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Identification of the SSB Binding Site on *E. coli* RecQ Reveals a Conserved Surface for Binding SSB's C Terminus

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RecQ DNA helicases act in conjunction with heterologous partner proteins to catalyze DNA metabolic activities, including recombination initiation and stalled replication fork processing. For the prototypical *Escherichia coli* RecQ protein, direct interaction with single-stranded DNA-binding protein (SSB) stimulates its DNA unwinding activity. Complex formation between RecQ and SSB is mediated by the RecQ winged-helix domain, which binds the nine C-terminal-most residues of SSB, a highly conserved sequence known as the SSB-Ct element. Using nuclear magnetic resonance and mutational analyses, we identify the SSB-Ct binding pocket on *E. coli* RecQ. The binding site shares a striking electrostatic similarity with the previously identified SSB-Ct binding site on *E. coli* exonuclease I, although the SSB binding domains in the two proteins are not otherwise related structurally. Substitutions that alter RecQ residues implicated in SSB-Ct binding impair RecQ binding to SSB and SSB/DNA nucleoprotein complexes. These substitutions also diminish SSB-stimulated DNA helicase activity in the variants, although additional biochemical changes in the RecQ variants indicate a role for the winged-helix domain in helicase activity beyond SSB protein binding. Sequence changes in the SSB-Ct element are sufficient to abolish interaction with RecQ in the absence of DNA and to diminish RecQ binding and helicase activity on SSB/DNA substrates. These results support a model in which RecQ has evolved an SSB-Ct binding site on its winged-helix domain as an adaptation that aids its cellular functions on SSB/DNA nucleoprotein substrates.

Keywords: RecQ; helicase; single-stranded DNA-binding protein; genome maintenance; protein complex

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Introduction

RecQ DNA helicases are evolutionarily conserved enzymes with diverse roles in genome maintenance.[1–5] In bacteria, RecQ proteins aid in recombination, stalled replication fork processing, SOS signaling, and antigen variation pathways.[6–12] In humans, individual mutation of three of five RecQ helicase genes (*BLM*, *WRN*, and *RECQ4*) gives rise to Bloom, Werner, and Rothmund–Thompson syndromes, respectively.[13–15] These conditions are marked by genome instability manifested as chromosome breakage and genomic rearrangements that are linked to cancer predisposition, underscoring the importance of the RecQ family of enzymes in maintaining the integrity of genomic DNA.

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Abbreviations used: SSB, single-stranded DNA binding protein; RPA, replication protein A; OB, oligosaccharide binding; ssDNA, single-stranded DNA; RecQ-WH, RecQ winged-helix; HSQC, heteronuclear single quantum coherence; 3D, three-dimensional; NOESY, nuclear Overhauser enhancement spectroscopy; dsDNA, double-stranded DNA; EMSA, electrophoretic mobility shift assay; EDTA, ethylenediaminetetraacetic acid; NIH, National Institutes of Health.

RecQ proteins associate with a large number of heterologous proteins, often functioning as components in multiprotein complexes.[1,5,16–18] Direct interaction with single-stranded DNA-binding protein (SSB) [called replication protein A (RPA) in eukaryotes] is a shared feature among bacterial and eukaryotic RecQ proteins. RecQ proteins known to associate with their cognate SSB include *E. coli* RecQ,[19] and human BLM,[20] WRN,[21] and RecQ1.[22,23] In addition, human RecQ5β is strongly stimulated by, and most likely associates with, RPA.[24] Due to the conservation of RecQ interactions with SSB, a physical and functional understanding of how these complexes assemble to act on DNA substrates is critical for appreciating the broad cellular roles of RecQ proteins.

In *E. coli* and nearly all other bacteria, SSB function as homotetramers, each subunit of which comprises an N-terminal oligonucleotide/oligosaccharide binding (OB) fold linked to a structurally dynamic C-terminal tail[25–29] (Fig. 1a). Whereas the OB fold is responsible for tetramerization and single-stranded DNA (ssDNA) binding, the C-terminal tail region ends with a highly conserved amphipathic peptide sequence (SSB-Ct) that associates with a diverse array of genome maintenance enzymes[31,32] (Fig. 1a). Interactions made with the SSB-Ct element are critical to proper genome maintenance, and alterations in the SSB-Ct sequence can severely affect *E. coli* viability.[33–38] To date, every bacterial SSB-interacting protein examined binds to SSB-Ct, including *E. coli* RecQ,[19,31] although structural data indicating how such complexes are formed are only available for *E. coli* exonuclease I.[32] Additional studies are necessary to map the conserved structural features that define SSB-Ct binding sites.

E. coli SSB is important for stimulating *E. coli* RecQ helicase activity and for aiding in joint activities between RecQ and other genome maintenance enzymes. SSB stimulates RecQ in at least two ways. First, SSB passively stimulates RecQ activity by binding and sequestering ssDNA generated by RecQ helicase activity and by preventing the formation of nonproductive ssDNA/RecQ complexes that inhibit the enzyme.[39,40] Second, SSB actively stimulates RecQ by recruiting RecQ to its DNA substrate and/or by helping retain the enzyme on DNA through direct physical interaction.[19] For active stimulation, interaction between SSB and RecQ is mediated by direct contacts made between the SSB-Ct sequence and the RecQ winged-helix (RecQ-WH) domain (Fig. 1a).[19] Interestingly, the winged-helix domain of WRN is also important for contacting RPA;[41] however, this interaction presumably occurs via a different mechanism, as RPA lacks the amphipathic SSB-Ct sequence that defines the bacterial SSB protein interaction site. Nonetheless, it is striking that, for both *E. coli* RecQ and WRN, their respective winged-helix domains play important roles in complex formation with cognate SSB. SSB is also a component in more complicated reactions, including recombination initiation with RecQ and RecA,[42] DNA catenation and supercoiling, and converging replication fork resolution with RecQ and topoisomerase III.[6,43,44]

In this study, we have used NMR to identify the SSB-Ct binding site on the RecQ-WH domain. This site, which is on the opposite face of the proposed DNA binding site of the domain,[30] forms a pocket that includes nonpolar and electropositive elements that resemble those found in the exonuclease I SSB-Ct binding site.[32] Using NMR data and additional structural information from the *E. coli* RecQ catalytic core X-ray crystal structure[30] as guides, we constructed a panel of RecQ variant proteins with amino acid substitutions that were predicted to impair association with SSB. This panel was used to determine the extent to which alteration of particular residues influences RecQ binding to SSB, and RecQ DNA binding and unwinding in the presence and in the absence of SSB. Additional experiments using SSB variant proteins with altered SSB-Ct elements assessed the effect of altering SSB in the same reactions. Each of the RecQ variant proteins had deficiencies in binding SSB protein, with several arginine and glutamine substitutions having the greatest impact. RecQ proteins bearing one or two amino acid changes that diminish SSB binding exhibited modest nucleic acid binding defects, but more pronounced defects in binding to SSB-coated DNA. These RecQ variants retained the ability to be stimulated by SSB, albeit to a lesser extent than wild-type proteins. Nonstandard SSB-Ct elements also mildly inhibited the helicase activity of wild-type RecQ protein on a partial-duplex DNA substrate. We postulate that SSB-Ct binding to the site identified in this study influences RecQ-mediated DNA unwinding of cellular nucleoprotein substrates.

Results

NMR studies identify RecQ-WH domain residues involved in SSB-Ct binding

Our earlier results indicated that *E. coli* RecQ and SSB form a complex that is mediated by stoichiometric binding of the RecQ-WH domain to the SSB-Ct element,[19] although the precise SSB-Ct binding site on the RecQ-WH domain was not defined. A structural approach was therefore taken to map the SSB-Ct binding site on RecQ. We initially attempted to map the site by soaking the SSB-Ct peptide into crystals of the *E. coli* RecQ catalytic core domain (which includes the RecQ-WH domain), but electron density for the peptide was never observed in these experiments (data not shown). Since the isolated RecQ-WH domain binds the SSB-Ct peptide with the same specificity and with nearly the same affinity as the full-length RecQ protein,[19] we used NMR to define the SSB-Ct binding site on this small (116-residue) domain.

^{15}N- and ^{15}N-,^{13}C-labeled RecQ-WH domain samples provided well-dispersed NMR spectra that allowed determination of over 90% of the

backbone resonance assignments from the domain, as described in Materials and Methods (Supplementary Fig. 1 and Supplementary Table 1). To map the SSB-Ct binding site, we titrated the SSB-Ct peptide into the RecQ-WH NMR samples and measured changes in the chemical shift positions of the domain's backbone amides in two-dimensional ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) experiments. We anticipated that amides in residues directly involved in SSB-Ct binding would undergo significant chemical shift changes relative to those that are not involved in binding. Consistent with this hypothesis, the addition of the SSB-Ct peptide dramatically changed the chemical shifts of several HN resonances from the RecQ-WH domain (Fig. 1b). Nineteen HN chemical shifts were

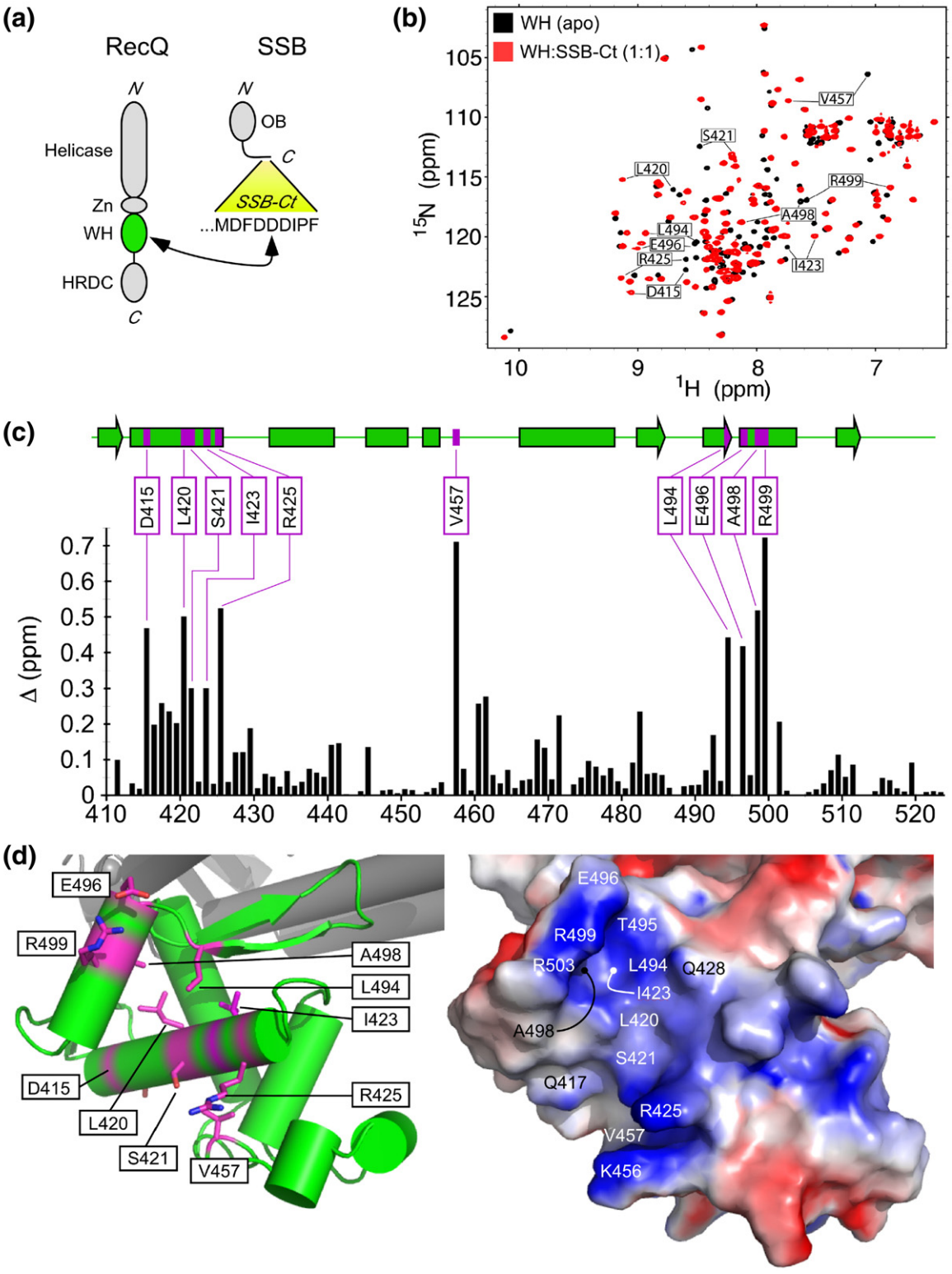

Fig. 1 *(legend on next page)*

substantially altered (≥0.2 ppm) by SSB-Ct addition, as follows: D415, A416, Q417, I418, A419, L420, S421, I423, R425, V457, M460, G461, S471, V482, L494, E496, A498, R499, and V501. Among these residues, the largest chemical shift changes (≥0.3 ppm) occurred for D415, L420, S421, I423, R425, V457, L494, E496, A498, and R499 (Fig. 1b and c). For many of these latter residues, the corresponding peak intensities disappear at intermediate titration points (1:0.5 RecQ-WH:SSB-Ct) due to exchange between the free and the bound conformations occurring during the NMR experiment. Therefore, a detailed analysis of the three-dimensional (3D) ^{1}H–^{15}N and ^{1}H–^{13}C nuclear Overhauser enhancement spectroscopy (NOESY)-HSQC spectra (1:1 RecQ-WH:SSB-Ct) was required to assign the new HN chemical shifts for the bound form of the RecQ-WH domain. Eleven amide residues (Y410, G412, N443, H452, K456, V472, A487, T495, R503, G504, and V512) could not be unambiguously identified after a 1:1 molar addition of SSB-Ct peptide to the RecQ-WH domain.

Each of the residues that undergoes a ≥0.3-ppm chemical shift upon binding to the SSB-Ct peptide maps to a single cleft on the *E. coli* RecQ-WH domain (Fig. 1d). The cleft has electrostatic characteristics that include a hydrophobic pocket (L420, I423, L494, and A498) flanked by positively charged or polar elements (R499 and R503, with additional residues Q417, R425, and K456 near the pocket) (Fig. 1d). As described in Discussion, these electrostatic features are strikingly similar to those found in the *E. coli* exonuclease I SSB-Ct binding site, which is the only protein for which the SSB-Ct binding site has previously been defined.[32] This cleft is positioned on the opposite face of the RecQ-WH domain relative to the proposed double-stranded DNA (dsDNA)-binding surface of the domain.[30] Interestingly, this cleft also forms a crystal contact in RecQ catalytic core domain crystals,[30] which could explain why SSB-Ct peptide soak experiments failed to produce the complex.

In addition to residues that comprise the putative SSB-Ct binding cleft on the *E. coli* RecQ-WH domain, HN chemical shift perturbation data revealed several other regions that undergo more minor chemical shift changes (0.1–0.2 ppm) (Fig. 1c). Although some of these smaller shift changes correspond to residues adjacent to the putative SSB-Ct binding site (N427, Q428, R429, and L492), several others do not (D411, R440, G441, Q445, H468, W469, Q509, and V512). These chemical shift changes are dispersed across loop and helical regions of the RecQ-WH domain. Therefore, it is likely that the observed minor chemical shift perturbations reflect small structural changes that are imparted upon SSB-Ct binding.

Panel of RecQ variant proteins

Based on the above structural studies, we hypothesized that the RecQ-WH domain cleft that was sensitive to the addition of the SSB-Ct peptide forms its SSB-Ct binding site. To test this idea, we created overexpression plasmids to produce a panel of single-site alanine RecQ variant proteins that individually alter several of the residues with large (≥0.3 ppm) SSB-Ct-dependent chemical shift changes: D415A, S421A, R425A, V457A, and R499A (Fig. 1). Examination of the crystal structure of the RecQ catalytic core[30] revealed additional neighboring residues that form part of the putative SSB-Ct binding cleft for which expression vectors were also created: Q417A, Q428A, K456A, T495A, and R503A. Based on the importance of basic residues in the SSB-Ct interface of exonuclease I,[32] charge-reversal variants were also made for R425, R499, and R503 (changing each to glutamate), along with each permutation of double and triple mutation for each arginine residue. Finally, a control variant was made in which an arginine distal to the putative SSB-Ct binding site was altered to alanine (R448A). Four of the RecQ variants (T495A, R425E/R499E, R499E/R503E, and R425E/R499E/R503E) could not be purified to homogeneity due to apparent proteolysis of the overexpressed proteins and were not pursued further.

To determine whether any of the RecQ variants was misfolded, we measured the ssDNA-dependent ATP hydrolysis rates of each protein. Wild-type RecQ hydrolyzed ATP at a rate of 1210± 140 min^{-1}, in agreement with previously published measurements.[45,46] Of the 14 variant RecQ proteins studied, five were indistinguishable from wild-type RecQ (Q428A, R448A, V457A, R499A, and R499E), and the remaining nine were only modestly reduced in ATP hydrolysis rate (ranging from 1.3-fold to 1.6-fold reductions) (Table 1). With

Fig. 1. Structural basis of RecQ SSB-Ct binding. (a) Schematic domain diagram of *E. coli* RecQ and SSB. Domains in RecQ [helicase, zinc binding (Zn), winged helix (WH; green), and helicase and RNase D C-terminal (HRDC)] and SSB (OB) are labeled. The sequence of the residues from SSB comprising the SSB-Ct element is given. An arrow indicates the elements of RecQ and SSB that are sufficient for complex formation.[19] (b) ^{15}N HSQC spectral overlay of the RecQ-WH domain (black) and a 1:1 mixture of the RecQ-WH domain and SSB-Ct peptide (red). The 10 largest chemical shift differences between the spectra are labeled. (c) Histogram of the difference in amide proton and nitrogen resonance chemical shifts of the RecQ-WH with and without the addition of a 1:1 mixture of the SSB-Ct peptide. The largest differences (>0.3 ppm) are labeled and shown in magenta on secondary structural elements (helices as boxes; strands as arrows) of the RecQ-WH domain in the context of the crystal structure of the RecQ catalytic core.[30] (d) Structure and electrostatics of the putative RecQ SSB-Ct binding site. Left: The 10 RecQ-WH domain residues with the highest chemical shift differences upon SSB-Ct binding are labeled and shown in magenta on a ribbon diagram of the RecQ catalytic core structure. Right: Electrostatic representation of the surface of the RecQ catalytic core (red, blue, and white for negative, positive, and neutral, respectively) is shown in the same perspective as the ribbon diagram. Residues, in addition to those identified by NMR to be altered in biochemical studies, are labeled in the electrostatic diagram.

Table 1. Biochemical parameters of RecQ variant proteins

RecQ variant	ATP hydrolysis (min^{-1})	Apparent relative DNA binding affinity	Apparent relative SSB/DNA binding affinity	[RecQ] for half-maximal unwinding (nM)	[RecQ] for half-maximal unwinding with SSB (nM)	Fold stimulation of helicase activity with SSB
RecQ	1210±140	+++	+++	2.9±0.1	0.3±0.1	9.7
R448A	1210±170	+++	+++	3.5±0.4	1.5±0.1	2.3
D415A	740±130	++	+	13.9±0.7	6.8±1.5	2.0
Q417A	910±70	++	+	6.4±1.8	2.0±0.2	3.2
S421A	940 ±100	+	+	9.5±1.1	4.7±1.6	2.0
R425A	730±60	++	+	4.5±0.6	2.3±0.5	2.0
R425E	810±100	++	++	8.1±0.3	5.4±0.6	1.5
Q428A	1310±110	+++	++	8.1±1.2	2.0±0.6	4.0
K456A	840±60	++	+	7.4±3.0	2.3±0.3	3.2
V457A	1050±80	++	++	3.0±0.4	1.0±0.1	3.0
R499A	1250±140	++	+	4.9±0.8	3.5±0.1	1.4
R499E	1100±110	+	+	8.7±1.2	4.3±0.3	2.0
R503A	770±60	+	+	9.8±2.7	6.0±0.8	1.6
R503E	770±90	+	+	6.8±1.1	4.1±0.6	1.7
R425E/R503E	960±80	++	+	21.6±4.6	13.2±2.1	1.6

the very modest effects on ATPase activity, these studies indicate that each of the RecQ variants is properly folded.

Amino acid substitutions in the putative SSB-Ct binding site impair SSB protein binding

Ammonium sulfate coprecipitation experiments were used to determine whether alterations in the RecQ variant proteins affect SSB binding. This method was developed originally to characterize SSB interactions with exonuclease I and has been adapted to analyze SSB binding to other partners as well, including RecQ.[6,19,38] The assay relies on the fact that SSB is precipitated in low concentrations of ammonium sulfate (150 g/L), whereas RecQ remains soluble in this condition. However, when SSB is mixed with RecQ prior to the addition of ammonium sulfate, RecQ that is in complex with SSB is coprecipitated, and the amount of RecQ found in the insoluble fraction provides a relative measure of SSB protein binding.

Consistent with the identification of the SSB-Ct binding site by NMR, each of the RecQ variants with altered residues in the putative SSB-Ct binding site showed deficiencies in SSB binding compared to wild-type RecQ in the coprecipitation assay (Fig. 2a). These binding defects could be divided into two groups: those with modest defects, which coprecipitated 20–50% as efficiently as wild-type RecQ (D415A, Q417A, S421A, K456A, and V457A), and those with severe defects, which coprecipitated 0–20% as efficiently as wild-type RecQ (R425A, R425E, Q428A, R499A, R499E, R503A, R503E, and R425E/R503E) (Fig. 2a). In all cases, the solubility of the RecQ variant proteins in the absence of SSB was

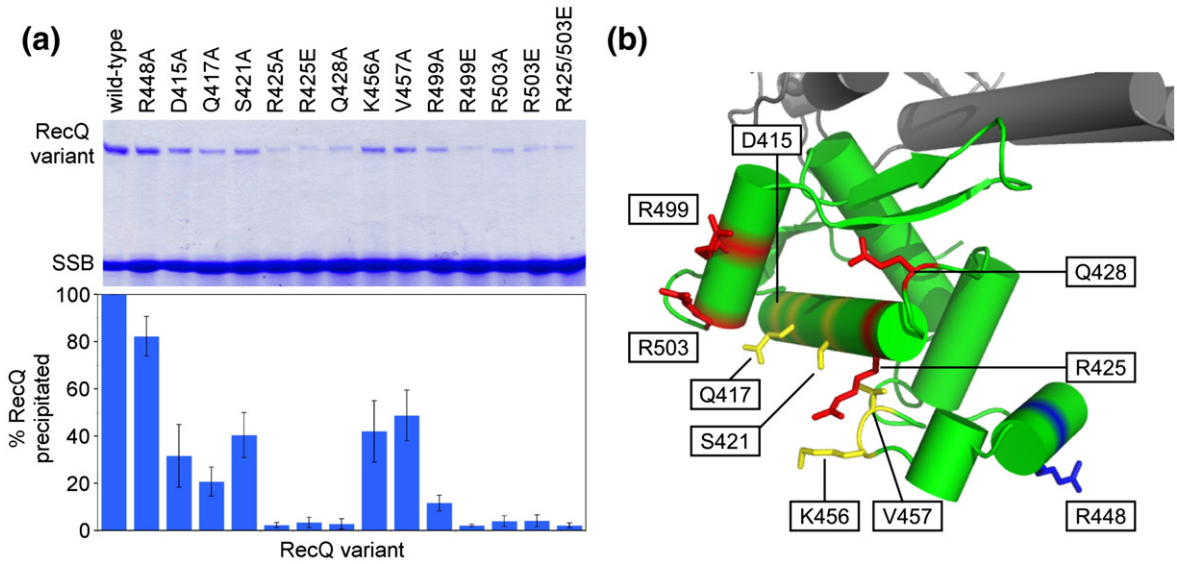

Fig. 2. RecQ variant binding to SSB. (a) Ammonium sulfate coprecipitation pellets of RecQ variant and SSB mixtures were resolved by PAGE (top). The quantitation of the intensity of the RecQ variant band relative to wild-type protein is shown below the gel. Values are the mean of three measurements, with 1 SD as error. (b) Alanine-substituted residues with modest (yellow) and severe (red) defects in SSB coprecipitation are shown on a ribbon diagram of the RecQ catalytic core structure.

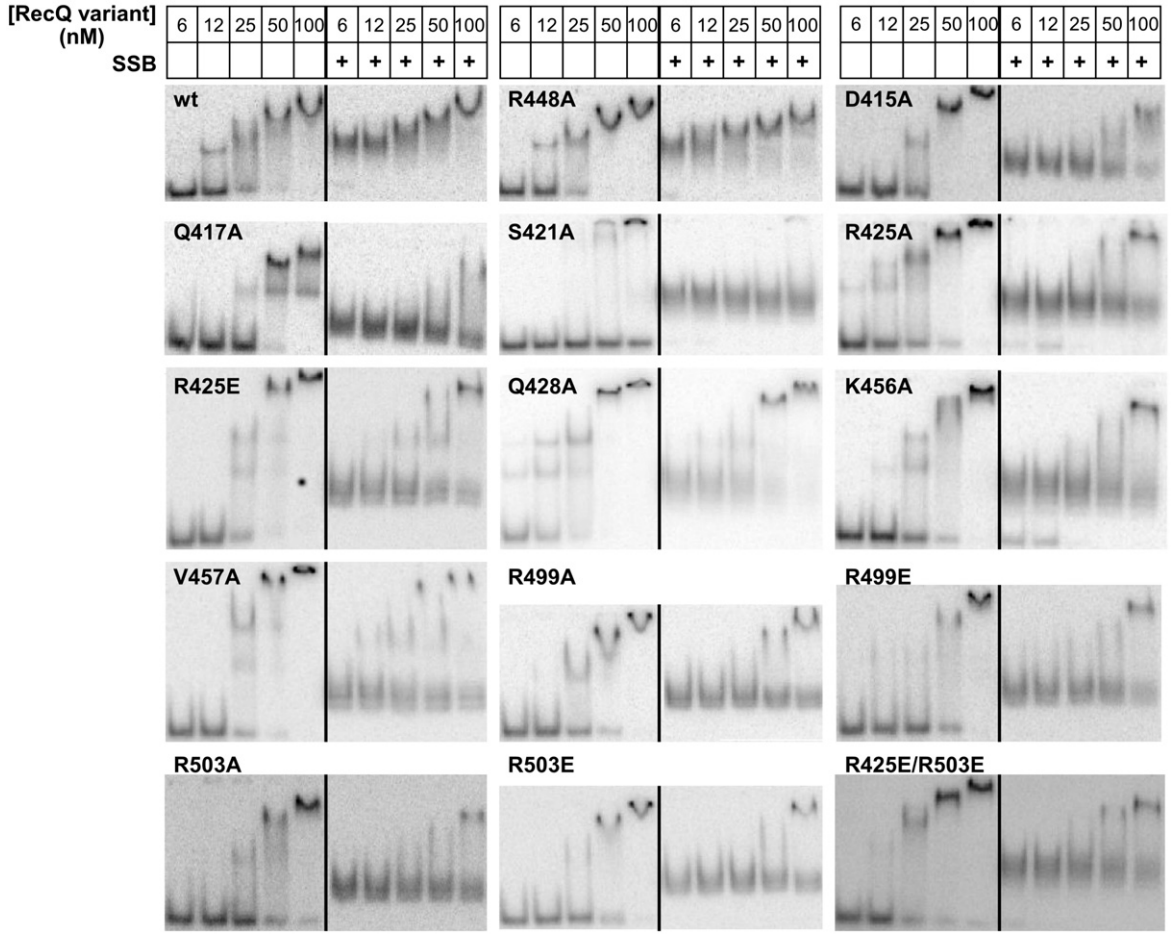

Fig. 3. DNA and SSB/DNA binding by RecQ variants. The electrophoretic mobility shift analysis of RecQ variant proteins binding to partial-duplex DNA is shown in the absence (left; each panel) and in the presence (right; each panel) of SSB.

unaltered relative to wild-type RecQ (data not shown). Notably, all but one of the substitutions that resulted in the largest deficiencies in SSB protein binding were those made in arginine residues at the periphery of the putative SSB-Ct binding pocket (Fig. 2b). These residues could provide positive charges to bind the highly electronegative SSB-Ct element. Additionally, the R448A control RecQ variant coprecipitated nearly as efficiently as wild-type RecQ (~80%). These data support identification of the SSB-Ct binding site on RecQ by NMR.

RecQ variants have reduced DNA and SSB/DNA binding affinity

Previous electrophoretic mobility shift assays (EMSAs) have shown that RecQ can bind to a partial-duplex DNA structure (a 30-bp duplex with a 70-base 3′ ssDNA overhang) and to a nucleoprotein complex formed between SSB and DNA.[19] However, substitution of an SSB lacking the SSB-Ct element occludes RecQ binding, indicating that RecQ recognizes both DNA and SSB in the nucleoprotein complex.[19] The same assay was used to test whether the RecQ variant proteins have impaired DNA or SSB/DNA binding.

Most of the RecQ protein variants had modestly weakened DNA binding affinities relative to wild-type RecQ (Fig. 3, left, and Table 1). Given the presence of multiple bands that likely correspond to different stoichiometries in the protein/DNA complexes, we could not accurately estimate dissociation constants for the protein variants. However, the EMSA experiments provided qualitative evidence that the substitutions in the panel of proteins alter DNA binding. Most of the variants are only modestly defective in DNA binding (e.g., D415A, which required ~2-fold higher concentrations to shift DNA mobility), but the S421A protein, which formed very-low-mobility DNA complexes only at very high protein concentrations (50–100 nM), was strongly defective in DNA binding. The control variant (R448A) bound the DNA and wild-type RecQ. The modest DNA binding defects of most of the RecQ variants might arise from substitution-induced changes in a DNA binding site distal to the SSB-Ct binding site, although a direct role for the site in DNA binding cannot be ruled out. Accordingly, studies with *E. coli* RecQ, WRN, and BLM proteins indicate that the winged-helix domain in each protein plays a direct role in DNA binding.[47–50]

We next tested the binding of the panel of RecQ variants to the same DNA prebound by SSB to examine the effects of the mutations on a nucleoprotein complex. Because our previous results indicated that RecQ recognizes both the DNA and the SSB components in SSB/DNA structures, variants that were defective in binding to either element should have weakened binding in this assay. Consistent with this prediction, each of the RecQ variant proteins (except for the R448A control) bound the SSB/DNA substrate with lower apparent affinity than the wild-type protein (Fig. 3, right, and Table 1). The S421A variant, which had the weakest DNA binding, also bound the SSB/DNA substrate most poorly, requiring 100 nM protein to elicit a mobility shift. Several other RecQ variants predominantly formed smears in the SSB/DNA EMSA (D415A, Q417A, V457A, R499A, R499E, R503A, and R503E), which could be related to weakened complex formation. Nearly all of the RecQ variants formed distinctive low-mobility bands, but required higher protein concentrations than wild-type RecQ or the R448A control variant to do so. Notably, the effects of the RecQ-WH domain substitutions on SSB/DNA binding were less pronounced than those observed in the RecQ/SSB protein binding assay (Fig. 2), which could be due to local concentration effects arising from RecQ and SSB binding to a common DNA substrate in the former assay. This possibility is discussed further below.

RecQ variants have reduced SSB-dependent helicase activity stimulation

Because SSB stimulation of RecQ DNA unwinding depends on direct physical interaction between the two proteins,[19] we predicted that disrupting the SSB/RecQ interface would inhibit SSB-mediated stimulation of RecQ helicase activity. To test this hypothesis, the SSB-dependent helicase activity of each RecQ variant protein was measured by utilizing the same partial-duplex DNA substrate used in the EMSA experiments. The 70-base 3′ ssDNA on the substrate provides a binding site for up to two tetramers of *E. coli* SSB.[51,52] Consistent with previously published data,[19] wild-type RecQ unwinds this substrate in a reaction that is stimulated ~10-fold by SSB (Table 1).

The variant RecQ proteins showed differential effects in unwinding the partial-duplex DNA substrate. In each case, RecQ helicase activity was stimulated in the presence of SSB, albeit to a lesser extent than the ~10-fold increase observed with wild-type RecQ (Table 1). The degree of stimulation ranged from 4-fold in the case of the most stimulated variant (Q428A) to 1.4-fold in the case of the least stimulated variant (R499A). Interestingly, each variant's ability to bind SSB in solution did not correlate with SSB stimulation of its helicase activity. This is perhaps best illustrated by the R448A control variant, which bound SSB and SSB/ssDNA with apparent wild-type affinity but was only stimulated 2.3-fold by SSB. The S421A variant exhibits an intermediate ability to bind SSB in solution (~40%, as well as wild-type), but its helicase activity is only stimulated ~2-fold by SSB, presumably because its ability to bind the DNA substrate both in the presence and in the absence of SSB is greatly diminished (Fig. 3). In contrast, the Q428A variant is an extremely poor SSB-binding protein in solution (~3% of wild-type levels), but displays the greatest degree of stimulation by SSB of any of the variants tested (Table 1). Therefore, because it appears that mutations in the RecQ-WH domain affect both SSB and DNA binding (and perhaps other functions of RecQ as well), the helicase results could not be correlated in a simple way to defects in SSB binding alone.

SSB variants with altered SSB-Ct sequences modify RecQ/SSB functions

To better evaluate the roles of the SSB/RecQ protein interface in their joint function, we employed several SSB variants with altered SSB-Ct elements. These included: (1) SSB113, which changes the penultimate proline of the wild-type SSB-Ct sequence (MDFDDDIPF) to serine; (2) SSB-mixed, which shuffles the order of the amino acids comprising the SSB-Ct sequence to DFMDDPFID; and (3) SSBΔC1, which removes the C-terminal-most phenylalanine residue. We have previously shown that RecQ is unable to bind SSB-Ct peptide equivalents of SSB113 and SSB-mixed sequences[19] and, based on structural studies of exonuclease I bound to the SSB-Ct peptide, we predicted that removing the C-terminal phenylalanine would disrupt binding as well.[19,32]

We first carried out ammonium sulfate coprecipitation and EMSA studies to determine the effects of the SSB mutations on complex formation with RecQ. Wild-type RecQ does not coprecipitate with any of these SSB variants (Fig. 4a), confirming the importance of the SSB-Ct amino acid sequence for binding RecQ in the absence of DNA. RecQ was also diminished in its ability to bind DNA precoated with either the SSB-mixed or SSBΔC1 variant SSBs, whereas binding to SSB113-coated DNA was not significantly diminished relative to that observed with SSB-coated DNA (Fig. 4b, compare with Fig. 3). This difference may reflect the more modest alteration of SSB113 compared to the other SSB variants. Nonetheless, these results support the notion that RecQ recognizes both DNA and SSB-Ct features in SSB/DNA substrates.

When RecQ helicase activity was tested on DNA substrates prebound to the SSB variants, we observed that higher concentrations of RecQ protein were required to reach half-maximal unwinding when SSB-mixed or SSBΔC1 was included than was necessary for wild-type SSB-bound DNA substrates, suggesting that the SSB variants modestly inhibit helicase activity (Fig. 4c and Table 2). Taken together, these results suggest that the RecQ/SSB-Ct interaction is dependent upon an intact SSB-Ct element. It is interesting to note, however, that none

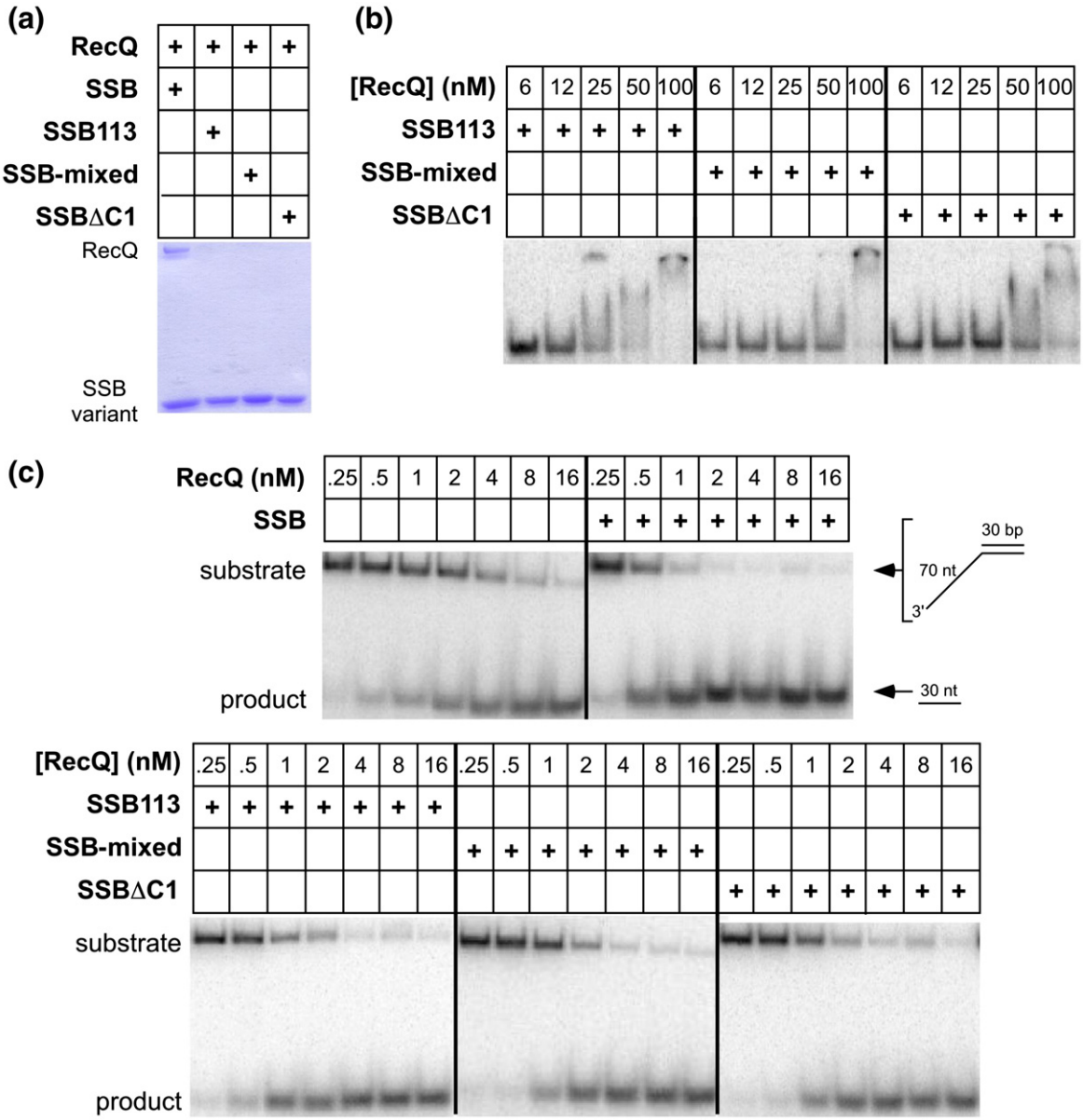

Fig. 4. Effects of SSB-Ct sequence changes on RecQ binding. (a) Ammonium sulfate coprecipitation pellets of RecQ and SSB variant mixtures were resolved by PAGE. (b) Electrophoretic mobility shift analysis of RecQ binding to partial-duplex DNA prebound by SSB protein variants. (c) DNA unwinding by wild-type RecQ in the absence and in the presence of SSB and SSB protein variants.

of the SSB variants tested entirely blocked RecQ DNA binding and unwinding in the way that complete deletion of the SSB-Ct element was observed earlier.[19] This could indicate that multiple elements of the SSB-Ct are important for associating with RecQ, and subtle alterations of the SSB-Ct do not eliminate all of the contact points used to form the RecQ/SSB complex.

Table 2. DNA helicase activity of RecQ with SSB variants

SSB variant	[RecQ] for half-maximal unwinding (nM)	[RecQ] for half-maximal unwinding with SSB (nM)	Fold stimulation
SSB	2.9±0.1	0.3±0.1	9.7
SSB113	2.9±0.1	3.2±0.5	0.9
SSB-mixed	2.9±0.1	4.0±0.7	0.7
SSBΔC1	2.9±0.1	4.7±0.8	0.6

Discussion

More than a dozen proteins involved in DNA replication, repair, recombination, and replication restart in *E. coli* have been found to interact directly with SSB, establishing it as an important scaffold for a large number of genome maintenance processes.[31] In all cases examined to date, interactions with SSB depend on the SSB-Ct element, an evolutionarily conserved amphipathic sequence of acidic and

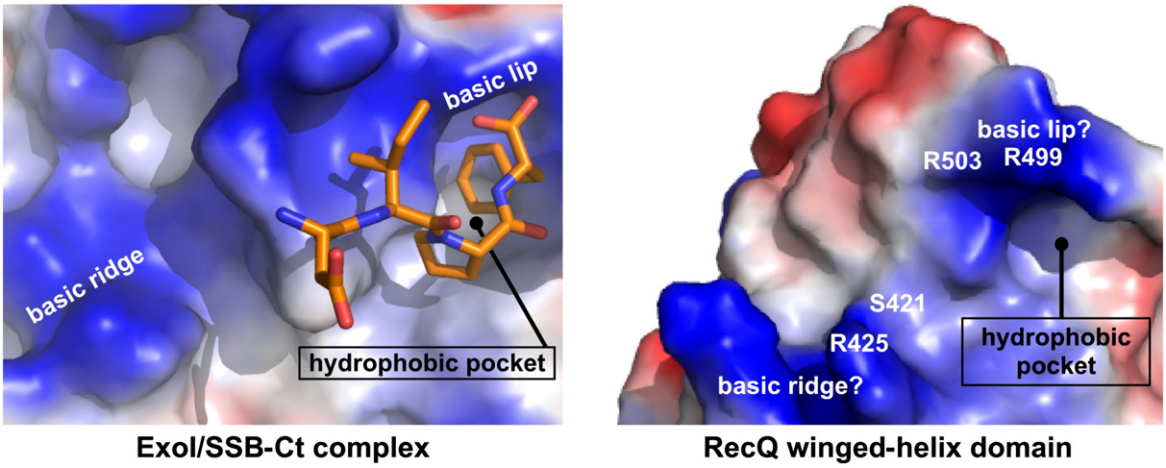

Fig. 5. Comparison of the SSB-Ct binding site from exonuclease I (left)[32] and RecQ (right). Surface representations of each molecule are shown by electrostatic features (red, blue, and white for negative, positive, and neutral, respectively). The SSB-Ct peptide is shown in orange on the exonuclease I structure. The basic ridge, the arginine at the lip of the binding pocket, and the hydrophobic pocket are highlighted in both images.

hydrophobic amino acids found at the C terminus of SSB.[31–37,53] Studies that offer structural and mechanistic insights into how the SSB-Ct element binds to heterologous proteins and into the biochemical consequences of these associations are important for understanding the structural architecture that underlies several bacterial genome maintenance processes.

Stimulation of DNA helicase activity by SSB was noted early in the characterization of RecQ.[39] This and subsequent studies concluded that this stimulation resulted from SSB binding to and sequestering liberated ssDNA following RecQ helicase activity, or from SSB preventing the formation of nonproductive ssDNA/RecQ complexes, but more recent investigations have shown that direct contact between the two proteins also plays an important role.[19,39,40] Here, we have combined structural and biochemical approaches to identify the SSB-Ct binding site on RecQ. Altering residues that comprise this site in the RecQ-WH domain results in greatly impaired binding to SSB and leads to more modest changes in SSB/DNA binding. This difference could be due to local concentration effects arising from SSB and RecQ binding to a common DNA substrate in the SSB/DNA experiments. Because it is involved in several distinct activities, alterations of the RecQ-WH domain affect biochemical functions of the RecQ variant proteins beyond just impairment of SSB binding. We have also shown that SSB variants with subtle changes in the SSB-Ct element generally inhibit RecQ substrate recognition and RecQ unwinding of SSB/ssDNA complexes.

Conservation of an SSB interaction site among genome maintenance enzymes

Our identification of the SSB-Ct binding site on RecQ allowed us to compare the RecQ site to that of exonuclease I, which is the only other SSB-associated protein for which structural information on the complex is available.[32] The exonuclease I SSB-Ct binding site is defined by hydrophobic and basic chemical features that function to accommodate several determinants from the SSB-Ct peptide (Fig. 5, left). These include a hydrophobic pocket on exonuclease I that engulfs the side chain of the C-terminal phenylalanine of the SSB-Ct. Binding to the phenylalanine is further promoted by an arginine residue from exonuclease I (the "basic lip" of the hydrophobic pocket), which complements the phenylalanine's α-carboxyl oxygen atoms via ionic interactions. Additional basic residues from exonuclease I form a "basic ridge" that is thought to be important for binding the more N-terminal acidic residues of the SSB-Ct. Comparison of the SSB-Ct binding sites from exonuclease I and RecQ reveals a number of structural features shared between the two sites, including a hydrophobic pocket lined by two potential "basic lip" residues (R499 and R503) and an adjoining "basic ridge" on RecQ (Fig. 5). In all cases, alanine substitutions of residues in this region produce proteins with weakened SSB and SSB/DNA binding (Figs. 2 and 3), consistent with this being the *bona fide* SSB-Ct binding site in RecQ. Remarkably, these common surface features in exonuclease I and RecQ arise from unrelated protein folds, which could indicate that the two enzymes have evolved similar SSB-Ct binding sites independently. Because many of the other known SSB-associated proteins also lack structural homology with exonuclease I and RecQ, these proteins appear to have independently evolved SSB binding sites as well. Future studies will be required to determine whether the features common to exonuclease I and RecQ SSB-Ct binding sites are conserved among other SSB partner proteins. The similar SSB-Ct binding site structures in exonuclease I and RecQ provide an electrostatic template that could prove useful for identifying SSB-Ct binding sites in these proteins.

The RecQ-WH domain as a node that integrates DNA and SSB protein binding

Winged-helix domains have been identified as common DNA-binding elements in many different proteins.[49,54–59] In addition, a growing number of winged-helix domains with protein interaction roles have also been noted.[49,60–62] Based upon its structural similarity to the winged-helix domain of several dsDNA-binding proteins, a DNA binding role for the RecQ-WH domain has been predicted in addition to its role in SSB binding.[19,30] Several studies indicate that the winged-helix domains from *E. coli* RecQ and its eukaryotic homologs directly bind to DNA,[47–50] and recent work has shown that the winged-helix domain of human RECQ1 plays an integral role in the DNA unwinding mechanism of the protein.[63] Furthermore, experiments with the WRN winged-helix domain[49] and preliminary NMR experiments from our group using the RecQ-WH domain (data not shown) indicate that their respective winged-helix domains bind DNA using sites that are distinct from the SSB-Ct binding site described herein.

Identification of the SSB-Ct binding site in this study shows that SSB binds at a position in the RecQ-WH domain that is on the opposite face relative to the predicted dsDNA-binding surface, which highlights a potential integrative role for the domain. Related to this centrality, nearly every RecQ-WH variant tested exhibited modest DNA binding defects even in the absence of SSB (Fig. 3). Likewise, single amino acid changes that are predicted to affect only SSB binding also alter RecQ helicase activity even in the absence of SSB (Table 1). These defects are distinct from the more dramatic defects in complex formation with SSB (Fig. 2), but nonetheless could reflect larger roles for the RecQ-WH domain in RecQ activity. Given the compact size of the RecQ-WH domain, these observations could relate to changes on other surfaces on the domain from the amino acid substitutions or could reflect possible secondary roles for the SSB protein binding site in RecQ activity, such as DNA binding during helicase functions. Such interrelated effects could point to an integrative role for the RecQ-WH domain in coordinating joint DNA and SSB protein binding, as has been hypothesized for the WRN winged-helix domain.[49] Interrelated binding could reflect an evolved coordination that is important for targeting the activity of RecQ within the cell.

Materials and Methods

Proteins, synthetic peptides, and DNA substrates

Full-length E. coli *RecQ and variant proteins*

Hexahistidine-tagged *E. coli* RecQ and single- and double-site variant proteins were expressed, purified, and quantitated as described previously.[45] Alanine and glutamic acid variants were generated by the QuikChange site-directed mutagenesis scheme (Stratagene). All plasmids were sequenced at the University of Wisconsin Biotechnology Center.

RecQ-WH domain

A T7 overexpression plasmid encoding the hexahistidine-tagged RecQ-WH domain (pET28-RecQ-WH; residues 408–523 of *E. coli* RecQ) has been described previously.[19] *E. coli* BL21(DE3) cells transformed with pET28-RecQ-WH were cultured at 30 °C in M9 minimal media containing 150 μg/ml ampicillin, $^{15}NH_4Cl$, and, in some cases, [^{13}C]glucose, to late-log phase ($OD_{600} \sim 1.0$). Protein expression was induced for 10 h by the addition of isopropyl β-D-thiogalactopyranoside to 1 mM, and the domain was purified as previously described.[19] Purified RecQ-WH was dialyzed against 20 mM Hepes (pH 6.5), 0.1 M KCl, and concentrated to 0.3–0.8 mM. All NMR samples contained 5–10% D_2O.

SSBs

T7 overexpression plasmids for *E. coli* SSB, SSB113, and SSB-mixed were described previously.[32] An overexpression plasmid encoding SSBΔC1 (*E. coli* SSB protein lacking its C-terminal-most residue) was made using the QuikChange site-directed mutagenesis method (Stratagene) and sequenced at the University of Wisconsin Biotechnology Center. *E. coli* SSB and SSB variant proteins were purified as described,[64] with the following modifications: following ammonium sulfate fractionation, SSB was dialyzed into low-salt buffer [20 mM Tris (pH 8.0), 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10% vol/vol glycerol] and purified chromatographically over Heparin Fastflow and Sephacryl S-100 size exclusion columns (GE Healthcare). Pure fractions were pooled, concentrated, dialyzed into storage buffer [20 mM Tris (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 50% vol/vol glycerol], and stored at −20 °C.

SSB-Ct peptide

A peptide comprising the *E. coli* SSB C-terminal sequence with an additional N-terminal Trp residue (SSB-Ct; Trp-Met-Asp-Phe-Asp-Asp-Asp-Ile-Pro-Phe) was synthesized and purified by the University of Wisconsin Peptide Synthesis Facility as described previously.[19]

Nucleic acid substrates

Oligonucleotides o30 (5′-CTAATGACGGTCCTAGAC-GAACCGAGCGTC-3′) and o100 (5′-GACGCTCGGTTC-GTCTAGGACCGTCATTAGTATGTTGATATACATA-GACCTTACCGCAGTGATTCGCTTGTCAGTCCATT-GAAGCACAATTACCCACGC-3′) were synthesized and purified by Integrated DNA Technologies and have been described previously.[19] o30 was phosphorylated by T4 polynucleotide kinase (New England Biolabs) with [γ-^{32}P] ATP (GE Healthcare) and annealed to unlabeled o100 to form a partial-duplex DNA (3′-OH) consisting of a 30-bp duplex with a 70-base 3′-overhang. The substrate was resolved by native polyacrylamide gel electrophoresis (PAGE), electroeluted, and dialyzed against 20 mM Tris (pH 8.0) and 50 mM NaCl.

Determination of NMR resonance assignments

Backbone ^{1}H, ^{13}C, and ^{15}N resonance assignments were carried out at 25 °C using Varian Inova 800- and 600-MHz spectrometers equipped with cryogenic probes. The ^{13}C and

^{15}N chemical shifts were referenced indirectly to 2,2-dimethyl-2-silapentane-5-sulfonic acid, as described previously.[65] Sequence-specific backbone assignments were achieved using two-dimensional ^{1}H–^{15}N HSQC, 3D HNCO, 3D HNCA, 3D HNCACB, 3D CBCA(CO)NH, and 3D HBHA(CO)NH experiments, as previously described.[66,67] In addition, 3D ^{1}H–^{15}N NOESY-HSQC and ^{1}H–^{13}C NOESY-HSQC (mixing time, 60 ms) spectra were acquired to confirm backbone resonance assignments. All spectra were processed and initially analyzed with NMRPipe[68] and Sparky† software packages, respectively. The automated assignment program PINE‡[69] was used to initially assign the backbone resonances. Near-complete (~90%) backbone resonance assignments for the RecQ-WH domain were obtained, and HN shifts were labeled on the ^{1}H–^{15}N HSQC spectrum (Supplementary Fig. 1). Eight-microlomar SSB-Ct peptide aliquots in 10 mM Hepes (pH 6.5) were titrated into the RecQ-WH domain NMR sample at room temperature and incubated for 15 min at each titration stage. Spectra were collected at 1:0, 1:0.1, 1:0.2, 1:0.5, 1:0.8, 1:1, 1:1.2, 1:1.5, 1:2, 1:2.5, and 1:5 RecQ-WH domain:SSB-Ct peptide molar ratios. Three-dimensional ^{1}H–^{15}N NOESY-HSQC spectra were acquired and analyzed at 1:1 and 5:1 RecQ-WH domain:SSB-Ct peptide molar ratios. Normalized changes in chemical shift between the 1:0 and 1:1 spectra were calculated from the equation: $\Delta ppm = [(\Delta^{1}H\ ppm)^2 + (\Delta^{15}N\ ppm\ X\ \alpha N)^2]^{0.5}$, where Δppm is the difference (in ppm) between the chemical shifts of the RecQ-WH domain and the RecQ-WH domain:SSB-Ct complex, and 0.1 serves as the scaling factor (based on the approximately 10-fold lower gyromagnetic ratio of ^{15}N relative to ^{1}H) of the nitrogen chemical shift changes (αN).[70]

DNA-dependent ATPase assays

ATP hydrolysis was measured by a standard assay as previously described.[71] RecQ variants (5 nM) were added to reactions containing dT_{35} (1 nM nucleotides) and an ATP regeneration system that allows for spectrophotometric observation of ATP hydrolysis by coupling this process to the oxidation of NADH.[72] Steady-state rates of ATP hydrolysis were calculated and normalized to the concentration of RecQ in order to determine the rate of ATP hydrolyzed per RecQ molecule per minute. Rates are reported as the mean of three measurements, with 1 SD as error.

Ammonium sulfate coprecipitation

Coprecipitation experiments were performed as described previously,[19,38] except that pellet fractions were suspended in 30 μl of loading buffer prior to SDS-PAGE on 4–15% polyacrylamide gradient gels (Bio-Rad). Assays were performed in triplicate, and relative binding was quantified by setting the intensity of the wild-type RecQ band to 100% using ImageQuant software (GE Healthcare). Values are reported as the mean of three measurements, with 1 SD as error.

Electrophoretic mobility shift assays

3′-OH DNA substrate radiolabeled with ^{32}P was diluted to 1 nM, added to reaction buffer [final concentrations: 20 mM Tris (pH 8.0), 50 mM NaCl, 1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 g/L bovine serum albumin, and 5.5% vol/vol glycerol], and incubated with 20 nM SSB tetramers or reaction buffer at room temperature for 5 min. RecQ (or a RecQ variant protein) was added to a final concentration of 0–1000 nM, incubated for 5 min at room temperature, and resolved by 6% native PAGE. Gels were run at 90 V for 3 h at 4 °C, dried, and imaged using a Molecular Dynamics Storm 820 PhosphorImager. Assays were conducted multiple times, with representative images shown. Qualitative assessments of the relative strengths of RecQ DNA and SSB/DNA binding are given in Table 1.

Helicase assays

Twenty nanomolar of SSB (or SSB variant) and 1 mM ATP were added to 1 nM 3′-OH DNA substrate in the reaction buffer described above. Reactions were started by the addition of RecQ protein (0–16 nM, as indicated) and incubated at room temperature for 20 min. Reactions were stopped by addition of stop solution (10 μg of proteinase K, 0.25% SDS, 30 mM EDTA, and 8.6 nM unlabeled o30) and incubated at 37 °C for 30 min. Reaction products were resolved by 10% native PAGE (140 V for 1.5 h at 4 °C), dried, and imaged using a Molecular Dynamics Storm 820 PhosphorImager. Reactions were performed in triplicate and quantified using ImageQuant 5.1 software. Half-maximal unwinding values were determined using CurveExpert software. Values are reported as the mean of three measurements, with 1 SD deviation as error.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.12.065

† http://www.cgl.ucsf.edu/home/sparky
‡ miranda.nmrfam.wisc.edu/PINE/

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