this region is responsible for RecR dimerization (Fig. 1D, right panel).
To confirm the above idea, we prepared two \( \text{ttRecR} \) mutants: \( \text{ttRecR}_{75-194} \), in which the N-terminal HhH motif had been truncated; and \( \text{ttRecR}_{1-173} \), in which the C-terminal region corresponding to the C-terminal swapping region of \( \text{drRecR} \) had been truncated. Using circular dichroism spectroscopy (Supplemental Fig. 2), we confirmed that these mutant proteins are not unfolding. Then, we examined their oligomeric state using size-exclusion chromatography (Fig. 1E). \( \text{ttRecR} \) (21.2 kDa) and \( \text{ttRecR}_{1-173} \) (19.2 kDa) were eluted at positions corresponding to 48.5 kDa and 44.6 kDa, respectively, showing that they form dimers. In contrast, \( \text{ttRecR}_{75-194} \) (15.3 kDa) was eluted at a position corresponding to 18.2 kDa, showing that it exists as a monomer. These results indicate that the N-terminal region (1-74) and not the C-terminal region (174-194) is responsible for \( \text{ttRecR} \) dimerization.

Although the \( \text{ttRecR} \) C-terminal region (174-194) was not the interface of the \( \text{ttRecR} \) dimer, the secondary structure of the C-terminal regions was conserved in both \( \text{ttRecR} \) and \( \text{drRecR} \) (Supplemental Fig. 1). In order to examine the local conformation of the C-terminal region of the \( \text{ttRecR} \) dimer, we subsequently carried out \( ^{15} \text{N} \)-separated 3D NOESY-HSQC experiments on \( ^{2} \text{H}^{15} \text{N} \)-labelled \( \text{ttRecR} \). Crosspeaks due to NOE were observed between amide protons of the 192/165, 194/165 and 164/163 residue pairs, indicating that these proton pairs are located within a region of approximately 5 Å. These results suggest that residues 192-194 of \( \text{ttRecR} \) form an anti-parallel \( \beta \)-sheet with residues 163-165 (Fig. 1F). In the case of \( \text{drRecR} \), the corresponding \( \beta \)-sheet is formed between two \( \text{RecR} \) molecules (Fig. 1B, C-terminal swapping region). Since the C-terminal regions of the \( \text{ttRecR} \) dimer did not form an interface, we concluded that the \( \text{ttRecR} \) C-terminal regions fold back to their own subunit and have a similar structure to that of \( \text{drRecR} \).

We employed two \( \text{drRecR} \) monomers capable of interfacing with each other via the N-terminal HhH motif (subunits colored in blue and green in Figure 1B). Then, the C-terminal swapping regions of residues 173-196 were replaced by the same region of subunits, which are colored in red and yellow, respectively, in Figure 1B. We used this structure as a template for homology modeling of the \( \text{ttRecR} \) dimer. The homology modeling was performed using the MODELLER program with the experimentally determined secondary structure of \( \text{ttRecR} \) (23) as an additional constraint. Finally, a dependable \( \text{ttRecR} \) dimer model was constructed (Fig. 1G). The root mean square deviation of \( C^\alpha \) between the derived \( \text{ttRecR} \) structure and the template structure was 0.38 Å.

The \( \text{RecR} \) dimer model was divided into three regions (Fig. 1G): 1) Residues 1-52, termed the HhH motif region after the HhH motif (residues 5-34) it contains; 2) Residues 53-76, termed the Zinc finger region after the zinc finger motif (residues 55-75) it contains; and 3) Residues 77-194, termed the Toprim domain region and comprised of the Toprim domain (residues 77-164) and the Walker-B motif (residues 165-180).

Unless otherwise stated, the designations RecR, RecO, RecF, RecA and SSB refer to the \textit{T. thermophilus} proteins after this section.

Chemical Shift Mapping of DNA Binding Sites onto the \( \text{RecR} \) Dimer – We sought to determine whether the \( \text{RecR} \) dimer interacts with DNA and to identify its DNA binding sites. In this assay, we added unlabeled DNA substrates to \( ^{15} \text{N} \)-labeled \( \text{RecR} \) protein and compared the \( ^{1} \text{H}^{15} \text{N} \) HSQC spectrum of \( \text{RecR} \) alone (black) with that in the presence of DNA (green). Figure 2A shows the superimposed patterns of the two spectra for \( ^{15} \text{N} \)-labelled \( \text{RecR} \) in the absence or presence of 15 bp dsDNA (upper panel) and in the absence or presence of 15 mer ssDNA (lower panel). Since each \( ^{1} \text{H}^{15} \text{N} \) crosspeak in the two panels is derived from the amide proton of a \( \text{RecR} \) amino acid as described in Figure 1C, the amino acids corresponding to the crosspeaks affected by the addition of DNA can be presumed to be involved in DNA binding. To examine the amino acid residues involved in DNA binding, we expressed the data shown in Figure 2A as a change in chemical shift per residue (Fig. 3A, left panels). Major chemical shift changes of residues more than 20 Hz were observed for the G21, G19 and A25 with 53, 41 and 20Hz, respectively (Fig. 2A, upper panel and Fig. 3A, upper left panel). In contrast, when ssDNA was added to \( \text{RecR} \), no major changes in chemical shift were observed (Fig. 2A, lower panel and Fig. 3A, lower left panel).

We then color-coded the amino acid residues that exhibited a change in chemical shift
greater than 20 Hz as red and those that exhibited a change between 5 and 20 Hz as yellow and mapped them onto the molecular surface of the RecR dimer (Fig. 2B). The result shows that the two G19 and two G21 residues showing high levels of chemical shift changes gather on the interface between the two HhH motifs of the RecR dimer, forming a patch with other residues whose chemical shifts are highly affected by the addition of dsDNA. As shown in Figures 2B and 2C, residues G19 and G21 correspond to the hairpin region of the HhH motif, which is located at the center of the inner surface of the RecR dimer. In addition, two Y178 residues in the Toprim domain are situated in the RecR inner surface, forming a cavity with the hairpin region that can accommodate dsDNA. These results suggest that RecR binds dsDNA in this cavity via G19 and G21 of the HhH motif of the RecR dimer (circled area in Fig. 2C).

The Toprim domains of topoisomerase or primase have an acidic cluster region that catalyzes nucleotidyl transferase activity in an Mg$^{2+}$-dependent manner. Since RecR also has a Toprim domain, it has been speculated that it recognizes specific features of damaged DNA via a similar mechanism (33). Therefore, we further investigated RecR-DNA interactions using 3' -tailed and 5' -tailed DNA substrates possessing a base-paired 5' - or 3' -terminus at junction, respectively, in the presence or absence of Mg$^{2+}$. As shown in Figure 3, RecR did not show major chemical shifts at the Toprim domain region, either in the absence (Fig. 3A) or in the presence (Fig. 3B) of the Mg$^{2+}$ ion. These results suggest that a role of Toprim domain of RecR differ from that of topoisomerase or primase. The chemical shift change patterns for dsDNA, ssDNA and dsDNA-ssDNA junctions all differ, but the last is particularly distinctive as the site where dsDNA and ssDNA interactions simultaneously occur.

Investigation of the RecO Binding Site on the RecR Dimer – The E. coli RecOR complex has been reported to facilitate the loading of RecA onto SSB-coated ssDNA (15). Since similar results were obtained for T. thermophilus (J. Inoue et al., unpublished results), we tried to examine the RecO binding site of the RecR dimer using NMR titration analysis. When we sequentially added 0.025, 0.025, 0.05 and 0.1 mM of RecO into 0.2 mM $^{15}$N-labelled RecR dimer, we observed a significant decay in signal intensity for all the crosspeaks of the RecR dimer (data not shown). This decay is not due to aggregation of NMR samples, because RecR and RecO did not form any precipitate after NMR measurements and no aggregates were detected in the NMR sample using size-exclusion chromatography. The most likely reason for these phenomena is the increased molecular weight of RecR due to complex formation with RecO. To compensate for the decreased sensitivity of RecR signals in the presence of 0.2 mM RecO, we repeated the scans four more times and compared the two $^{1}$H-$^{15}$N HSQC RecR dimer spectra (Fig. 4A). Although most crosspeaks were recovered, some were still weak in the presence of RecO (Fig. 4A, right panel). Although we cannot exclude other possibilities, we speculate that these weaknesses are caused by the interaction of RecO with RecR. Therefore, the relative intensity of each crosspeak per residue was plotted as a histogram (Fig. 4B). The relative intensity of crosspeaks for residues 84, 85, 89, 124, 144, 145, 146, 148, 153, 154, 168, 173, 174 and 175 dropped to less than 10% when RecO was added. All these residues are located in the Toprim domain of the RecR dimer. Then, we color-coded residues exhibiting a relative intensity below 10% as red and those between 10% and 20% as yellow, and mapped them onto the structure of the RecR dimer (Fig. 4C). The colored residues were observed to cluster primarily on the inner region of each Toprim domain in the RecR dimer, suggesting that this may be responsible for RecO binding (two circled areas in Fig. 4C, right panel). Interestingly, the proposed RecO binding sites and the acidic cluster regions of the RecR Toprim domain largely overlapped (Fig. 4C and 4D, left panels).

Introduction of Mutation into the RecR Acidic Cluster Region – The acidic cluster region of the RecR Toprim domain consists of three conserved acidic residues, E84, D88 and E144 (Fig. 4D, right panel). In the presence of an equimolar amount of RecO, the E84 and E144 signals were lost though D88 could not be analyzed because of the overlapping crosspeaks (data not shown). The results from these NMR titration experiments suggest that the acidic cluster region may be a binding site for RecO. To investigate the functional significance of these regions, we generated the RecR mutants E84A,
D88A and E144A by substituting each residue with alanine. The elution profiles obtained by size-exclusion chromatography for purified RecR E84A, D88A and E144A matched that obtained for wild-type RecR, suggesting that each mutant protein folds properly and forms a dimer (data not shown).

The RecR E144A and E84A Mutations Affect Complex Formation with RecO – To clarify the functional role of the RecR E84, D88 and E144 residues in RecO binding, the RecR E84A, D88A and E144A mutant proteins and wild-type RecR were incubated with RecO, and the resulting RecOR complex formation was analyzed using native PAGE (Fig. 5A). RecO did not migrate into the gel since RecO has a positive charge (pI = 10.7) in the running buffer, while RecR (pI = 5.5) was observed as a single band (Fig. 5A, lanes 1 and 2). The mobility of E84A, D88A and E144A differed slightly from that of wild-type RecR, perhaps due to the substitution of alanine for an acidic residue. When RecR and RecO were present at a molar ratio of 5:1, the band corresponding to the RecOR complex was observed (Fig. 5A, lane 3). When the molar ratio of RecR and RecO was 1:1, the band of RecR became faint, while the band of the RecOR complex was stained more clearly (Fig. 5A, lane 4). In contrast, when E144 was mutated to alanine, the complex formation with RecO was clearly abrogated even with a molar ratio of 1:1 (Fig. 5A, lanes 11-13). The band for the RecOR complex was faintly visible when E84 was mutated to alanine (Fig. 5A, lanes 5-7) and the D88A mutation had almost no effect on RecOR complex formation (Fig. 5A, lanes 8-10). To confirm the specificity of the RecR-RecO binding observed by native-PAGE, we electrophoresed the mixture of RecR and lysozyme (pI = 11.8) possessing a similar isoelectric point as RecO. As a result, RecR was not affected and migrated to the original position (Fig. 5A, lane 14). These results suggest that E144 and E84 residues of RecR are important for RecO binding.

We next examined the effect of these mutants on RecA activity (Fig. 5B). RecA forms a filament on ssDNA that hydrolyzes ATP in a time-dependent manner (Fig. 5B, grey line A). This ssDNA-dependent ATPase activity is suppressed when SSB is pre-incubated with ssDNA (Fig. 5B, grey line A+SSB). However, the addition of RecO and RecR prior to addition of RecA to the reaction mixture enhances the loading of RecA onto SSB-coated ssDNA and restores its ATPase activity (Fig. 5B, grey line A+SSB+OR[WT]). Since RecO or RecR alone could not restore the ATPase activities (Fig. 5B, grey line A+SSB+O and A+SSB+OR), the complex formation of RecR and RecO seems to be important. As expected from the results shown in Figure 5A, the substitution of the E144A mutant for wild-type RecR in the reaction mixture clearly inhibited RecA-mediated ATPase activity, causing levels of hydrolyzed ATP to drop from 70 to 20 nmol after a 30 min reaction (Fig. 5B, line A+SSB+OR[WT] and A+SSB+OR[E144A], respectively). A lesser reduction in ATPase activity, from 70 to 58 nmol of hydrolyzed ATP, was observed for the E84A mutant. D88A did not have a suppressive effect. Since RecFOR proteins function at the nucleation step of the RecA protein, it would be important to examine the effect of RecR mutants on the initial rate of RecA-mediated ATPase activity. Therefore, we calculated the rate of ATP hydrolysis at 5 minutes and described it as a histogram (Figure 5C). In accordance with the results of the complex formation by native PAGE, the mutants that could form RecOR complex tended to restore the ATPase activity of RecA. These results suggest that the E144 residue is significantly important for and that E84 also participates in RecO binding.

The RecR E144A Mutation Suppresses the RecFR Complex Formation – We also used native PAGE to analyze the RecFR complex formation of the RecR acidic cluster mutants E84A, D88A and E144A (Fig. 6A). RecF did not migrate into the gel since RecF has a positive charge (pI = 9.09) in the running buffer (Fig. 6A, lane 1). As shown in lanes 3 and 4 of Figure 6A, wild-type RecR formed a complex with RecF (Fig. 6A, lane 3, 4). The mutation of the E144 residue to alanine inhibited complex formation (Fig. 6A, lanes 11-13). In contrast, the E84A and D88A mutations did not affect RecFR complex formation (Fig. 6A, lanes 5-7 and 8-10, respectively).

We further analyzed the effect of these mutations on dsDNA binding using an agarose gel retardation assay (Fig. 6B). dsDNA binding of RecR was not observed in this assay perhaps because of its week affinity (Fig. 6B, lane 2) although we revealed interactions between RecR
RecF was capable of binding dsDNA, but most of the RecF formed aggregates that were trapped in the well of the agarose gel (Fig. 6B, lane 3). When RecF, RecR and dsDNA were all present, RecR formed a complex with RecF and bound dsDNA (Fig. 6B, lane 4). When the E144A mutant was substituted for wild-type RecR in the reaction mixture, the band corresponding to the RecFR-dsDNA complex could not be observed and the RecF formed aggregates, just as it had when it had alone been present (Fig. 6B, compare lanes 3 and 7). Both E84A and D88A mutants allowed RecFR-dsDNA complex formation (Fig. 6B, lanes 5 and 6, respectively), although RecF showed a slight tendency to aggregate in the presence of the E84A mutant (Fig. 6B, lane 5). These results suggest that the E144 residue of RecR plays a significant role in RecFR complex formation and in dsDNA binding by the complex and that the E84 residue of RecR also helps contribute to dsDNA binding. Interestingly, the tendency of the RecR mutants for RecF binding paralleled those for RecO binding.

RecR and RecF Form a 4:2 Heterohexamer – We next used size-exclusion chromatography to further investigate the complex formation of RecR, RecF and RecO. The RecR dimer, RecF and RecO eluted as a single peak, in general agreement with their calculated molecular masses of 42, 38 and 25 kDa, respectively (Fig. 7A, 7B, 7D and 7F). The trailing shoulder peaks of RecO may be due to an interaction between RecO and the column resin. When a mixture of RecF and RecR at a molar ratio of 1:2 was subjected to chromatography, a single peak corresponding to a molecular mass of 157 kDa was preferentially observed (Fig. 7C and 7F). The trailing shoulder peaks of RecO may be due to an interaction between RecO and the column resin. When a mixture of RecF and RecR at a molar ratio of 1:2 was subjected to chromatography, a single peak corresponding to a molecular mass of 157 kDa was preferentially observed (Fig. 7C and 7F). When analyzed by sedimentation equilibrium analysis, this fraction was found to contain a protein complex of 158 kDa (Supplemental Fig. 3) and a molecular mass approximately the same as that described above.

When a mixture of RecF, RecR and RecO at a molar ratio of 1:2:1 was subjected to the chromatography, two peaks of 158 kDa and 25 kDa were observed which molecular masses identical to those of the RecFR complex and RecO, respectively (Fig. 7C, 7D and 7E). Next, fractions 1-13 (elution volume 8-16 ml) were analyzed using SDS-PAGE (Fig. 7G). The initial peak contained RecF and RecR (Fig. 7G, lanes 5 to 7) but not RecO, with RecO eluting at its original position (Fig. 7G fractions 11 to 13). The molecular ratio of RecR to RecF in the RecFR complex was estimated using densitometric analysis, in which we compared the densities of two of the bands in SDS-PAGE (Fig. 7G, lanes 5 to 7) with those of known amounts of RecR and RecF. The molar ratio of RecR to RecF was found to be 2:1. On the basis of these findings, we propose that RecR and RecF form a 4:2 heterohexamer (160 kDa) that is not dissociated by the addition of RecO to the solution. When subjected together to size-exclusion chromatography, RecR and RecO were eluted separately at their original position. This result suggests that RecO and RecR are unable to form or maintain the complex through the elution process (data not shown).

**DISCUSSION**

The Toprim domain of RecR was found to be important for binding RecO and RecF. Previously, the acidic cluster regions of the Toprim domain in topoisomerase or primase were reported to be the active center for nucleotidyl transferase activity, which needs the interaction of a conserved glutamate and two aspartate residues with Mg$^{2+}$ (32-34). However, the two aspartate residues are not conserved in the Toprim domain of RecR (35). Recently, structural analysis of drRecR showed that the RecR Toprim domain commonly has a RecR-specific acidic cluster region that consists of three acidic residues (E84, D88 and E144 in case of *T. thermophilus* RecR) (22). In this study, we used NMR titration analysis to reveal that the Toprim domain of RecR is not involved in DNA binding through Mg$^{2+}$. Instead, we revealed that the region was involved for binding RecO and RecF. When the E144 residue of RecR was mutated to the uncharged residue alanine, the ability of RecR to bind RecO and RecF was severely diminished, showing that E144 plays a significant role in the formation of both the RecOR and RecFR complexes. We also demonstrated that E144 is important for RecOR or RecFR complex functions, as described in Figures 5 and 6. Since E144 is the most externally positioned of the three acidic residues and located at the greatest distance from the HhH motif of the
dsDNA binding site (Fig. 4D, right panel), its location may be favorable for interaction with RecF and RecO.

RecF and RecO may compete for their overlapping RecR binding sites. Indeed, we found that the RecOR-enhanced binding of RecA to SSB-coated linear ssDNA (as shown in Fig. 5B) was reduced by the addition of RecF in a concentration-dependent manner (1 to 10 µM RecF, data not shown). Our results are partly supported by the work of Bork et al., which showed that the E. coli RecF protein competes with RecO for RecR protein association with the RecA filament by analyzing its ATPase activity in the presence of SSB-coated ssDNA (16).

The manner in which RecR proteins bind DNA has not been well described, although the drRecR tetramer has been reported to bind both dsDNA and ssDNA (22). The dsDNA binding of the RecR dimer could not be observed by a gel retardation analysis (Fig. 6B, lane 2) as shown previously in similar assay using E. coli RecR dimer (20). However, NMR titration analysis showed that the hairpin region of the HhH motif in the RecR dimer is responsible for dsDNA binding. The dissociation constant for the binding of RecR to dsDNA was considered to be in the mM range, because the chemical shift changes of both the G19 and G21 residues of RecR (0.4 mM) were not saturated by the addition of 1.6 mM dsDNA (data not shown). Although the HhH motif is a positively charged region and a nonspecific interaction between the motif and a negatively charged dsDNA might occur, we believe the binding to be specific for the following reasons: 1) We showed that two neutral residues of G19 and G21 are most affected; 2) Our results accord well with findings that two glycine residues in the hairpin region of the HhH motif were critical for interaction with dsDNA (36,37). Moreover, Glycine-Isoleucine-Glycine in the hairpin region has been suggested to bind to dsDNA through hydrophobic interactions with the bases in the grooves (38); 3) The hairpin region of the HhH motif is highly conserved among known RecRs, including the RecR, drRecR and E. coli RecR (Supplemental Fig.1); 4) The mutational work of drRecR also shows that the HhH motif is critical for DNA binding (22). On the basis of these findings, we concluded that the hairpin region of the HhH motif is an important region for dsDNA binding of RecR. It should be noted, however, that dsDNA binding of RecR could not be observed at low protein concentrations. The dsDNA binding by RecR alone may not be significant in vivo. Since RecR is able to bind dsDNA tightly by forming a complex with RecF as described in this study (Fig. 6B, lane 4) and in a previous report in E. coli (14,20), the HhH motif of RecR may be important when it exists as the RecFR complex.

RecR forms a stable dimer in solution, which is supported by the dimerization property of E. coli RecR (15,20). In contrast, ultracentrifugation analysis has revealed that both drRecR and Helicobacter pylori RecR form a tetramer in solution (22). Moreover, crystallized drRecR forms a ring-like structure involving both the N- and C- terminal interfaces. The structural properties of the C-terminal regions (see Fig.1), i.e., their capacity for interacting with each other to form tetramers, may determine why some RecR proteins form dimers and others tetramers. Given that the drRecR tetramer binds dsDNA more tightly than does the RecR dimer, our finding that RecR and RecF spontaneously form a 4:2 heterohexamer complex which stably binds dsDNA suggests that, like drRecR, the RecR dimer functions as a ring-like tetramer in the presence of RecF. Although the conformation of the four RecR monomers in the RecFR heterohexamer is not known at present, RecR proteins may have the ability to form a ring-like structure that enables them to bind dsDNA tightly. RecR preferentially formed a RecFR complex in the presence of RecF, RecO and RecR (Fig. 7). The RecF or RecFR complex has been reported to bind dsDNA-ssDNA junctions (4,39). Although it has been suggested that RecO (or the RecOR complex) binds the RecFR complex (or RecF) via RecR at the dsDNA-ssDNA junction site (4), the process of RecFOR assembly at the dsDNA-ssDNA junction is not yet understood in detail. In this study, RecR and RecF formed a 4:2 heterohexamer in solution that was unaffected by adding RecO, indicating a preference by RecR for RecF over RecO. RecO is thought to be localized at ssDNA regions that are coated with SSB, because E. coli RecO prefers ssDNA to dsDNA (40) and also has a high affinity for SSB (15). These properties were conserved in the T. thermophilus RecO (J. Inoue et al., unpublished...
results). Therefore, the RecO localized at ssDNA region may interact with the Toprim domain of RecR that forms a complex with RecF at dsDNA-ssDNA junctions to ultimately form the RecFOR complex (or the RecOR complex) at the site, as described in Figure 8.

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**FOOTNOTES**

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1. The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SSB, single-stranded DNA-binding protein; HhH, helix-hairpin-helix; HSQC, heteronuclear single quantum coherence; NOE, Nuclear Overhauser Effect; NOESY, Nuclear Overhauser Effect Spectroscopy; and PAGE, polyacrylamide gel electrophoresis.

**FIGURE LEGENDS**

Fig. 1. Structural analysis of ttRecR dimer. (A) Sedimentation equilibrium analysis of ttRecR at 2 mg/ml protein concentration. (B) The three-dimensional structure of the *D. radiodurans* RecR (drRecR) tetramer (Protein Data Bank, structure 1VDD). Individual subunits are colored in blue, green, red and yellow. (C) Kinetics of $^1$H-$^{15}$N HSQC spectra of $^2$H/$^{15}$N-labelled ttRecR after exposure to D$_2$O. All spectra were processed using identical parameters. (D) Mapping of detectable amide protons onto a drRecR monomer after 4 h exposure to D$_2$O. Red spheres represent amide protons that were protected from deuterium exchange. The left panel, on which seven residues are labeled, shows amide protons located on the surface of the drRecR monomer. The right panel depicts the N-terminal interface of drRecR monomers. (E) The digomeric states of ttRecR, ttRecR$_{1-173}$ and ttRecR$_{75-194}$ proteins were determined by size-exclusion chromatography. The molecular mass was calibrated with the following size markers: albumin (67 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa) and RNaseA (13.7 kDa), as indicated. (F) 3D NOESY NMR analysis of the ttRecR C-terminal region. The anti-parallel β-sheet structure between ttRecR residues 163-165 and 192-194 is displayed as a topology diagram. Interstrand backbone NOEs are depicted as double-headed red arrows. (G) Ribbon model structure of the ttRecR dimer. Domains are colored as follows: HhH motif region (red); Zinc-finger region (blue); Toprim domain region (green); and Walker-B motif (orange).

Fig. 2. Chemical shift mapping of dsDNA binding sites onto the RecR dimer. (A) Overlay of the $^1$H-$^{15}$N HSQC spectra for RecR before (black) and after (green) addition of 15 bp dsDNA (top) or 15 mer ssDNA (bottom). (B) Mapping of chemical shift changes ($\Delta_\nu$) induced by dsDNA binding onto the molecular surface model of RecR dimer. Residues were color-coded as follows: red, $\Delta_\nu$ >20Hz; and yellow, 20Hz
$\Delta n > 5\text{Hz}$. The DNA binding patch consisted of two G19 and two G21 residues of the RecR dimer (labeled red). Residues that could not be analyzed are in blue. (C) The ribbon diagram of RecR is color-coded as in Figure 1B. The circled area represents a schematic diagram of the dsDNA that binds the RecR dimer.

**Fig. 3.** Analysis of RecR interaction with DNA in the presence or absence of Mg$^{2+}$. Chemical shift changes for RecR after addition of various DNA substrates were plotted against residue number. A schematic of the RecR domain structure aligned with the residue number is also shown. DNA substrates are as follows: dsDNA, d(CCGGTGATAGACTTG)/(CAAGTCTATCACCGG); ssDNA, d(CAAGTCTATCACCGG); 3’-tailed DNA, d(CCGGTGATAGACTTGAGCTAAC)/(CAAGTCTATCACCGG); and 5’-tailed DNA, d(CCGGTGATAGACTTG)/(GTTAGCTCAAGTCTATCACCGG). DNA was added from a concentrated stock to $^{15}$N-labelled RecR in the absence (A) or presence (B) of 40 mM MgCl$_2$. Data for overlapping crosspeaks and proline residues were excluded from the plots.

**Fig. 4.** NMR analysis of the RecO binding site on the RecR dimer. (A) $^1$H-$^{15}$N HSQC spectra of 0.2 mM $^{15}$N-RecR in the absence or presence of 0.2 mM RecO. (B) The relative intensities of RecR amide protons in the presence or absence of RecO. A schematic of the RecR domain structure aligned with the residue number is also shown. The crosspeaks with relative intensities below 10% are indicated in red. (C) Residues exhibiting significant differences in relative intensity ($\Delta_{\text{int}}$) are color-coded and mapped onto the structure of the RecR dimer ($\Delta_{\text{int}} < 10\%$, red; 10% < $\Delta_{\text{int}}$ < 20%, yellow). Residues that could not be analyzed are in blue. The ribbon model at right was generated by a 30° rotation around the vertical axis followed by a 90° rotation around the horizontal axis. The circled areas show the proposed RecO binding sites. (D) Electrostatic potential at the molecular surface of the RecR dimer (left panel). The potential was calculated using the GRASP program. Positive and negative potentials are indicated in blue and red, respectively. The conserved acidic cluster region of the RecR Toprim domain is circled. The right panel shows a ribbon diagram of the RecR dimer. E84, D88 and E144, three conserved acidic residues comprising the acidic cluster region, are labeled and depicted by space-filling model with red.

**Fig. 5.** Effects of the RecR E84A, D88A and E144A mutations on RecOR complex formation and function. (A) Analysis of the interaction between RecR mutants and RecO by native PAGE. Wild-type RecR, E84A, D88A or E144A mutants (10 $\mu$M) were incubated with 0, 2 or 10 $\mu$M RecO, respectively, and the complexes were resolved by electrophoresis. Wild-type RecR (10 $\mu$M) was also incubated with 10 $\mu$M lysozyme as a control. (B) Effects of mutant RecOR complexes on RecA filament formation on SSB-coated ssDNA. An ssDNA-dependent ATPase assay was used to monitor RecA filament formation. SSB protein (1 $\mu$M), RecO (1 $\mu$M) and RecR (1 $\mu$M) were pre-incubated with 340-mer poly(dC), before ATP (3 mM) and RecA (1 $\mu$M) were added to the reaction mixture. Control reactions are indicated in gray. Reactions with RecR mutants are indicated in black. (C) Rate of ATP hydrolysis of RecA at 5 minutes. The rate was calculated from the absorption change between 3 and 5 minute.

**Fig. 6.** Effects of the RecR E84A, D88A and E144A mutations on RecFR complex formation and function. (A) Analysis of the interaction between RecR mutants and RecF by native PAGE. RecR or mutant proteins (10 $\mu$M) were incubated with 0, 2 or 10 $\mu$M RecF, respectively, and the complexes were resolved by electrophoresis. Wild-type RecR (10 $\mu$M) was also incubated with 10 $\mu$M lysozyme as a control. (B) The agarose gel retardation assay used to detect the binding of dsDNA by RecFR complexes. RecF (10 $\mu$M), RecR (5 $\mu$M) or the mixture of RecF (10 $\mu$M) and RecR (5 $\mu$M) were incubated for 10 min in the presence of a 70 bp dsDNA (20 $\mu$M) and ATP (1mM), and each mixture was then subjected to electrophoresis as described in Experimental Procedures. The nucleotide sequence of the dsDNA substrates is GCCGTTCTTATTTAAAACCGCAACTCCCTCCG-GCGAAAGTCCTCCTCCAGTCTCAATGTCACCCCC/GGGGTGGACATTTGACGAAAGGCTTGG AAGACTTTCGCCGGAGGGAGTGTGCCGTTTTAATAAGGATCGC.
Fig. 7. Analysis of RecFOR complex formation by size-exclusion chromatography. Elution profiles of (A) RecR, (B) RecF, (C) RecF and RecR mixture, (D) RecO, and (E) RecF, RecR and RecO mixture by size-exclusion column chromatography. The elution volume is shown on the x axis, and eluted proteins are detected by absorption at 280 nm. The elution points of molecular mass standards are labeled a-e in Figure 7A. Molecular mass standards were (a) ferritin, 400 kDa; (b) catalase, 230 kDa; (c) aldolase, 130 kDa; (d) ovalbumin, 45 kDa; and (e) chymotrypsin, 25 kDa. (F) The molecular mass calibration to the retention volume, using the size-markers described above. The closed boxes indicate the RecFR complex, RecF, RecO and RecR. (G) SDS-PAGE of fractions depicted in Figure 7E.

Fig. 8. Model for RecFOR assembly at dsDNA-ssDNA junctions. (A and B) When dsDNA-ssDNA junctions are generated as a consequence of DNA damage, the RecFR heterohexamer complex binds to the 5’-base paired dsDNA-ssDNA junction and RecO interacts with SSB-coated ssDNA region. (C) RecO binds to a Toprim domain of RecR in the RecFR complex at the dsDNA-ssDNA junction. RecO may form a complex with RecR by competing with RecF for the RecR Toprim domain. (D) The RecOR complex, which may associate with RecF, facilitates the loading of RecA onto SSB-coated ssDNA. DNA damage is subsequently repaired by the homologous recombination mediated by RecA.
Figure 1
**Figure 2**

A. (ppm) 1H (ppm)

B. (ppm) 15N (ppm)

C. HhH motif

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http://www.jbc.org/Downloaded from
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A

RecFR complex $\rightarrow$ RecO

FR $\rightarrow$ SSB $\rightarrow$ SSB

B

RecFR complex $\rightarrow$ RecO

FR $\rightarrow$ SSB $\rightarrow$ SSB

C

RecO binds Toprim domain of RecR

FR $\rightarrow$ SSB $\rightarrow$ SSB

D

Rec(F)OR complex

Rec(F)OR facilitates RecA binding to SSB-coated ssDNA

RecA $\rightarrow$ ATP $\rightarrow$ ADP

Figure 8
Identification of the RecR toprim domain as the binding site for both RecF and RecO:  
A role of RecR in RecFOR assembly at dsDNA-ssDNA junctions  
Masayoshi Honda, Jin Inoue, Masatoshi Yoshimasu, Yutaka Ito, Takehiko Shibata and  
Tsutomu Mikawa  

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