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Mechanism of RecO recruitment to DNA by single-stranded DNA binding protein

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ABSTRACT

RecO is a recombination mediator protein (RMP) important for homologous recombination, replication repair and DNA annealing in bacteria. In all pathways, the single-stranded (ss) DNA binding protein, SSB, plays an inhibitory role by protecting ssDNA from annealing and recombinase binding. Conversely, SSB may stimulate each reaction through direct interaction with RecO. We present a crystal structure of *Escherichia coli* RecO bound to the conserved SSB C-terminus (SSB-Ct). SSB-Ct binds the hydrophobic pocket of RecO in a conformation similar to that observed in the ExoI/SSB-Ct complex. Hydrophobic interactions facilitate binding of SSB-Ct to RecO and RecO/RecR complex in both low and moderate ionic strength solutions. In contrast, RecO interaction with DNA is inhibited by an elevated salt concentration. The SSB mutant lacking SSB-Ct also inhibits RecO-mediated DNA annealing activity in a salt-dependent manner. Neither RecO nor RecOR dissociates SSB from ssDNA. Therefore, in *E. coli*, SSB recruits RMPs to ssDNA through SSB-Ct, and RMPs are likely to alter the conformation of SSB-bound ssDNA without SSB dissociation to initiate annealing or recombination. Intriguingly, *Deinococcus radiodurans* RecO does not bind SSB-Ct and weakly interacts with the peptide in the presence of RecR, suggesting the diverse mechanisms of DNA repair pathways mediated by RecO in different organisms.

INTRODUCTION

Homologous recombination (HR) is the most efficient pathway for chromosomal damage repair in all organisms (1–4). Deficient HR renders cells unable to repair chromosome breaks and to reinitiate stalled replication (2), while hyperactivity leads to illegitimate recombination and chromosomal abnormalities (5,6). Ubiquitous recombination mediator proteins (RMPs) together with single-stranded (ss) DNA binding proteins regulate recombinational DNA repair. HR is initiated by binding of the RecA-like recombinase to ssDNA, forming the extended nucleoprotein filament called the presynaptic complex. The ssDNA-binding proteins inhibit this step, while the RMPs overcome their inhibitory effect. In addition to HR, RMPs are also important for translesion synthesis (TLS) (7,8) and DNA annealing (9–11). RMPs include phage UvsY (12), prokaryotic RecBCD and RecFOR proteins (7,13–15), and numerous eukaryotic members (16). Mutations of human RMPs are associated with cancer predisposition, mental retardation, UV sensitivity and premature aging (17–20). The mechanism of RMP's interaction with DNA, recombinases and ssDNA binding proteins is poorly understood.

RecF, -O and -R proteins are the most common RMPs in bacteria. They are involved in the restart of stalled replication, in ssDNA gaps (SSGs) repair, in TLS and in double-strand (ds) DNA breaks (DSB) repair (21–24). RecFOR are the only RMPs found in the radiation resistant bacteria, *Deinococcus radiodurans* (25,26). In *Escherichia coli*, both RecO and RecR are essential for the RecA loading on SSB-protected ssDNA (27), while RecF is likely to play a regulatory role (28,29) or is involved in an alternative pathway of HR initiation (30). RecO interacts with SSB and DNA, while RecR binds either RecO or RecF (27,31–33). RecO anneals complimentary ssDNA strands protected by cognate SSB (34,35), resembling the properties of the eukaryotic RMPs, Rad52 and BRCA2 (9,10). The crystal structures of all three proteins from *D. radiodurans* have been previously reported, as well as a structure of the RecOR complex (36–40). The crystal structure of RecR revealed a DNA clamp-like tetramer architecture (36); however, the role of a potential DNA clamp in RMPs-mediated reaction is unknown. The crystal structure of RecO did not resemble any structural features of the functional eukaryotic analog, Rad52 (38,40,41). RecO is a basic protein with an extensive, positively charged surface area. It is

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comprised of three domains: the N-terminal OB-fold domain, which is characteristic for DNA binding proteins, the central α-helical domain and the zinc binding domain. RecO binds both ss- and dsDNA substrates, likely by the OB-fold domain and, potentially, positively charged surface areas of other domains. The RecR binding interface is also localized on the OB-fold domain of RecO (39). Interestingly, in the BRCA2 structure there are three OB-fold domains, which bind either ssDNA or other proteins (42).

Overall, the mechanism of RecFOR interactions with recombinase, DNA and SSB during presynaptic complex formation remains unknown. In addition to binding RecR and ss- and dsDNA, RecO also interacts with SSB (27). The SSB lacking eight C-terminal residues (SSBΔC8) inhibits RecOR-mediated HR initiation in *E. coli*, suggesting that RecO binds the C-terminus of SSB (SSB-Ct) (43). In addition to protecting ssDNA, the SSB specifically interacts with multiple proteins of various DNA metabolism events through the conserved SSB-Ct (44,45). Therefore, SSB plays two seemingly opposing roles: preventing recombinase and RMPs from interaction with ssDNA and potentially recruiting RMPs to ssDNA. It is unknown whether the interaction of RecOR(F) with SSB should either completely or partially remove SSB from ssDNA (46) or alter the conformation of SSB-bound ssDNA, making it available for RecA nucleation and annealing. The structure of RecO from *D. radiodurans* did not reveal any potential SSB-Ct binding sites resembling those previously reported in other SSB-Ct binding proteins. To understand the mechanism of RMPs' interactions with SSB and DNA, which is likely to describe the common features of SSB interactions with multiple DNA metabolism proteins, we solved a high resolution crystal structure of the *E. coli* RecO bound to SSB-Ct, and demonstrated that SSB recruits RecO to ssDNA in both annealing and recombination initiation reactions. Intriguingly, *D. radiodurans* RecO does not bind SSB-Ct, suggesting that the role of RecO interaction with SSB may differ in the two organisms.

MATERIALS AND METHODS

DNA, peptides and proteins

For simplicity, *E. coli* homologs will be referred to by protein names without prefixes in the rest of the paper. DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Peptides were initially purchased from GenScript USA, Inc. (Piscataway, NJ, USA) and later from LifeTein LLC (South Plainfield, NJ, USA). SSB-Ct peptide had an additional N-terminal Trp residue for quantification (WYMDFDDDIPF-177). SSB-Ct-113 had a Pro176Ser mutation, SSB-Ct-3ΔD had Asp172Ala, Asp173Ala, Asp174Ala mutations and SSB-Ct-ΔF lacks the C-terminal Phe177. RecO protein was cloned and purified as previously described for DrRecO with an additional purification step on a heparin column (38). SSB protein was purified from a plasmid provided by Dr M. Cox (University of Wisconsin) according to a previously described protocol (43). All RecO mutants were characterized by the comparable wild-type protein solubility and affinity toward the heparin resin. A few RecO mutants were tested for DNA annealing. The *E. coli* SSB mutant lacking the last eight amino acids (SSBΔC8) was a gift from Dr M. Cox.

Crystallization and structure determination

RecO protein was concentrated in solution with 0.2 M NaCl, 10 mM Tris–HCl, pH 7.5, 0.5 mM TCEP, 5% DMSO, 0.2 mM CHAPS and with 1.5 molar excess of SSB-Ct, up to a concentration of 4 g/l. Crystals were grown by the vapor diffusion method with sitting drops using 96-well Corning plates. Initial crystals diffracting to 3.5 Å resolution were obtained with buffer containing 20–25% PEG 4K and Bis–Tris Propane, pH 5.5. Initial attempts to solve the structure with the molecular replacement method were not successful, partially due to crystal twinning. Later, crystals were also obtained with 1.9–2.2 M DL-Malic acid, pH 7.0, using both native protein and selenomethionine derivative. Final data sets were collected on a GM/CA-CAT beam line at APS, ANL. Initial phases were obtained using 2.8 Å resolution single wavelength data set (SAD) collected from Se-Meth derivative crystal and 2.3 Å native data set using Phenix software (47) (Supplementary Table S1). The initial map obtained from the SAD experiment followed by the phases extension to 2.3 Å using the native data set was of excellent quality, sufficient for automated building of ~80% of the structure with the Arp/Warp program (48). The model building and refinement were completed using the Coot (49) and Refmac (50) programs. The CNS program was implemented to calculate composite omit maps during model building (51). There were two RecO molecules per asymmetric unit. Analysis of electron density maps revealed the presence of SSB-Ct bound to both molecules and a CHAPS molecule (detergent used in crystallization) bound to one subunit only. All substrates were built using experimental electron density maps. Non-crystallography averaging was not utilized during initial model building and refinement steps.

Peptide and DNA binding assays

Proteins were dialyzed from storage buffer against assay buffers with the addition of 50 mM Arg-HCl and 50 mM NaGlu. Concentrations were determined by absorbance at 280 nm and extinction coefficients of 24 595 $M^{-1} cm^{-1}$ (RecO), 22 600 $M^{-1} cm^{-1}$ (DrRecO), 6335 $M^{-1} cm^{-1}$ (RecR), 9000 $M^{-1} cm^{-1}$ (DrRecR) and 27 960 $M^{-1} cm^{-1}$ (SSB). Fluorescein-labeled (FAM) peptides were solubilized in DMF (dimethylformamide), diluted in assay buffer and concentrations determined by absorbance at 280 nm and extinction coefficient of 5,500 $M^{-1} cm^{-1}$. Fluorescein-labeled DNA was solubilized in Mili-Q H_2O. The reaction volume of all binding assays was 100 µl. All reactions were run in triplicate and independently repeated two or more times. Two different reaction buffers were utilized for various assays: buffer A with 25% glycerol, 50 mM NaCl, 20 mM Bis–Tris Propane, pH 8.0, 1 mM TCEP and similar buffer B with

200 mM NaCl. Fluorescent assays were read at 485/528 nm using a BioTek Synergy 2 plate reader. Fluorescent polarization anisotropy values were normalized using the following equation: $A = (A_i - A_0)/A_0$, where A_0 and A_i are fluorescence anisotropy values for free substrate and for each titration point correspondingly. Binding constants were determined with BioKin DynaFit using single-site binding approximation for all assays. Isotherms were plotted with the Systat SigmaPlot program.

Competition assay

RecO at 1 μM was pre-incubated with 0.9 μM of F-SSB-Ct in buffer B for 30 min at room temperature and was titrated by increasing concentrations of peptides, SSB and SSB bound to dT_{35} and dT_{70}.

DNA pull down

Biotin-labeled dT_{45} was immobilized on streptavidin beads and 50 μl of beads were incubated with 100 μl of a 0.5 μM solution of SSB or SSBΔC8 for 30 min, washed with excess buffer a few times and then incubated with RecO and RecOR proteins for 30–60 min (200 μl of total volume with 0.2 μM of RecO and 2 μM of RecR, when present, with 0.02 μM SSB and 0.1 mg/ml BSA). The beads were washed 2 times with 500 μl of the corresponding buffer and samples of all solutions and beads were analyzed on SDS–PAGE. The pull-down experiment was performed multiple times while varying the presence of BSA, detergent and small amount (5%) of SSB together with RecO and RecOR to compensate for potential dissociation. Neither of these conditions was found to alter final results.

DNA annealing

Oligonucleotide 1 with the sequence TCCTTTTGATAA GAGGTCATTTTTGCGGATGGCTTAGAGCTTAAT TGC was labeled by fluorescein (FAM) at the 5′-end, and the complimentary oligonucleotide 2 was labeled with Dabsyl at the 3′-end. Each oligonucleotide at the concentration of 25 nM was incubated separately with 250 nM of either SSB or SSBΔC8 in different buffers for 20 min and then solutions with complimentary DNA were mixed together. RecO equilibrated in corresponding buffer was added to the mixture with the final concentration of 250 nM. The concentrations of NaCl were 10, 20, 60 and 100 mM. Annealing of DNA was monitored by a decrease (quenching) of FAM emission as a function of time. The intensities were read on the BioTek plate reader (preheated to 27°C). Fluorescence intensity values were inverted and normalized using the following equation: $I = -1*(I_i - I_0)\ /I_0$, where I_0 and I_i are fluorescence intensity values at initial time point and the next time point, respectively.

RESULTS

Structure of RecO complex with SSB-Ct

To understand the mechanism of RecO interaction with SSB, SSB-Ct binding to RecO in solution was measured (described below) and the crystal structure of RecO bound to SSB-Ct (residues 169–177) was solved at 2.3 Å resolution. There were two RecO molecules per asymmetric unit. The overall architecture of RecO closely resembles that of the *D. radiodurans* homolog (DrRecO) (38,40), comprised of an OB-fold N-terminal domain, a central α-helical domain and a third domain with zinc binding site in DrRecO (Figure 1A–C). Two structures were aligned with a root mean square deviation of 3.1 Å for 215 Cα atoms. No zinc ion was found in the *E. coli* homolog. There is only one cysteine in the sequence of RecO versus four in DrRecO (Supplementary Figure S1), although the topology of these domains is very similar. Other differences include substitution of an α-helix E in DrRecO by a short loop and the presence of an additional C-terminal α-helix in RecO in place of the partially unstructured C-terminal loop in DrRecO. The later feature is important for differences in interaction of these homologs with SSB-Ct (see below).

Analysis of the electron density maps revealed additional densities corresponding to SSB-Ct in both molecules of the asymmetric unit and a CHAPS molecule in one monomer (Figure 1; Supplementary Figures S2 and S3). The two most C-terminal residues of SSB-Ct, Pro-176 and Phe-177, were fitted into the electron density located inside the hydrophobic pocket of the α-helical domain (Figure 1D and E; Supplementary Figures S2 and S3). The Ile-175 was poorly ordered and the rest of the tail was disordered. Peptides bound to each RecO monomer of the asymmetric unit were built using an electron density map independently without averaging, yet had very similar conformations. The SSB-Ct binding hydrophobic pocket is surrounded by a positively charged surface area resembling the architecture of the SSB-Ct binding site in the ExoI protein (52). The interior of the cavity is formed by hydrophobic side chains of Ile-84, Phe-228 and Tyr-91, and by aliphatic groups of Arg-229, Arg-225, Arg-210 and Lys-206. Amino groups of the latter residues, together with Arg-203 and Arg-229, form the positively charged edge of the cavity (Supplementary Figure S4). Interestingly, part of the cavity inner surface is formed by the polar groups of Glu-128, Arg-132 and Glu-135. Arg-132 forms ionic interactions with the C-terminal oxygens of Phe-177 of SSB-Ct.

The binding of SSB-Ct to RecO and its mutants was assayed using a fluorescence polarization technique with fluorescein-labeled SSB-Ct (F-SSB-Ct) (Figure 2A, Table 1). RecO binds F-SSB-Ct with an apparent $K_d = 0.06 \pm 0.01$ μM in buffer A with 50 mM NaCl. The strongest loss of SSB-Ct binding was observed in the following mutants: F228D, in which the polar group was introduced in the middle of the hydrophobic surface of the cavity; R132A, which lacks the guanidinium groups of Arg 132 anchoring the SSB-Ct carboxyl end; and R210E, with the reversed polarity of the basic side chain. Removal the Arg-210 side chain in the R210A mutant also had a strong inhibitory effect comparable to that of reversing the polarity of two arginines at the other end of the binding cavity (R203E and R229E). All mutants were characterized by solubility and affinity to heparin resin similar to that of a wild-type protein.

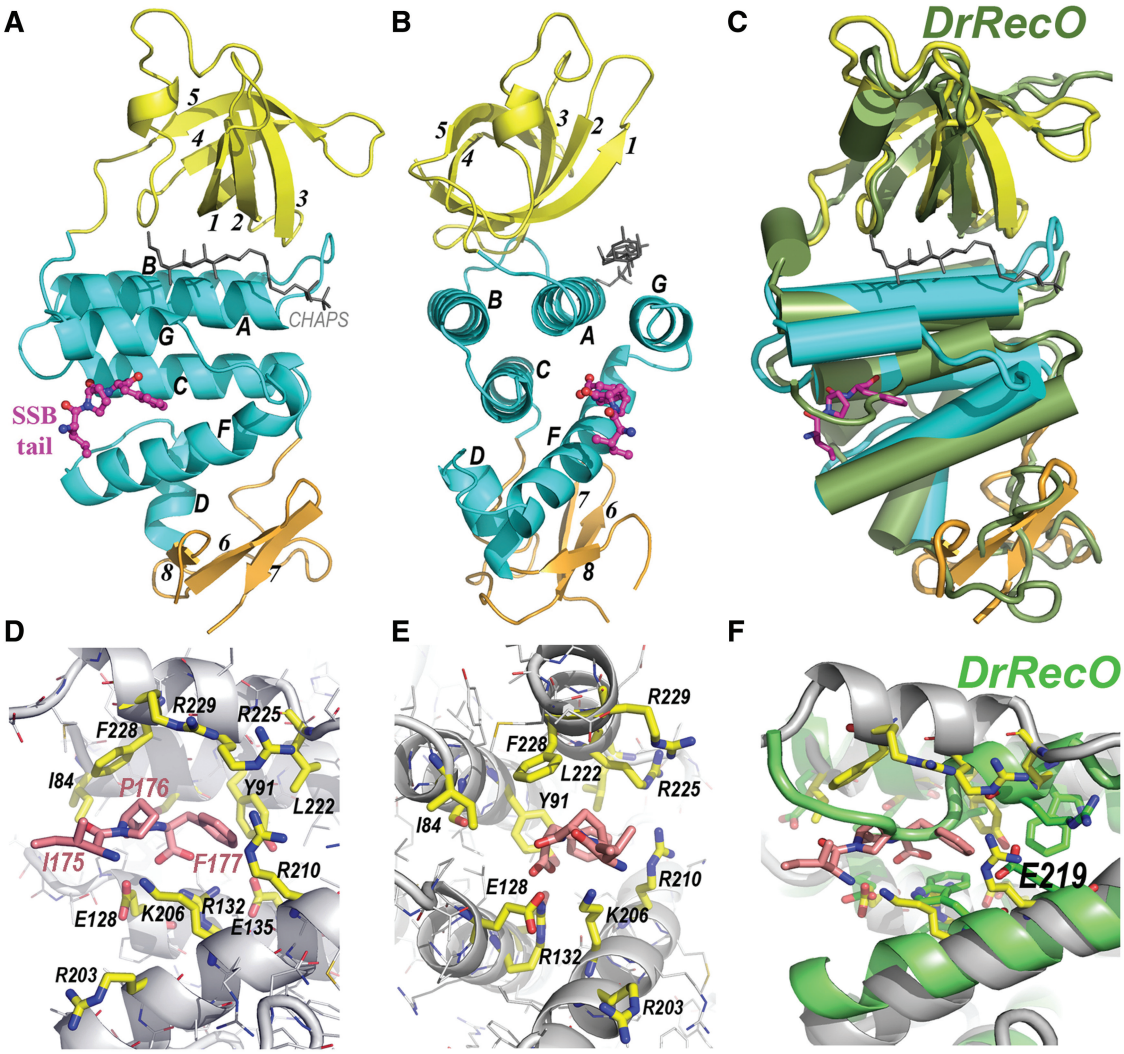

Figure 1. Crystal structure of RecO complex with SSB-Ct. (**A**) Ribbon representation of the RecO structure with the N-terminal domain shown in yellow, α-helical in cyan and the analog of DrRecO zinc binding domain in orange. β-Strands are numbered and α-helixes are lettered similarly to the nomenclature used for DrRecO structure. The analog of DrRecO α-helix E is missing. SSB-Ct is shown in stick representation with carbons, nitrogen and oxygen atoms colored in magenta, blue and red, respectively. The CHAPS molecule is shown by a thin gray sticks representation. (**B**) The RecO complex with SSB-Ct shown in the orientation rotated by 90° around a vertical axis. (**C**) The superposition of RecO and DrRecO structures shown in ribbon representation. DrRecO is shown in green and RecO is similar to that in A. (**D**) The partially hydrophobic SSB-Ct binding site in orientation similar to that in panel A. RecO ribbon representation is shown in gray. Side chains forming the peptide binding site are shown in a thick stick representation with the carbon atoms in yellow and the SSB-Ct in pink. (**E**) The SSB-Ct binding site is shown in an orientation similar to that in B. (**F**) Superposition of DrRecO structure with the SSB-Ct binding site of RecO shown by same color scheme as in C with the superimposed DrRecO structure shown in green. The orientation is similar to that in panel A.

Selected mutants were tested for DNA annealing properties as well (see below).

The role of the conserved residues of SSB-Ct and the binding of RecO to the full-length SSB was assayed in a competition experiment with F-SSB-Ct pre-bound to RecO (Figure 2B). The SSB-Ct variants without Phe-177(ΔF), with Pro-176 substituted by serine (113) and with the completely stripped negative charge by substituting all three aspartic acids with alanines (Δ3D) did not bind RecO. The wild-type peptide and the full-length protein interacted with similar affinity, and the presence of DNA did not significantly change SSB binding to RecO.

SSB recruits RecO to DNA at moderate salt concentration

To further test the role of ionic and hydrophobic interactions, we compared SSB-Ct binding to RecO in solutions with 50 mM and 200 mM NaCl (Figure 3A). At 200 mM NaCl, the RecO binds F-SSB-Ct 5 times weaker than at 50 mM NaCl with a $K_d = 0.34 \pm 0.02$ μM. Relatively strong binding at the elevated salt concentration is likely to reflect a significant contribution of hydrophobic interactions observed in the crystal structure. Similar binding was observed in the presence of RecR (apparent $K_d = 0.1 \pm 0.04$ μM in Buffer A and 0.64 ± 0.01 μM in Buffer B), which forms a complex

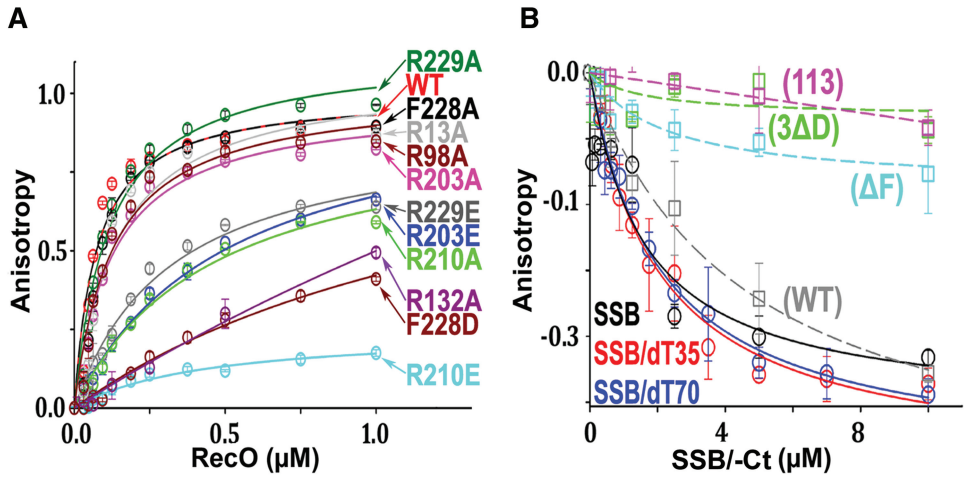

Figure 2. Equilibrium binding of RecO and RecO mutants to SSB-Ct and SSB. (**A**) F-SSB-Ct (20 nM) was titrated by wild-type RecO (red) and its mutants designated on the right side of the plot in buffer A (50 mM NaCl). Increase in anisotropy corresponds to F-SSB-Ct binding to RecO. (**B**) Competition assay between RecO binding to F-SSB-Ct and various substrates. RecO (1 μM) was incubated with 0.9 μM of F-SSB-Ct and the mixture was titrated by the following substrates: SSB (solid lines and circles) alone (black) and with dT_{35} (red) and dT_{70} (blue); SSB-Ct variants designated by labels in parentheses (dashed lines and squares): SSB-Ct (gray), SSB-Ct -113 (magenta), SSB-Ct-3ΔD (green) and SSB-Ct-ΔF (cyan). Reactions were performed in moderate salt concentration buffer B (200 mM NaCl) due to low solubility of RecO in the presence of dT_{70} used in a control experiment (data not shown). Decrease in fluorescence anisotropy corresponds to F-SSB-Ct release from RecO.

Table 1. Apparent equilibrium dissociation constants of F-SSB-Ct binding to RecO and RecO mutants

Mutant	K_d (μM)	SD (μM)
WT	0.06	0.01
R203A	0.13	0.01
R203E	0.62	0.04
R210A	0.56	0.02
R210E	>10	
F228A	0.10	0.01
F228D	>10	
R229A	0.11	0.01
R229E	0.46	0.03
R132A	>10	
R98A	0.13	0.01
R13A	0.11	0.01

with RecO and is essential for HR initiation (27,29,53–56). RecO binding to ss- and dsDNA (34,38,40) is important for DNA annealing and, likely, for the recombination initiation in the presence of RecR. Yet, previous binding studies were performed only at low salt concentrations. We found that RecO binding to 15-mer ss- and dsDNA was completely inhibited at 200 mM NaCl (Figure 3B) and significantly weakened with longer DNA substrates (Supplementary Figure S5). RecR does not bind DNA at submillimolar concentrations, but stimulates RecO binding to DNA, at least at 200 mM NaCl. However, interaction of RecOR with DNA at 200 mM NaCl was also significantly weaker than at 50 mM. The difference in the salt dependence of RecO binding to SSB-Ct and DNA suggests that under physiological conditions, with the concentration of monovalent metal ions between 100 and 200 mM (57), interaction of RecO with SSB may stimulate subsequent steps of RecO and RecOR interaction with ssDNA.

Neither RecO nor RecOR complex dissociates SSB from ssDNA

To address RecO and RecOR binding to the SSB/DNA complex, a pull-down assay with dT_{45} oligonucleotide immobilized on streptavidin beads was performed (Figure 4). Immobilized DNA was bound to either SSB or SSBΔC8 and then incubated with an excess of RecO or RecOR. At 50 mM NaCl, RecO and RecOR interacted with both free DNA and SSB-bound ssDNA and, to a smaller extent, with SSBΔC8-bound ssDNA. In spite of the excess of RecO(R) in solution, there was no noticeable dissociation of SSB from DNA. Similar results were obtained at 200 mM NaCl, with the stronger binding of RecO and, particularly, of RecOR to SSB-bound ssDNA than to free DNA and to SSBΔC8-bound DNA. Similar results were obtained with dT_{70} (data not shown).

SSBΔC8 inhibits RecO-mediated DNA annealing

To further verify the role of SSB-Ct in RecO recruitment to DNA, a DNA annealing assay was performed at different salt concentrations with ssDNA bound to either SSB or SSBΔC8 (Figures 5 and Supplementary Figure S6). At low salt concentrations (10 and 20 mM NaCl), RecO stimulated DNA annealing of ssDNA protected by both SSB and SSBΔC8. At 60 mM NaCl, DNA annealing was observed only in the presence of SSB, while SSBΔC8 inhibited the reaction. NaCl at 200 mM inhibited the reaction with both SSB variants (data not shown), similar to previously published results (34). The reverse experiment with RecO mutants R132A and F228D, deficient in SSB-Ct binding, confirmed the importance of SSB-Ct for RecO-mediated strand annealing at moderate

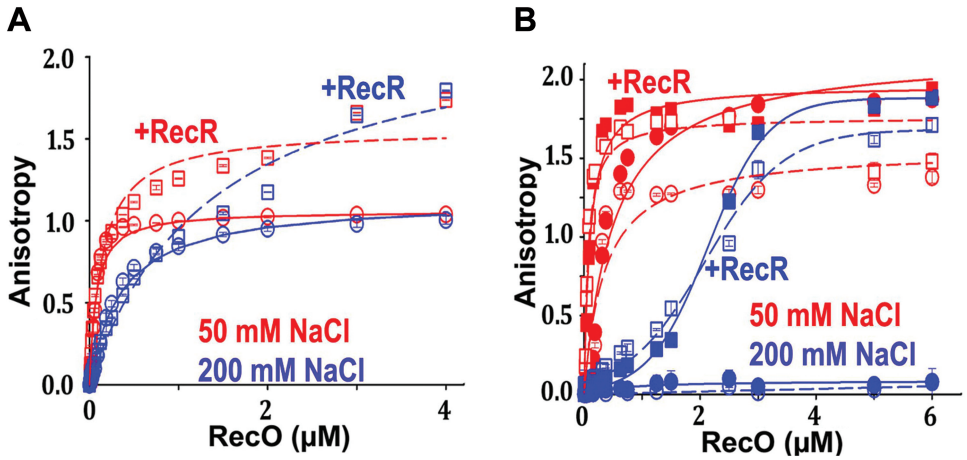

Figure 3. Different salt dependence of RecO binding to SSB-Ct and DNA. (**A**) The binding of F-SSB-Ct to RecO alone (solid lines, circles) or in the presence of 10 μM of RecR (dashed lines, squares) in low salt buffer A (red) and in moderate salt buffer B (blue) measured as a function of the fluorescent anisotropy change. Binding to the RecOR complex resulted in a larger signal than in the case of RecO due to the larger size of the complex. However, the beginning of both isotherms coincides, reflecting the similar binding constants of SSB-Ct to RecO and RecOR. (**B**) DNA binding by RecO at low (red) and moderate (blue) salt concentrations. FAM-labeled dT_{15} (5 nM) (solid lines and solid symbols) and 5 nM of 15 bp dsDNA labeled with FAM (dashed lines and open symbols) were titrated by RecO alone (circles) and in the presence of 10 μM of RecR (squares). Note the shift of red isotherms for the RecR complex to the left, reflecting stimulation of RecO DNA binding by RecR. The shape of the isotherm for the RecOR binding at 200 mM NaCl is likely to reflect a two-step mechanism of RecO binding to RecR and binding of the complex to DNA, rather than cooperative binding to DNA.

salt concentration (Figure S6). Both mutants annealed SSB-bound ssDNA at low salt concentrations, but were unable to anneal SSB-bound ssDNA at 60 and 100 mM NaCl.

Deinococcus radiodurans RecO does not bind SSB tail

Structural comparison of *E. coli* and *D. radiodurans* RecO revealed a different conformation of the SSB tail binding site (Figure 1C and F). In DrRecO, the potential peptide binding cavity is occupied by the C-terminus. Moreover, the residues forming the SSB-Ct binding pocket are not conserved. For example, there is Glu219 in DrRecO instead of the Arg210 in RecO. This substitution (R210E) completely inhibited SSB-Ct binding by RecO (Figure 2 and Table 1). Correspondingly, DrRecO did not bind DrSSB-Ct (Figure 6). Both SSB-Ct are highly conserved, and the *E. coli* homolog binds peptides from both organisms with comparable affinity, in agreement with earlier studies which demonstrated the functionality of DrSSB in recombination initiation with *E. coli* proteins (58). The only weak interaction was observed with the DrRecOR complex in the presence of magnesium at 50 mM NaCl, leaving open the possibility of similar interactions between DrRecOR and DrSSB-Ct during recombination initiation. Due to the lack of a hydrophobic cavity on DrRecO surface, this binding is likely to be mediated by ionic interactions between the acidic part of SSB-Ct and the DrRecOR complex, which may resemble that of ssDNA binding to DrRecOR.

DISCUSSION

SSB protects ssDNA and specifically binds numerous proteins (44). These two functions are somewhat exclusive since protein interactions eventually lead to SSB removal from ssDNA. The relationship between these events is essential for understanding the mechanisms of DNA replication, recombination and repair. During recombination, SSB plays a dual role by inhibiting the initiation and facilitating the extension of RecA nucleoprotein filament. RecOR overcomes the first inhibitory step of loading RecA on SSB-protected ssDNA, and this function depends on SSB recognition by RecO. The structural and equilibrium binding data presented here describe the mechanism of RecO interaction with SSB mediated by SSB-Ct and suggest that SSB facilitates the RecO binding to ssDNA through SSB-Ct under physiological conditions in both DNA annealing and RecR-mediated recombination initiation pathways.

Hydrophobic interactions facilitate RecO recruitment to ssDNA by SSB at physiological conditions

SSB-Ct binding to RecO depends on both hydrophobic and electrostatic interactions. The importance of hydrophobic interactions is underlined by the structural data, where only the hydrophobic part of SSB-Ct is well ordered in structures of RecO and ExoI complexes, by mutagenesis and by the moderate salt dependence of RecO binding to SSB-Ct. The acidic residues of SSB-Ct are disordered in both structures. Only substitution of all three aspartates decreases interaction in a similar degree to that of a single mutation of Pro176 or Phe177. Thus, hydrophobic interactions should play a major role in SSB-Ct binding to RecO and other proteins. SSB binds ssDNA at a wide range of salt concentrations (59), suggesting that it facilitates RecO interaction with ssDNA at physiological conditions. A similar recruitment mechanism may be common for other DNA replication and repair proteins known to

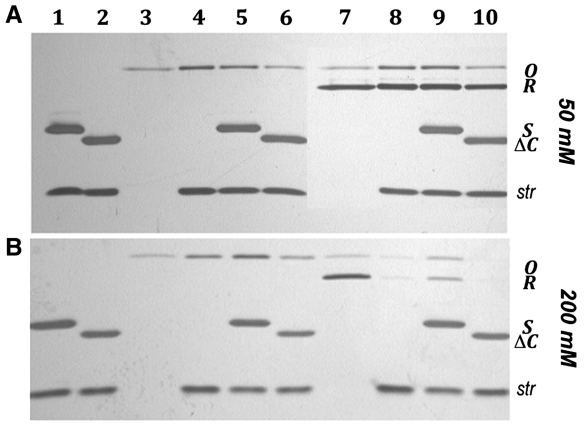

Figure 4. Binding of RecO and RecOR to ssDNA in the presence of SSB and SSBΔC8. Streptavidin beads with immobilized dT_{45} were incubated first with SSB or SSBΔC8, then with either RecO or RecOR proteins, and immobilized proteins were detected on silver-stained 15% SDS–PAGE. (**A**) Pull down assay was performed in buffer A (50 mM NaCl) with the addition of 0.1 mM CHAPS and 0.1 mg/ml BSA. Proteins immobilized on DNA-bound beads are shown in the following lanes: 1, SSB; 2, SSBΔC8; 4, RecO; 5, RecO in the presence of SSB; 6, RecO in the presence of SSBΔC8; 8, RecOR; 9, RecOR in the presence of SSB; 10, RecOR in the presence of SSBΔC8. The loading solutions for RecO and RecOR are shown on lanes 3 and 7, respectively. (**B**) Similar assay performed in buffer B (200 mM NaCl) with similar order of lanes. Protein bands are marked on the right side with O corresponding to RecO, R to RecR; S to SSB; Δ*C* to SSBΔC8; str to streptavidin. Unlike the results from the equilibrium binding experiments (Figure 3 and Supplementary Figure S5), in the absence of RecR, RecO binds to DNA immobilized on beads relatively strong at moderate salt concentration (B, lane 4), which may be an artifact of protein aggregation due to high local concentration of immobilized DNA. However, SSB-bound DNA still remains the preferred substrate for RecO in contrast to low salt conditions in A.

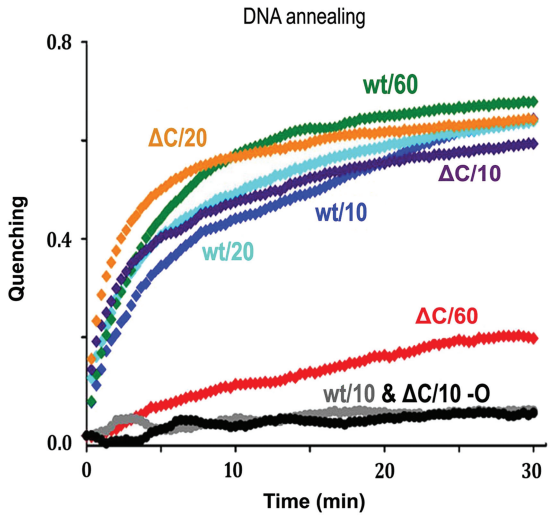

Figure 5. SSBΔC8 inhibits RecO-mediated annealing at an elevated salt concentration. Complementary ssDNA oligonucleotides labeled with FAM and Dabsyl were incubated with SSB or SSBΔC8 in buffers of variable NaCl concentration and mixed together. FAM quenching was plotted as a function of time after addition of RecO. Blue, cyan and green colors correspond to SSB-bound DNA at 10, 20 and 60 mM NaCl; purple, orange and red colors correspond to SSBΔC8-bound DNA at 10, 20 and 60 mM NaCl. Gray and black labels correspond to the incubation of SSB and SSBΔC8 at 10 mM NaCl without RecO.

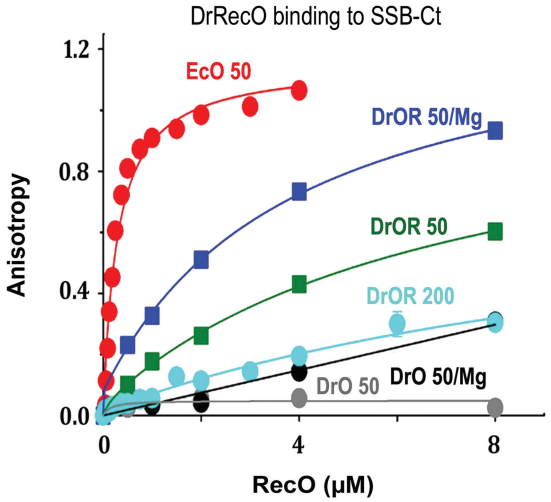

Figure 6. DrRecO does not bind SSB-Ct. Equilibrium binding of DrSSB-Ct to DrRecO and RecO assayed using FAM-labeled DrSSB-Ct (10 nM). Binding to RecO (EcO) in buffer A is shown in red, to DrRecO (DrO) in buffer A in gray, and in buffer A with 5 mM $MgCl_2$ in black, to DrRecOR in the presence of 20 μM of DrRecR (DrOR) in buffer A in green, in buffer A with 5 mM MgCl2 in blue, and in buffer B in cyan.

bind SSB and which are characterized by a strong dependence of DNA binding on the ionic strength of solution. For example, the χ subunit of the DNA polymerase III binds SSB-Ct, and this interaction stimulates both clamp loading and polymerase processivity only at elevated ionic strength (60,61). Similarly, we found that RecO-mediated DNA annealing depends on the presence of SSB-Ct only at moderate salt concentrations.

Previously, SSB was proposed to limit interaction of RecO with ssDNA due to a time delay in the RecA loading reaction mediated by RecOR in the presence of SSB (43). In pull-down and DNA annealing assays, we also observed an inhibitory role of the SSBΔC8 core protein on RecO interaction with ssDNA. At the same time, the presence of SSB-Ct clearly stimulated both annealing and ssDNA binding by RecO at elevated salt concentrations. The relationship between the protective role of the SSB core structure and the recruitment role of SSB-Ct should be further investigated with a more quantitative approach. Interestingly, overexpression of RecO partially compensated temperature sensitivity of SSBΔ35 *Bacillus subtilis* cells expressing SSB without the C-terminus (45).

The recruitment mechanism suggests a synergy between DNA and SSB binding. PriA binds ssDNA-bound SSB 10 times stronger than free SSB (62). Interaction of χ with SSB was not affected by the presence of DNA, but χ does not bind DNA. However, DNA stimulates χ binding to SSB by 1000-fold in the presence of DNA binding subunit of Pol III (61). No significant enhancement RecO binding to DNA-bound SSB versus free SSB was observed in a competition assay, at least at 200 mM NaCl (Figure 2B). However, in a pull-down assay at 200 mM NaCl, the binding of RecO and RecOR to DNA was stronger in the case of SSB-bound DNA (Figure 4).

RecO and RecOR form a complex with DNA-bound SSB

Another consequence of a synergy or cooperativity between SSB and DNA binding is that the SSB-mediated recruitment of proteins to ssDNA should not cause its own removal from DNA. In the case of recombination, RMPs facilitate loading of recombinase on SSB-protected ssDNA, which is likely accompanied by at least partial removal of SSB from ssDNA. Previously, it was suggested that RecOR either form a complex with ssDNA-bound SSB (27) or remove SSB from ssDNA (46). Our data demonstrated that RecO binding to SSB-Ct and DNA are not exclusive (Figure 2B). None of our pull-down assays revealed a noticeable dissociation of SSB as well as SSBΔC8 from DNA, even when they were incubated with an excess of RecO and RecOR (Figure 4). We hypothesize that RecO and RecOR do not remove SSB from ssDNA, but instead alter the conformation of ssDNA. SSB binds ssDNA in two different modes: SSB_{65}, where all four SSB monomers are wrapped by 65-mer ssDNA, and SSB_{35}, with only two monomers bound to 35-mer ssDNA (63,64). Even removing the unstructured C-termini affects DNA binding (65) and the equilibrium between these two modes (66). Binding of SSB-Ct to positively charged RecO is likely to change the conformation of bound ssDNA. Such an altered conformation should be compatible with the DNA annealing process in the presence of complimentary ssDNA. The completion of DNA annealing can force the dissociation of SSB from DNA. RecR inhibits DNA annealing by RecO and is essential for RecA loading, even though its interaction with RecA had never been shown. Therefore, RecR should also change the conformation of ssDNA or of the whole RecO/SSB/ssDNA assembly to prevent DNA from annealing and to make it available for RecA binding. This hypothesis is supported by our data showing similar binding of RecO and RecOR to SSB-Ct and a stimulatory effect of RecR on RecO binding to DNA. Theoretically, even partial unwrapping of ssDNA from the SSB tetramer by the RecOR complex, for example, by changing SSB_{65} to the SSB_{35} mode, may free ssDNA of a sufficient length for RecA nucleation to occur (67).

Differences of SSB interaction with RecO in *D. radiodurans* and *E. coli*

The bacterium, *D. radiodurans*, is characterized by a unique ability to efficiently repair thousands of chromosome breaks caused by radiation and desiccation (68–71). RecFOR are important for the rapid reconstitution of a shredded genome, which is thought to occur through a synthesis-dependent strand annealing process followed by DNA recombination (26). Previously, we demonstrated that DrRecO does stimulate DNA annealing in the absence of SSB (38). The lack of DrRecO binding to DrSSB-Ct suggests that such annealing is SSB-independent in this organism, which may be important for annealing of relatively short ssDNA fragments insufficient for SSB binding. In the case of recombination initiation, we cannot rule out DrRecOR interaction with SSB-Ct. However, previous observations imply the existence of alternative pathways of recombination initiation. First, significant differences in properties of RecA from two organisms have been reported (72). Second, RecF was shown to rescue RecOR function in the presence of SSBΔC8 mutant in *E. coli* (30), suggesting an alternative SSB-Ct independent pathway of RecOR-mediated RecA loading. This data also suggests that, in addition to SSB binding, other functions of RecO are essential for recombination initiation.

The prediction of similar SSB-Ct binding properties of RecO homologs is complicated by the overall low sequence homology and the lack of conserved motifs (38). Even the identification of RecO homologs in some genomes is challenging (73,74). Likewise, the prediction of an SSB-Ct binding site formed mostly by C-terminal α-helixes is difficult, unless overall sequences are considerably shorter, as is the case of *Helicobacter pylori*. Thus, in some species, the RecO-mediated strand annealing may be independent of interaction with SSB-Ct. Strand annealing and HR initiation activities may also reside in separate proteins. Interestingly, DdrB was recently identified as a ssDNA annealing protein in *D. radiodurans* as well (75). The RPA-dependent strand annealing property is not conserved between Brh2 and BRCA2 homologs in eukaryotes (10,76).

Overall, a large number of known SSB binding partners implies a multifunctional role for SSB-Ct. In addition to its role in stabilization of DNA–protein complexes reported here, SSB-Ct was suggested to perform a switch function for two proteins exchanging places on DNA via a mutually exclusive interaction with SSB-Ct (77). Interestingly, other RecF pathway proteins, including RecQ and RecJ, also bind SSB-Ct (78–80), suggesting that a similar switch mechanism regulates the transition from the RecQJ-mediated step of DNA processing to the RecFOR-mediated step of RecA loading (78).

ACCESSION NUMBER

PDB ID 3Q8D.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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