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Peptide inhibitors identify roles for SSB C-terminal residues in SSB/Exonuclease I complex formation†

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Abstract

Bacterial single-stranded (ss) DNA-binding proteins (SSBs) facilitate DNA replication, recombination, and repair processes in part by recruiting diverse genome maintenance enzymes to ssDNA. This function utilizes the C-terminus of SSB (SSB-Ct) as a common binding site for SSB's protein partners. The SSB-Ct is a highly conserved, amphipathic sequence, comprising acidic and hydrophobic elements. A crystal structure of *E. coli* Exonuclease I (ExoI) bound to a peptide comprising the *E. coli* SSB-Ct sequence shows that the C-terminal-most SSB-Ct Phe anchors the peptide to a binding pocket on ExoI and implicates electrostatic binding roles for the acidic SSB-Ct residues. Here, we use SSB-Ct peptide variants in competition experiments to examine the roles of individual SSB-Ct residues in binding ExoI in solution. Altering the C-terminal-most Pro or Phe residues in the SSB-Ct strongly impairs SSB-Ct binding to ExoI, confirming a major role for the hydrophobic SSB-Ct residues in binding ExoI. Alteration of N-terminal SSB-Ct residues leads to changes that reflect cumulative electrostatic binding roles for the Asp residues in SSB-Ct. The SSB-Ct peptides also abrogate SSB stimulation of ExoI activity through a competitive inhibition mechanism, indicating that the peptides can disrupt ExoI/SSB/ssDNA ternary complexes. Differences in the potency of the SSB-Ct peptide variants in the binding and nuclease inhibition studies indicate that the acidic SSB-Ct residues play a more prominent role in the context of the ternary complex than in the minimal ExoI/SSB-Ct interaction. Together, these data identify roles for residues in the SSB-Ct that are important for SSB complex formation with its protein partners.

Unwinding genomic DNA to form single-stranded (ss) DNA is an obligatory step in many DNA replication, recombination, and repair pathways. However, because ssDNA is sensitive to chemical and nucleolytic attack and can self-associate to form structures that impede genome maintenance, DNA unwinding also presents a potential threat to genomic integrity. To help mediate this risk, cells have evolved ssDNA-binding proteins (SSBs) that bind and protect ssDNA from biochemical attacks and maintain its single stranded structure (1-3). SSB/ssDNA nucleoprotein complexes thus form a common substrate utilized in genome maintenance reactions. Defining how DNA replication, recombination, and repair enzymes recognize and process SSB/ssDNA structures is key to understanding cellular genome maintenance mechanisms.

Bacterial SSBs function as homooligomers (most often tetramers) in which each monomer contributes a ssDNA-binding/oligomerization domain and a structurally dynamic C-terminal tail (1-3). The nine C-terminal residues of SSB (SSB-Ct, Met-Asp-Phe-Asp-Asp-Asp-Ile-Pro-

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Phe in *E. coli*) form an amphipathic peptide that serves as the binding site for several genome maintenance proteins (3,4). Among 280 bacterial species, the C-terminal-most Pro and Phe SSB-Ct residues are invariant in 98% and 100% of the SSB sequences, respectively. In addition, the SSB-Ct acidic tripeptide is conserved in ~75% of SSB sequences. In accordance with its evolutionary conservation and roles in protein interactions, mutations that alter the SSB-Ct sequence have drastic effects on SSB function. One such mutation in *E. coli* (*ssb113*) alters the SSB-Ct Pro to Ser, leading to temperature-sensitive lethality caused by failure of the SSB113 protein to interact with the cellular replication machinery (5-9). The *ssb113* mutation also leads to hypersensitivity to DNA damage in permissive conditions (10-14), which could reflect a general failure of the SSB variant to interact properly with other genome maintenance proteins in vivo. Weakened interactions of the SSB113 protein with heterologous proteins have been observed previously for Exonuclease I (ExoI) (4,15), the chi subunit of DNA polymerase III (8,9,16), PriA (17), RecQ (18,19), Topoisomerase III (20), RecG (21), and DNA polymerase V (22) in vitro. Additional experiments indicate that altering the C-terminal Phe residue from *E. coli* SSB to Cys disrupts heterologous protein interaction and is lethal to *E. coli* (15). Although these studies show that the SSB-Ct is important for SSB's interactions with other proteins, the roles of individual SSB-Ct residues in heterologous protein binding have not been well defined.

Several observations indicate that *E. coli* ExoI provides an excellent model for studying interactions between SSB and its protein partners. ExoI is a DnaQ-family 3' ssDNA exonuclease (23-26). Its enzymatic activity is important in several genome maintenance pathways, including mismatch repair (27-29), frameshift mutation suppression (30,31), and removal of abasic sites (32). In contrast to most nucleases, ExoI enzymatic activity is stimulated by SSB in a manner that depends on interaction with the SSB-Ct element of SSB (4,15,33, 34). A recent crystal structure of *E. coli* ExoI bound to a peptide comprising the SSB-Ct sequence has shown that the side chain of the C-terminal-most SSB-Ct Phe docks into a hydrophobic pocket on the surface of ExoI and that an Arg side chain from ExoI forms a critical interaction with the α-carboxyl group of the SSB-Ct Phe (4) (Figure 1). Additional electrostatic binding roles for the SSB-Ct acidic tripeptide element docking on a "basic ridge" element of ExoI were also proposed, although the crystal structure did not provide direct evidence of this interface. Mutational studies based on the ExoI/SSB-Ct structure confirmed the importance of elements in ExoI for binding the SSB-Ct, but did not address the roles of individual SSB-Ct residues in binding to ExoI (4). Because these sequence elements are likely to be important for SSB binding to most, if not all, of its protein partners, a systematic study that delineates the roles of each residue of the SSB-Ct is critical for understanding SSB's activities in nucleating cellular genome maintenance reactions.

In this paper, two competition assays are established in which SSB-Ct peptide variants are used to define the importance of each residue of the sequence for binding *E. coli* ExoI. In the first, the abilities of the peptide variants to compete with a fluorescein-labeled SSB-Ct peptide for binding ExoI are assessed. These experiments indicate a strong dependence on the presence of the C-terminal Pro-Phe dipeptide in the SSB-Ct for binding ExoI. Additionally, they indicate that the net negative charge of the N-terminal Asp residues is critical for binding to ExoI but that no single Asp residue plays a dominant individual role. In the second assay, the abilities of the SSB-Ct variant peptides to disrupt ExoI interactions with full-length SSB are tested in exonuclease assays. Interestingly, SSB-Ct variant peptides with intact Asp residues but altered hydrophobic elements are more potent inhibitors in the nuclease assays than in the minimal peptide competition binding assays. These differences may reflect more prominent roles for the acidic residues in the context of the ExoI/SSB/ssDNA ternary complex than in the ExoI/ SSB-Ct peptide interaction. Taken together, these studies highlight the importance of both the acidic and hydrophobic portions of the SSB-Ct in mediating SSB/protein interactions.

Experimental Procedures

Peptides and Proteins

SSB-Ct (Trp-Met-Asp-Phe-Asp-Asp-Asp-Ile-Pro-Phe), F-SSB-Ct (SSB-Ct with an N-terminal fluorescein), and variant peptides in which the C-terminal Pro or Phe were altered to Ser or Tyr (respectively) were synthesized and purified by the University of Wisconsin Biotechnology Center. The N-terminal reporter Trp residue, which is not part of the natural *E. coli* SSB-Ct sequence, was added to each peptide for quantitation. All other peptides were synthesized on solid phase using a Symphony automated synthesizer (Protein Technologies, Inc.). Coupling cycles were completed with a standard coupling time of 30 minutes using Obenzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (4 equivalents) in Dimethylformamide (DMF) solvent. Four equivalents of Fmoc amino acid were used for each coupling cycle. Deprotection steps used 20% piperidine in DMF for 20 minutes. C-terminal acid peptides were prepared on phenylalanine-loaded Wang resin. Peptides were cleaved and/or deprotected by shaking with trifluoroacetic acid:H_2O:triisopropylsilane:ethanedithiol:thioanisole, 80:5:5:5:5,v/v/v/v/v (1.25 mL/25 mmol) for 4 hours, followed by precipitating into cold diethyl ether. The precipitate was collected by centrifugation/decantation prior to purification. All peptides were purified by reverse-phase HPLC and characterized by analytical HPLC and matrix assisted laser desorption ionization mass spectrometry for purity and identity, respectively. Pure peptides were lyophilized to yield a white powder. All peptides were stored in 100% dimethly sulfoxide (DMSO) and freshly diluted into appropriate buffers. Peptide concentration was determined spectrophotometrically (ε_{275} = 1420 M^{-1} cm^{-1} in water). In all cases, the peptides were soluble in the conditions tested (data not shown). *E. coli* ExoI and SSB were purified as described previously (4).

Peptide competition-binding assay

E. coli ExoI (1 μM) was incubated with 10 nM F-SSB-Ct peptide and 0-200 μM unlabeled SSB-Ct (or a variant) peptide for 5 minutes at room temperature in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 g/L bovine serum albumin, 4% (vol/vol) glycerol, 1% (vol/vol) DMSO. The apparent dissociation constant for the ExoI/F-SSB-Ct complex is 261 +/- 22 nM under these conditions (data not shown). Fluorescence polarization (FP) was measured at 25°C using a Panvera Beacon 2000 FP system with 490 nm excitation and 535 nm emission wavelengths for three replicates; the average FP value was plotted with one standard deviation of the mean shown as error. Fluorescence intensity did not vary by greater than 15% across the range of competitor concentrations tested, indicating that quantum yield differences between free and ExoI-bound F-SSB-Ct and did not significantly influence the FP measurements. IC_{50} values are the concentrations of unlabeled peptides necessary for 50% inhibition of F-SSB-Ct binding. Data were fitted with a single binding-site model using the GraphPad Prism software package; lower baseline values were fixed at 43 mP to allow IC_{50} determination in cases where 100% dissociation was not observed with the peptide concentrations tested. Apparent inhibitor dissociation constant values from the competitive binding assay (K_{bind}) were calculated using the following equation: $K_{bind} = [I]_{50}/([L]_{50}/K_d + [P]_o/K_d + 1)$, where $[I]_{50}$ is the concentration of free inhibitor at 50% inhibition, $[L]_{50}$ is the concentration of free F-SSB-Ct at 50% inhibition, K_d is the apparent dissociation constant of the ExoI/F-SSB-Ct complex, and $[P]_o$ is the concentration of free ExoI at 0% inhibition (35). It should be noted that K_{bind} values are not true K_i measurements because ExoI is not saturated with F-SSB-Ct in these experiments.

Exonuclease assay

Amplification and purification of a radiolabeled 1,369-base ssDNA substrate was carried out as described previously (4). For specific activity measurements, substrate (1.1 μM

(nucleotides)) was mixed with 0 or 200 nM *E. coli* SSB and 0-100 μM SSB-Ct peptide (or a variant) in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 3 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 g/L bovine serum albumin, 10% (vol/vol) glycerol, 5% (vol/vol) DMSO and incubated for 10 minutes at 37 °C. The apparent dissociation constant for the ExoI/F-SSB-Ct complex is 178 +/- 10 nM under these conditions (data not shown). 200 nM SSB saturates the ssDNA substrate, which is required for maximal stimulation of ExoI activity (4). For Michaelis-Menten kinetic measurements, DNA substrate concentrations were varied from 0.5 to 160 μM (nucleotides) and the SSB concentration was altered to maintain a constant ratio of 200 nM SSB/1.1 μM (nucleotides) DNA. Reactions were initiated by the addition of *E. coli* ExoI at a final concentration of 30-100 pM and incubated at 37 °C. At several time intervals, reaction aliquots were quenched by adding EDTA to 0.05 M and the remaining substrate was precipitated by adding Torula RNA (1 g/L, final concentration) and perchloric acid (0.25 M, final concentration) followed by incubation on ice. The precipitated substrate was pelleted and 80% of the supernatant was removed for liquid scintillation counting to measure the acid-soluble cpm. Reaction velocities were determined by measuring the time-dependent generation of acid-soluble cpm using 3-4 linear time points. One unit of exonuclease activity is the amount of enzyme required to generate 1 μmol of acid-soluble products per min at 37°C; specific enzyme activity is the units per mg of protein. Specific activity data are shown as the mean of three experiments. Error bars are omitted for clarity except for full-length SSB-Ct peptide titrations, which are representative of typical experimental error. The concentrations of SSB-Ct variant peptides required to reduce SSB-stimulated ExoI specific activity to the midpoint between maximal and SSB-free levels (50% inhibition) is estimated from trend lines in the nuclease figures. For Michaelis-Menten kinetic experiments, SSB-Ct peptide was omitted or added at 2 μM or 8 μM and data were globally fit using GraphPad Prism.

Results

Competition-binding studies reveal important features of the SSB-Ct peptide

Previous studies have indicated that the SSB-Ct element is necessary and sufficient for interaction with ExoI (4,15) and numerous other bacterial genome maintenance enzymes (3). However, systematic experiments that account for the roles of each SSB-Ct residue in protein interactions have not been carried out. To better understand the roles of individual SSB-Ct residues in SSB protein interactions, the *E. coli* SSB-Ct peptide and a series of 16 SSB-Ct variant peptides with altered sequences were produced and used in competition-binding experiments.

Wild-type SSB-Ct peptide competition assay—An initial experiment testing the ability of the unlabeled *E. coli* SSB-Ct peptide to compete with a fluorescein-labeled SSB-Ct peptide (F-SSB-Ct) for binding to ExoI was used to establish a minimal competition-binding assay (Figure 2A). Complexes formed between ExoI and F-SSB-Ct were challenged with 0.03 to 200 μM SSB-Ct peptide. Consistent with competitive binding of the two peptides, the FP signal of F-SSB-Ct was reduced in an SSB-Ct peptide-dependent manner (Figure 2B). The inhibition constant (IC_{50}) for SSB-Ct was 5.2 +/- 0.2 μM (Table 1). As described in *Experimental Procedures*, this IC_{50} translated to an apparent inhibition constant (K_{bind}) of 0.9 +/- 0.1 μM. We note that this value is not a true K_i for the SSB-Ct peptide because the experiment could not be performed with saturating F-SSB-Ct ligand concentrations due to constraints in the binding assay. Nonetheless, the K_{bind} value for the SSB-Ct peptide is reasonably close to the K_d for the F-SSB-Ct/ExoI interaction under identical conditions (0.3 μM, data not shown). These data confirm the competition-binding assay's utility for investigating ExoI binding by the SSB-Ct and other binding inhibitors.

N-terminal SSB-Ct peptide deletions—SSB-Ct peptide variants with one to six N-terminal residues deleted were used in the competition-binding assay to test the importance of each N-terminal residue in ExoI binding. Deleting the N-terminal-most Met from the SSB-Ct sequence had a negligible effect on apparent binding to ExoI, however each of the subsequently shorter SSB-Ct variant peptides competed for binding ExoI more poorly than the wild type SSB-Ct peptide (Figure 2B and Table 1). The effect of deleting each residue was cumulative, with ~2-3 fold weaker competition observed with each additional residue removed from the N-terminus. Notably, removal of all Asp residues from the peptide led to ~50-fold weaker apparent binding to ExoI relative to the wild type SSB-Ct peptide.

Acidic SSB-Ct residue substitutions—The N-terminal SSB-Ct peptide deletion data presented above indicated a possible role for the Asp residues of the SSB-Ct in ExoI binding. However, because the data were obtained from deletion peptides, it was also possible that the weakened apparent binding of the peptides was due to their reduced length rather than to changes in the number of Asp residues in the peptides. To test the importance of the Asp residues in the SSB-Ct peptide more directly, peptide variants in which each Asp residue was replaced with Ala individually or *en masse* were examined in the competition-binding assay. Each of the single Asp-to-Ala variant peptides competed for ExoI binding with only modestly reduced apparent binding affinity (Figure 2C and Table 1). However, the SSB-Ct variant peptide with all four Asp residues altered to Ala competed ~35-fold more poorly than the wild type SSB-Ct peptide in the binding assay. This ~35-fold difference was similar to the ~50-fold weaker competition observed for the N-terminal deletion variant where all of the Asp residues were removed, indicating that the charge of the peptide rather than its length is a major determinant of its competition efficiency in the peptide binding assay. Moreover, these data indicate that, whereas no single Asp residue is critical for interaction with ExoI, the net charge provided by the side chains plays a major role in ExoI binding.

C-terminal SSB-Ct peptide deletions and substitutions—SSB-Ct variant peptides with one to three C-terminal residues deleted were tested next in the competition-binding assay. Strikingly, removal of even one C-terminal residue led to a complete loss of competition in the assay, indicating a greatly diminished apparent binding (Figure 2D and Table 1). IC_{50} values could not be measured for any of the C-terminally deleted SSB-Ct peptide variants because little or no competition was observed, even at the highest competitor concentrations measured (200 μM). These observations confirm a prominent role for the hydrophobic element in the SSB-Ct in ExoI binding in this assay.

To better define the importance of the C-terminal residues of the SSB-Ct in ExoI binding, two additional SSB-Ct variant peptides were tested in the competition-binding assay. The first substitutes a Ser for the penultimate Pro of the SSB-Ct. This variant mimics the well-studied *E. coli ssb113* mutation, which causes temperature-dependent lethality and DNA damage hypersensitivity in permissive conditions (5-14). The second peptide substitutes a Tyr for the C-terminal Phe. This conservative substitution tests whether addition of a hydrophilic hydroxyl group to the C-terminal-most side chain alters binding to ExoI. In both cases, the variant SSB-Ct peptides competed more efficiently than any of the C-terminal deletion variants but significantly weaker than the wild-type SSB-Ct peptide (Figure 2D and Table 1). The Pro-to-Ser and Phe-to-Tyr SSB-Ct variants compete ~10-fold and ~75-fold more poorly than the wild type SSB-Ct peptide, respectively. Consistent with the structural information on the ExoI/SSB-Ct complex (4) and the deletion peptide data above, these findings indicate that the hydrophobic C-terminal residues of the SSB-Ct play critical roles in ExoI binding.

Peptide inhibition of SSB-stimulated ExoI activity

ExoI nuclease activity is stimulated by SSB in a reaction that requires direct interaction between SSB and ExoI (4,15,33). Inhibitors that can block SSB/ExoI complex formation, such as the SSB-Ct variant peptides described above, are therefore predicted to abrogate SSB stimulation of ExoI activity (Figure 3A). Because the SSB-Ct binding site is distinct from the active site and predicted substrate-binding channel of ExoI (Figure 1) (4,36), such inhibitors would be expected to have no effect on ExoI activity in the absence of SSB. The SSB-Ct peptide variants were tested in SSB-stimulated ExoI assays to determine whether they conform to this model of inhibition. Because this assay tests each peptide's ability to inhibit the interaction of ExoI with SSB/ssDNA complexes, these studies also provide insights into the importance of each residue in the SSB-Ct peptide for ExoI binding in a more physiologically relevant condition.

Wild-type SSB-Ct peptide inhibition assay—An initial experiment with the wild-type SSB-Ct peptide was used to establish whether the peptide could specifically block SSB-stimulated ExoI nuclease activity in a reconstituted ExoI/SSB nuclease assay without altering activity in the absence of SSB. As predicted, titration of the SSB-Ct peptide into SSB-stimulated ExoI assays reduced nuclease activity to SSB-free levels (Figure 3B). SSB-Ct peptide inhibition was dose-dependent, requiring ~1 μM peptide to achieve 50% inhibition and ~10 μM to reduce ExoI activity to SSB-free levels (Figure 3B and Table 1). Notably, the ~1 μM peptide concentration required for 50% inhibition closely matched the value that would be predicted from the K_{bind} measurement (0.9 +/- 0.1 μM). The addition of up to 100 μM SSB-Ct did not inhibit ExoI activity to levels below that of ExoI with free ssDNA and addition of the peptide to SSB-free reactions had no measurable effect on ExoI nuclease activity (Figure 3B), consistent with the inhibition model posited above. Tests of each of the other SSB-Ct peptide variants similarly showed no effects on ExoI activity in the absence of SSB (data not shown).

Kinetic studies were undertaken to determine the mechanism by which the SSB-Ct peptide abrogates SSB-stimulated ExoI activity. If the peptide blocks ExoI binding to SSB in the SSB/ssDNA substrate as described above, then we anticipated that it would inhibit activity by a competitive mechanism. ExoI hydrolysis was measured as a function of substrate concentration and the resulting data were fitted to a Michaelis-Menten kinetic scheme (Figure 3C). In the absence of SSB-Ct peptide, ExoI hydrolyzed ssDNA in SSB/ssDNA complexes with a k_{cat} of 6930 +/- 260 min^{-1} and a K_m of 16.0 +/- 2.1 μM (nucleotides). These values were similar to previous estimates of k_{cat} (10,400 and 16,500 min^{-1}) and K_m (11 μM (nucleotides)) for *E. coli* ExoI (37, 38). Interestingly, the addition of 2 or 8 μM SSB-Ct peptide led to increases in apparent K_m values with no measurable effects on apparent k_{cat}, consistent with the SSB-Ct peptide acting as a competitive inhibitor (Figure 3C). Statistical comparisons of fits of the data to competitive, non-competitive, and mixed inhibition models confirmed that the SSB-Ct peptide inhibits by a competitive mechanism. A global fit of the kinetic data determined an apparent inhibitor dissociation constant ($K_{i, enz}$) of 2.9 +/- 0.5 μM for the SSB-Ct peptide. This $K_{i, enz}$ value is similar to both the K_{bind} measured in the peptide competition studies (0.9 +/- 0.1 μM) and the ~1 μM SSB-Ct peptide concentration that was required to reduce ExoI activity 50% on SSB/ssDNA substrates. These data support the model posited above in which the SSB-Ct peptide acts to block ExoI binding to SSB/ssDNA substrates.

N-terminal SSB-Ct peptide deletions—Inhibition of SSB-stimulated ExoI activity by N-terminal deletion variants of the SSB-Ct peptide was tested to assess the effects of altering individual residues of the SSB-Ct element. As was the case for the competition-binding assay, deletion of the N-terminal Met from the SSB-Ct sequence had no effect on inhibition of SSB-stimulated ExoI nuclease activity, however each of the subsequently shorter SSB-Ct variant peptides required higher concentrations of peptide to inhibit ExoI nuclease activity than the

wild type SSB-Ct peptide (Figure 3D and Table 1). Deletion of the two most N-terminal residues led to a ~10-fold reduction in inhibition relative to the wild-type SSB-Ct peptide and deletion of either three or four residues led to ~40-fold weaker inhibition relative to the wild-type peptide. Deletions beyond the first four N-terminal residues abolished inhibition. Interestingly, unlike the wild-type SSB-Ct peptide, the concentrations of the N-terminal SSB-Ct deletion variants required for 50% inhibition exceeded the values that would be predicted from the competition-binding studies by 5- to 8-fold (Table 1). Differences between the two assays that may lead to these discrepancies are considered in the *Discussion*.

Acidic SSB-Ct residue substitutions—The importance of the SSB-Ct peptide Asp residues in inhibiting SSB-stimulated ExoI activity were tested using peptide variants with neutralizing Asp-to-Ala substitutions. Each of the single variants inhibited SSB-stimulated ExoI activity as well as the wild-type SSB-Ct peptide (~1 μM needed for 50% inhibition), whereas the quadruple Asp-to-Ala variant peptide required ~20 μM to achieve 50% inhibition (Figure 3E and Table 1). The concentrations required for 50% inhibition are very similar to those that would be predicted from K_{bind} values derived from the competition-binding experiments.

C-terminal SSB-Ct peptide deletions and substitutions—The inhibitory properties of the C-terminal deletion variants of the SSB-Ct peptides were also tested in the nuclease assay. Peptides with one, two, or three residues removed from the C-terminus inhibited SSB-stimulated ExoI activity, requiring ~40 μM peptide to achieve 50% inhibition of the SSB stimulation (Figure 3F and Table 1). In contrast to the results with the N-terminal deletion peptides, the concentrations of the C-terminal deletion peptides required for 50% inhibition were less than what would be predicted from the competition-binding studies.

The single-site variant peptides altering the Pro or Phe residues at the SSB-Ct C-terminus each required ~10 μM peptide concentrations to inhibit SSB-stimulated ExoI activity by 50% (Figure 3F and Table 1). Similarly to the C-terminal deletion peptides, the Phe-to-Tyr variant is a more potent inhibitor of SSB-stimulated ExoI activity than would be predicted by the competition-binding studies in which it had a K_{bind} of 79 +/- 8 μM. Together with the C-terminal deletion peptide data, these findings indicate that alteration of the SSB-Ct C-terminus produces peptides that are more effective inhibitors against SSB-stimulated ExoI activity than F-SSB-Ct binding and implicate a major role for the N-terminal acidic residues from the SSB-Ct peptide in blocking ExoI/SSB complex formation.

Discussion

SSB interacts directly with at least a dozen different proteins involved in DNA replication, recombination, and repair. In all cases examined to date, these interactions occur by heterologous protein binding to the evolutionarily conserved SSB-Ct element of SSB (3). Mutations that alter the sequence of the SSB-Ct lead to profound genomic instability or death in bacterial cells (5-15). Thus, protein interfaces formed with the SSB-Ct element are key conserved features of bacterial genome maintenance processes.

The SSB-Ct sequence is amphipathic, containing a segment of residues with acidic side chains followed by a segment with hydrophobic side chains terminating in a highly conserved Pro-Phe dipeptide (3,4). Using a panel of peptide variants, we have established two competition assays that systematically test the importance of each SSB-Ct residue for interaction with ExoI. These include a minimal SSB-Ct peptide/ExoI competition-binding assay and a reconstituted ExoI/SSB nuclease assay in which inhibition by the peptide variants was used to assess the strength of binding to ExoI.

In both assays, nearly all of the SSB-Ct peptide variants with deleted N-terminal residues were significantly impaired for binding ExoI (Figure 2B and 3D). In the minimal competition-binding assay, SSB-Ct variants with larger N-terminal deletions produced progressively weaker competitors, indicating that these residues play important roles in ExoI binding. Although the same peptides also inhibited activity in the ExoI/SSB nuclease assay, the concentrations needed to achieve 50% inhibition were significantly higher than would be predicted from the competition-binding experiments (Table 1). For example, the peptide Trp-Asp-Ile-Pro-Phe, had a K_{bind} of 16.2 +/- 0.6 μM in the minimal peptide-binding assay but did not inhibit SSB-stimulated ExoI nuclease activity even at the highest concentration tested (100 μM). One explanation of this phenomenon is that N-terminal elements of the SSB-Ct (primarily residues with acidic side chains) are more important for binding to ExoI in the context of the ExoI/SSB/ssDNA ternary complex than in minimal ExoI/SSB-Ct interactions. Interestingly, as is described further below, deletions and modifications of the C-terminal hydrophobic residues on the SSB-Ct have the opposite effect, requiring higher concentrations of the variants to inhibit the minimal ExoI/SSB-Ct peptide interaction than the ExoI/SSB nuclease assay. These data support a model in which the acidic element of the SSB-Ct plays a more substantial role in mediating contacts at the ExoI/SSB interface in ExoI/SSB/ssDNA ternary complexes than in the minimal ExoI/SSB-Ct peptide interaction.

In agreement with the deletion peptide data, SSB-Ct peptide variants with modified Asp residues highlight the importance of net negative charge for binding ExoI. Our data suggest that no single Asp residue plays a dominant role in binding, because each of the variants with one Asp altered to Ala competes nearly as well as the wild type SSB-Ct peptide in both assays. However, simultaneous neutralization of all four Asp residues produces a peptide that competes ~35- to 50-fold more weakly in the two assays. Consistent with the noted salt-dependence of the SSB/ExoI interaction (15) and with the importance of the electropositive “basic ridge” portion of ExoI in interacting with the SSB-Ct peptide and SSB (4), these data show that the high net negative charge of the N-terminal portion of the SSB-Ct element is important for interaction with ExoI. A simple structural explanation of the data is that several Asp residues from the SSB-Ct peptide participate in electrostatic interactions with ExoI’s basic ridge surface. Because no single Asp residue appears to be more important than the others (Figures 2C and 3E), this electrostatic interface may not arise from a single unique conformation of the SSB-Ct but instead multiple arrangements of the Asp residues could potentially support the interface.

A prominent role in binding ExoI for the C-terminal-most Pro-Phe SSB-Ct dipeptide is confirmed in both the competition-binding and nuclease inhibition assays. Deletion of one, two, or three C-terminal residues produced SSB-Ct peptides that failed to compete for binding ExoI in the minimal ExoI/SSB-Ct competition-binding assay, indicating their clear importance in binding ExoI. This is consistent with the role played by the C-terminal-most Phe in anchoring SSB-Ct binding in the ExoI/SSB-Ct complex crystal structure (4). The C-terminal deletion SSB-Ct peptide variants could still inhibit SSB-stimulated nuclease activity, although they required ~40-fold higher concentrations than the wild-type SSB-Ct peptide to do so (Table 1).

SSB-Ct peptide substitutions changing the Pro residue to Ser and the C-terminal-most Phe residue to Tyr provided additional insights into the roles of the Pro-Phe dipeptide in SSB-Ct interactions. The SSB113 protein (Pro-to-Ser variant) has been widely used in SSB studies because it has been shown to interact more weakly with its binding partners than wild type SSB (4-22). Relative to the wild type SSB-Ct peptide, 10-fold higher concentrations of the Pro-to-Ser SSB-Ct peptide variant were required for inhibition in both the minimal competition-binding and the ExoI/SSB nuclease assays (Table 1). The second variant (Phe-to-Tyr) tested the extent to which introduction of a polar hydroxyl group onto the SSB-Ct side chain alters binding. This residue is invariant among 280 bacterial SSBs and the side chain is engulfed in a hydrophobic pocket on the surface of ExoI (3,4). This modification severely

weakened binding to ExoI in the contexts of both the competition-binding and ExoI/SSB nuclease assays. These effects could explain the extraordinary conservation of this residue among SSBs.

Taken together, the data presented herein support a model for interaction between ExoI and SSB in which both the acidic and hydrophobic segments of the SSB-Ct element play critical roles in forming an interface with ExoI. Our findings are in agreement with earlier complementary work showing that mutations in ExoI that altered the hydrophobic pocket, the Arg residue on the rim of the pocket, or basic ridge elements severely compromised SSB-Ct binding and SSB stimulation of ExoI nuclease activity (4). Recent mapping studies of the SSB-Ct binding site on *E. coli* RecQ, a second SSB-interacting protein, identified analogous surface features that are critical for its association with SSB (19). Thus, it is possible that most, if not all, SSB-binding proteins use similar strategies to bind to the SSB-Ct element and that SSB-Ct residues play conserved roles in SSB's associations with its many protein partners. Future studies with additional SSB-binding proteins will be required to assess the extent to which this SSB-Ct binding strategy is conserved among the SSB interaction network.

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Abbreviations

ss
: single-stranded

SSB
: ssDNA-binding protein

SSB-Ct
: C-terminal nine residues of SSB

ExoI
: Exonuclease I

DMF
: Dimethylformamide

DMSO
: Dimethyl sulfoxide

IC_{50}
: 50% inhibition concentration

K_{bind}
: apparent inhibitor dissociation constant (determined from competitive binding)

$K_{i,\ enz}$
: apparent inhibitor dissociation constant (determined from kinetic analysis)

FP
: Fluorescence polarization

$[I]_{50}$
: concentration of free inhibitor at 50% inhibition

$[L]_{50}$
: concentration of free ligand (F-SSB-Ct) at 50% inhibition

K_d
: apparent dissociation constant

$[P]_o$
: concentration of free ExoI at 0% inhibition

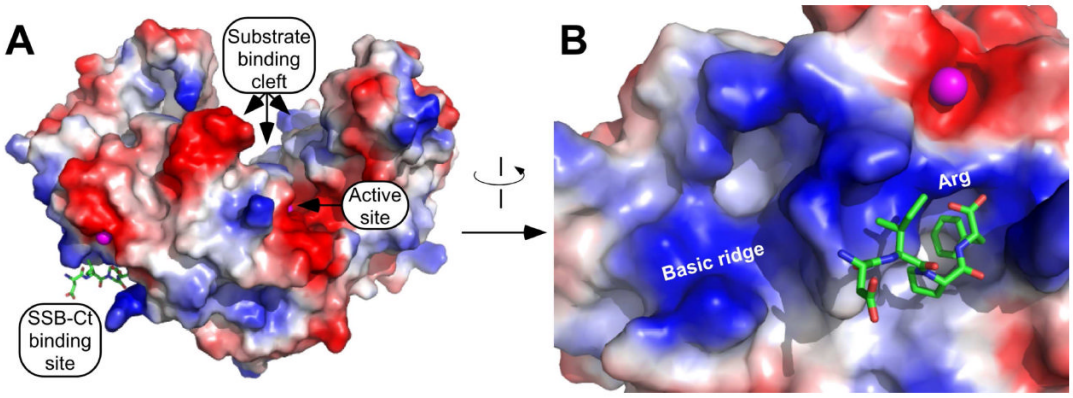

Figure 1. SSB-Ct binding site on *E. Coli* ExoI

Structure of SSB-Ct peptide-bound ExoI (4). ExoI is shown (surface representation; red = electronegative, blue = electropositive, white = neutral) with Mg^{2+} ions (magenta spheres) and the SSB-Ct peptide (sticks). **(A)** Global view of ExoI with labels indicating SSB-Ct binding site and substrate binding cleft/active site. **(B)** Close-up view of the SSB-Ct binding site on ExoI. The SSB-Ct peptide bound in the biologically-relevant site as determined in (4) is colored by atoms (green = carbon, red = oxygen, blue = nitrogen); a second SSB-Ct peptide that was observed in the structure but is non-functional has been removed for clarity. Only the C-terminal-most four residues were resolved in the crystal structure. Elements of ExoI that are important for SSB binding are labeled “Arg” and “Basic ridge” (4). Pymol (39) was used to render the structures.

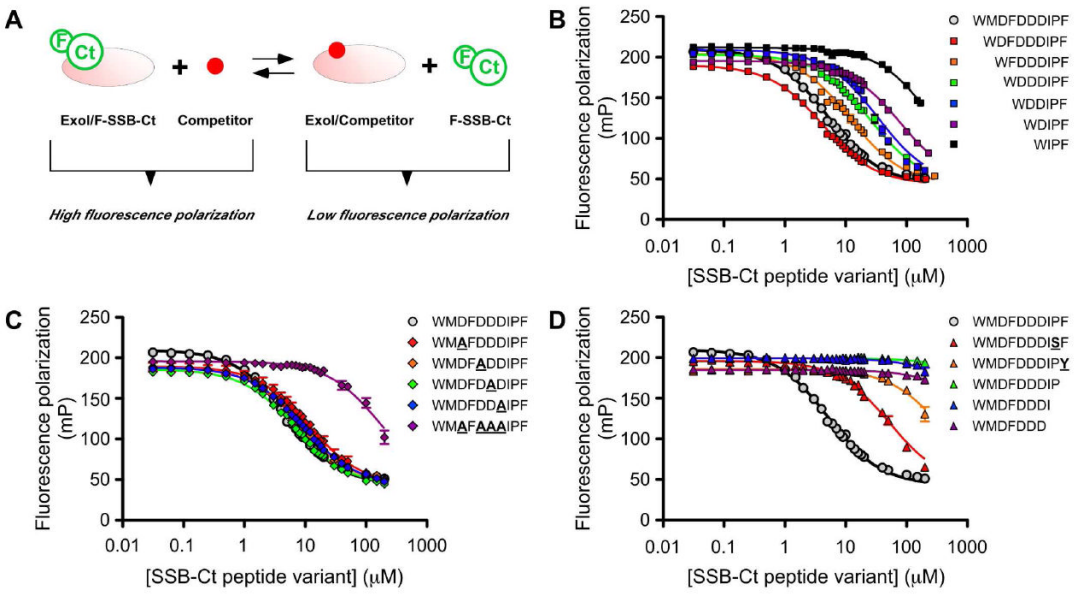

Figure 2. SSB-Ct variant peptides disrupt ExoI/F-SSB-Ct complexes

(A) Model of competitive binding by SSB-Ct variant peptides. ExoI/F-SSB-Ct complexes (high fluorescence polarization) and free competitor peptide are in equilibrium with ExoI/competitor complexes and free F-SSB-Ct (low fluorescence polarization). F-SSB-Ct and competitor peptides compete for binding a common site on ExoI. **(B, C, and D)** SSB-Ct peptide variants were incubated at indicated concentrations with ExoI/F-SSB-Ct complexes. Decreases in fluorescence polarization are attributed to the SSB-Ct peptide variant displacing the F-SSB-Ct peptide. Data points are reported as the mean of three independent measurements (error bars are one standard deviation of the mean) and lines are fits as described in *Materials and Methods*. Data for the SSB-Ct peptide control (grey circle) are shown in all plots with **(B)** N-terminal deletion SSB-Ct variant peptides (squares), **(C)** acidic SSB-Ct variant peptides (diamonds), or **(D)** C-terminal deletion/modified SSB-Ct variant peptides (triangles).

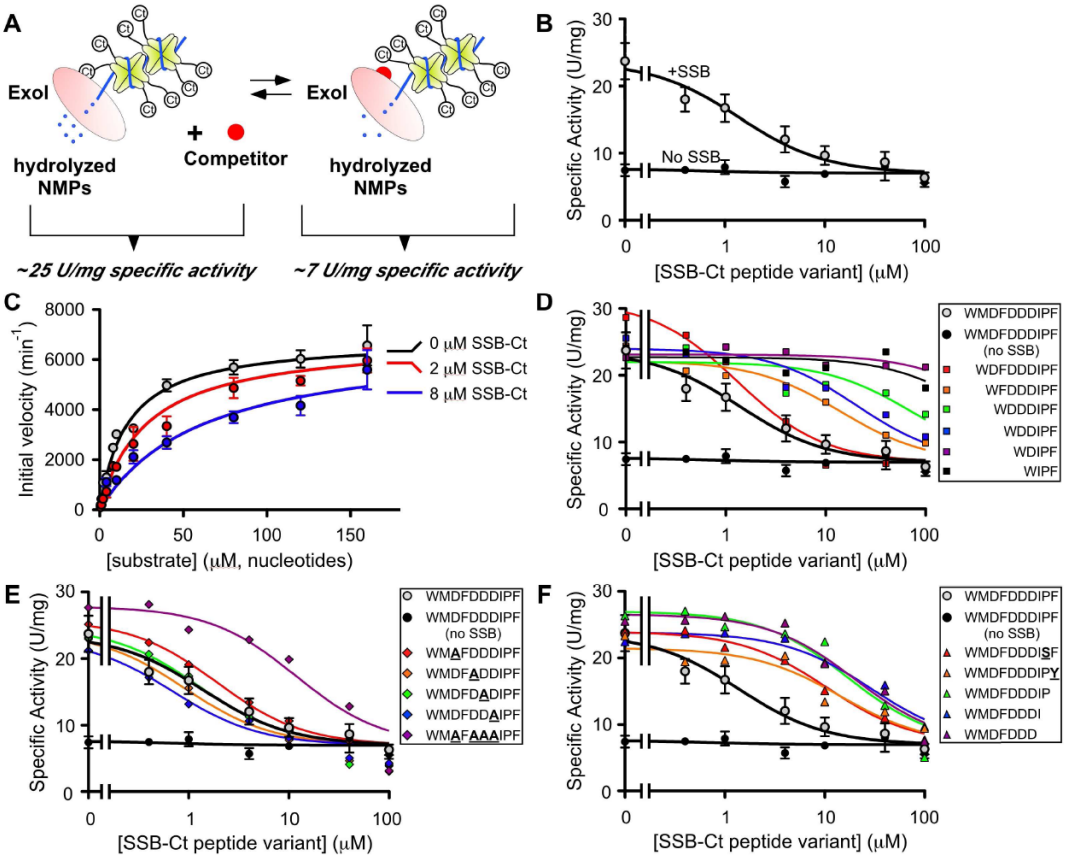

Figure 3. SSB-Ct variant peptides disrupt ExoI/SSB/ssDNA ternary complexes

(A) Model of nuclease inhibition by SSB-Ct variant peptides. The SSB-Ct/ExoI interface in high specific activity ExoI/SSB/ssDNA ternary complexes can be disrupted by competitive binding to the SSB-Ct binding site on ExoI, lowering the specific activity to SSB-free levels. **(B)** SSB-Ct peptide was incubated at indicated concentrations in ExoI nuclease assays in the presence (grey circles) or absence (black circles) of 200 nM SSB. Data points are reported as the mean of three independent measurements with error bars as one standard deviation of the mean. **(C)** Saturation kinetic data of ExoI nuclease activity on SSB/ssDNA substrate in the presence of 0 (grey), 2 (red), or 8 (blue) μM SSB-Ct peptide. Data points are reported as the mean of three independent measurements with error bars as one standard deviation of the mean. Lines depict a global fit of the data to a competitive inhibition model. **(D, E, and F)** Inhibition data for the SSB-Ct peptide (from (B)) are shown in all plots with **(D)** N-terminal deletion SSB-Ct variant peptides (squares), **(E)** acidic SSB-Ct variant peptides (diamonds), or **(F)** C-terminal deletion/modified SSB-Ct variant peptides (triangles). Error bars are shown for the SSB-Ct peptide data; these are representative of typical experimental error in the system.

Table 1

IC_{50}, K_{bind}, and 50% ExoI/SSB inhibition values for SSB-Ct peptide variants

	Minimal peptide-binding assay		ExoI/SSB activity assay
Peptide inhibitor	**IC_{50} (μM)**	**K_{bind} (μM)**	**50% inhibition (μM)**
WMDFDDDIPF (wild type)	5.2 +/- 0.2	0.9 +/- 0.1	~1
N-terminal truncations			
WDFDDDIPF	4.3 +/- 0.1	0.7 +/- 0.1	~1
WFDDDIPF	12.1 +/- 0.7	2.3 +/- 0.1	~10
WDDDIPF	25.3 +/- 0.9	5.1 +/- 0.8	~40
WDDIPF	33.5 +/- 2.6	6.8 +/- 0.1	~40
WDIPF	79.1 +/- 2.9	16.2 +/- 0.6	>100
WIPF	252 +/- 12	52 +/- 2	>100
Neutralizing modifications			
WM**A**FDDDIPF	11.6 +/- 0.9	2.2 +/- 0.2	~1
WMDF**A**DDIPF	8.5 +/- 0.3	1.6 +/- 0.1	~1
WMDFD**A**DIPF	6.7 +/- 0.3	1.2 +/- 0.1	~1
WMDFDD**A**IPF	9.3 +/- 0.3	1.8 +/- 0.2	~1
WM**A**F**AAA**IPF	169 +/- 15	35 +/- 3	~20
C-terminal modifications/truncations			
WMDFDDDI**S**F	52.9 +/- 3.5	10.8 +/- 0.7	~10
WMDFDDDIP**Y**	382 +/- 41	79 +/- 8	~10
WMDFDDDIP	>1000	>200	~40
WMDFDDDI	>1000	>200	~40
WMDFDDD	>1000	>200	~40