Interaction with Single-stranded DNA-binding Protein Stimulates *Escherichia coli* Ribonuclease HI Enzymatic Activity*

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**Background:** Bacterial single-stranded DNA-binding proteins (SSBs) interact with and stimulate many genome maintenance enzymes.

**Results:** SSB interacts with *E. coli* ribonuclease HI (RNase HI) to form a complex with heightened nuclease activity.

**Conclusion:** SSB stimulates RNase HI activity.

**Significance:** Formation of the RNase HI-SSB complex could be important for RNase HI-mediated removal of RNA/DNA hybrids that threaten genome stability.

Single-stranded (ss) DNA-binding proteins (SSBs) bind and protect ssDNA intermediates formed during replication, recombination, and repair reactions. SSBs also directly interact with many different genome maintenance proteins to stimulate their enzymatic activities and/or mediate their proper cellular localization. We have identified an interaction formed between *Escherichia coli* SSB and ribonuclease HI (RNase HI), an enzyme that hydrolyzes RNA in RNA/DNA hybrids. The RNase HI-SSB complex forms by RNase HI binding the intrinsically disordered C terminus of SSB (SSB-Ct), a mode of interaction that is shared among all SSB interaction partners examined to date. Residues that comprise the SSB-Ct binding site are conserved among bacterial RNase HI enzymes, suggesting that RNase HI-SSB complexes are present in many bacterial species and that retaining the interaction is important for its cellular function. A steady-state kinetic analysis shows that interaction with SSB stimulates RNase HI activity by lowering the reaction *Km*. SSB or RNase HI protein variants that disrupt complex formation nullify this effect. Collectively our findings identify a direct RNase HI/SSB interaction that could play a role in targeting RNase HI activity to RNA/DNA hybrid substrates within the genome.

RNA/DNA hybrids are essential nucleic acid structures that are formed during cellular DNA replication and transcription reactions. In replication, the RNA components of RNA/DNA hybrids serve as primers from which replicative polymerases extend DNA chains (1). Thousands of such RNA primers must be made and degraded during each round of genome replication in *Escherichia coli* (2). In transcription, RNA polymerase forms RNA/DNA hybrids as it synthesizes RNA using single-stranded (ss) DNA as a template (3). These hybrids are normally transient and occluded within the RNA polymerase active site. However, accumulating evidence suggests that the 5’ portions of nascent RNAs that have exited RNA polymerase can invade homologous duplex DNA, thereby displacing non-template ssDNA and generating hybrid structures called R-loops (4–9). Although RNA/DNA structures are essential, collisions between DNA replication complexes and hybrids can stall replication fork progression and increase the frequency of fork collapse and of double strand DNA break formation (10–14). Given these threats, several cellular mechanisms have evolved to remove hybrids from the genome (6, 15–19).

Ribonucleases H (RNases H) are endoribonucleases that help to prevent the accumulation of RNA within genomic DNA by degrading RNA/DNA hybrids (20, 21). Type 1 RNases H (RNase HI in bacteria) hydrolyze the RNA within hybrids that include four or more consecutive ribonucleotides, such as replicative primers and R-loops (4, 20, 22–28). In contrast, type 2 RNases H remove shorter segments of RNA, including single rNTPs that can occur from ribonucleotide misincorporation by replicative polymerases (29–32). Although the structures and enzymatic mechanisms of RNases HI have been examined in detail, insights into the mechanisms that regulate their cellular activities are less well understood. In particular, whether bacterial RNases HI function in higher order protein complexes or act exclusively in isolation is not known.

Bacterial ssDNA-binding proteins (SSBs) bind and protect exposed ssDNA within cells and have evolved a protein interaction network that includes over a dozen different protein partners (33, 34). The N-terminal domain of SSB mediates
ssDNA binding and homotetramer formation, whereas the C-terminal tail forms complexes with other proteins (33–37). The C-terminal-most six residues of the tail (Asp–Asp–Asp–Ile–Pro–Phe) in *E. coli* (referred to as the SSB-Ct) form a docking site for binding its protein partners. The SSB-Ct residues are evolutionarily well conserved, particularly in the final Pro-Phe dipeptide (33). In cases that have been examined, interactions with SSB appear to stimulate enzymatic activity and/or localize SSB binding partners to their cellular substrates (33, 38–43). Consistent with essential cellular roles for SSB/protein interactions, mutations that disrupt interactions with SSB are lethal in *E. coli* (39, 42, 44).

Using proteomic, structural, and biochemical approaches we have identified a complex formed between *E. coli* RNase HI and SSB. RNase HI co-purifies with affinity-tagged SSB expressed in *E. coli*, and the two proteins directly interact with one another in vitro. The RNase HI/SSB interaction is similar to other SSB protein complexes both in terms of the stability of the complex and its reliance on the SSB-Ct sequence. Structural analyses of *E. coli* RNase HI bound to a peptide comprising the SSB-Ct sequence reveal a conserved interface that mediates complex formation. Steady-state kinetic analysis of the effects of SSB on RNase HI activity show that interaction with SSB increases wild-type RNase HI catalytic efficiency through a 10-fold reduction in the enzyme *K*ₘ. Consistent with RNase HI-SSB complex formation being required for enzymatic stimulation, SSB or RNase HI variants that have lost the ability to interact with SSB appear to stimulate enzymatic activity and/or localize RNase HI-enriched fractions were pooled, dialyzed against Buffer A at 4 °C, and loaded onto an SP-Sepharose Fast Flow ion exchange column (GE Healthcare). Protein was eluted with a gradient from 0 to 100% Buffer B (20 mM HEPES-HCl, pH 6.8, 750 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) at 4 °C. The dialyzed sample was loaded onto a HiPrep Heparin Fast Flow column (GE Healthcare), and a gradient from 0 to 100% Buffer B (20 mM HEPES-HCl, pH 6.8, 750 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) was used to elute RNase HI at ~0.6 M NaCl. RNase HI-enriched fractions were pooled, dialyzed against storage buffer (20 mM Tris-HCl, pH 8.0, 50% (v/v) glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM DTT), and stored at ~20 °C. Purification of the RNase HI* variant in which the three natural Cys residues are replaced with Ala used in nuclear magnetic resonance (NMR) experiments was performed as described previously (49).

**SSB Purification—** *E. coli* SSB, SSBΔF, and SSB-113 were purified as described previously (50). SSB-Ct peptide (Trp-Met-Asp-Phe-Asp-Asp-Asp-Ile-Pro-Phe) was prepared as described previously (51).

**Isothermal Titration Calorimetry (ITC)—** *E. coli* RNase HI (or variants), SSB, SSB-113, and SSBΔF were dialyzed at 25 °C against 20 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 150 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol and brought to a final concentration of ~30 μM for RNase HI and 550 μM (monomers) for SSB, SSB-113, and SSBΔF. Titrations were performed at 25 °C using a MicroCal™ VP-ITC (GE Healthcare) as follows. SSB was titrated (1 × 1-μl, 4 × 4-μl, and 28 × 8-μl injections) in a thermostated sample cell containing RNase HI. Data were fit to a single site model (one SSB monomer binding to one RNase HI) using Origin software.

**NMR—** SSB-Ct peptide aliquots were titrated into 15N-labeled RNase HI* (1.34 mM) in 50 mM sodium acetate, pH 5.5 at 25 °C. 1H-15N heteronuclear single quantum coherence (HSQC) spectra were collected at 0:1, 0.5:1, 1:1, and 2:1 peptide:RNase HI* molar ratios. Normalized changes in chemical shift positions between the 0:1 and 2:1 spectra were calculated from the equation Δppm = [(ΔH ppm)² + (Δ15N ppm × αN)²]₀.⁵⁵ where Δppm is the difference in ppm between the chemical shifts of the RNase HI* and RNase HI*-SSB-Ct complex and 0.1 serves as the scaling factor (based on the ~10-fold lower gyromagnetic ratio of 15N relative to 1H) of the nitrogen chemical shift changes (αN) (52).

**Crystallization and Structure Determination of RNase HI-SSB-Ct—** *E. coli* RNase HI (9.6 mg/ml in 20 mM HEPES-HCl, pH 7.5) was combined with SSB-Ct peptide at a 1:1.2 molar ratio, and 1 μl of the protein-peptide sample was crystallized by mixing with 0.8 μl of well solution (1 M sodium citrate, 90 mM HEPES-HCl, pH 7.0, 11% (v/v) glycerol) and 0.2 μl of 3% (v/v) 1,4-dioxane in hanging drop vapor diffusion crystallization experiments. RNase HI-SSB-Ct crystals formed after several

**Structure and Function of the RNase HI-SSB Complex**

RNase HI Purification—*E. coli* BL21(DE3) pLysS cells and grown at 37 °C in Lysogeny broth (LB) (46) supplemented with 50 μg/ml kanamycin and 25 μg/ml chloramphenicol to midlog phase (*A₆₀₀nm ~ 0.6*). Protein expression was induced by the addition of 0.8 l, 4 μl, and 28 μl, and 28 μl 8 μl injections) in a thermostated sample cell containing RNase HI. Data were fit to a single site model (one SSB monomer binding to one RNase HI) using Origin software.

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days and were then transferred into 0.8 M sodium citrate, pH 7.0, 90 mM HEPES-HCl, pH 7.5, 25% (v/v) glycerol and flash frozen in liquid nitrogen. Diffraction data were indexed and scaled using HKL2000 (53). The RNase HI-SSB-Ct structure was determined by molecular replacement using Phaser (54) with a previously determined E. coli RNase HI structure (55) as a search model. Iterative model building with Coot (56) and refinement with REFMAC (57) generated the final model. The coordinates and structure factor files are available at the Protein Data Bank (code 4Z0U).

Determination of Evolutionary Conservation—ConSurf software (58, 59) was used to align 150 bacterial species using default settings (E-value, 0.0001; maximum percent identity between sequences, 95; minimum percent identity between homologs, 35) with the RNase HI B chain from the RNase HI-SSB-Ct co-crystal structure. The alignment results and conservation scores (actual E-value range, 2E−59–4E−47; average pairwise distance of DNA sequences, 0.47) were visualized with PyMOL (60).

RNase HI Activity Assay—Substrate was generated by annealing a 3’ fluorescein-labeled RNA oligonucleotide (rArGrCrGrArUrGrGrArGrArA-fluorescein) with a complementary DNA oligonucleotide containing a 5’ Iowa Black Hole Quencher (IBHQ) and (dT)70 tail at the 3’ end (IBHQ-dTdTdCdGdTdCdCdAdTdCdCdGdCdT-(dT)70). Oligonucleotides were mixed in a 1:1 molar ratio in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, incubated at 95 °C for 4 min; and then slow cooled to 25 °C over a ∼6-h period. For Michaelis-Menten kinetic measurements, the RNA/DNA hybrid substrate concentration was varied from 0.25 to 10 μM (nucleotides) (equivalent to 2.55–102 nM (molecules)), and the SSB concentration was varied from 0.25 to 10 μM (nucleotides) (equivalent to 2.55–102 nM (molecules)), and the SSB concentration was adjusted to maintain one SSB tetramer/substrate molecule in reaction buffer. The final reaction conditions included 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM DTT, 0.01% (v/v) Tween 20. Reactions were incubated on ice for 10 min prior to the addition of RNase HI, and the remainder of the experiment was performed at room temperature. The change in initial fluorescence intensity (∆F0) of the substrate was determined for 1 min with excitation at 493 nm and emission at 517 nm using a QuantaMaster™ 400 spectrophotometer (Photon Technology International). Reactions were then initiated by the addition of RNase HI (at 1.25–16.40 nM depending on the variant) and were analyzed at 395 nm using a QuantaMasterTM 400 spectrofluorometer. The change in fluorescence intensity was recorded for 5 min. The maximum fluorescence (Fmax) for each reaction was determined by incubating reactions with excess (1 μM) wild-type RNase HI and measuring the final fluorescence emission. Fmax values were assumed to represent 100% hydrolyzed product.

Time points used to determine the change in fluorescence in the presence of RNase HI (∆FRNH) were taken from the initial 10% of the linear increase in fluorescence after RNase HI addition. The following equation was used to convert the change in fluorescence emission (∆F) values to change in product formation (∆P).

\[
\Delta P = \frac{[\Delta F][S]}{F_{\text{max}}} \quad \text{(Eq. 1)}
\]

Initial velocity, \(V_0\), was defined as \(\Delta P/t\) where \(t\) is time in minutes. The equation below was used to determine the \(V_0\) for RNase HI activity and to adjust for any changes in fluorescence prior to the addition of enzyme (\(\Delta P_0/t_0\)).

\[
V_0 = \frac{(\Delta P_{\text{RNH}}/t_{\text{RNH}})-(\Delta P_0/t_0)}{K_{\text{cat}}/K_m} \quad \text{(Eq. 2)}
\]

\(V_0\) values of reactions were plotted against [S], and the kinetic data were fitted using the following Michaelis-Menten equation in GraphPad Prism.

\[
V_0 = \frac{V_{\text{max}}[S]}{K_m + [S]} \quad \text{(Eq. 3)}
\]

Data are presented as the mean of at least three reactions with error bars depicting one S.E. \(k_{\text{cat}}\) values were determined by dividing \(V_{\text{max}}\) by [RNase HI], and values from \(k_{\text{cat}}/K_m\) represent the “catalytic efficiency” of the reactions.

Results

RNase HI Interacts with SSB via the SSB-Ct—A TAP approach was used to identify proteins that interact with SSB from E. coli cells. SSB fused with an N-terminal dual affinity tag (NTAP-SSB) was expressed in E. coli, and TAP was performed to isolate TAP-SSB protein complexes as described previously (47). Although most co-purifying proteins were not visible as discrete bands via gel analysis, MALDI-TOF mass spectrometry of trypsin-derived peptides from the TAP eluant identified peptides from several known or putative SSB-binding proteins. Among the putative SSB binding partners, three unique peptides that comprised 27% of the RNase HI protein were identified, indicating that the two proteins co-purified. Additional peptides were derived from previously characterized SSB interaction partners: DNA topoisomerase III (53% sequence coverage) (61), PriA DNA helicase (41%) (62), exonuclease I (37%) (39, 63), RecQ DNA helicase (26%) (64), and PriC (12%) (47). Our results are consistent with an earlier proteome-wide study in E. coli (65).

Because TAP can identify both direct and indirect interactions, ITC was used to test whether purified RNase HI and SSB could directly interact in vitro and to determine the dissociation constant (\(K_d\)) and stoichiometry of the RNase HI-SSB complex. In this experiment, the change in heat associated with SSB titration into an RNase HI sample was measured and fit to a binding model as described under “Experimental Procedures.” The analysis showed that RNase HI and SSB interact directly with one another, forming a 1:1 (monomers) complex with a \(K_d\) of 2.0 ± 0.2 μM (Fig. 1A and Table 1). The stability of the RNase HI-SSB complex is within the range that has been observed with other SSB/protein interactions (0.1–10 μM) (33).

Because interactions between SSB and several of its protein partners are mediated by the SSB-Ct with a particularly strong dependence on the C-terminal Pro-Phe dipeptide (33, 40, 43, 47, 62–64, 66–68), we next examined whether the SSB-Ct is required for RNase HI-SSB complex formation. The RNase HI binding abilities of two SSB variants either lacking the C-terminal Phe residue (SSBΔF) or with a Pro-to-Ser change at the penultimate residue (SSB-113) were tested using ITC (Fig. 1, B and C, and Table 1). Neither SSB variant bound to RNase HI, consistent with the SSB-Ct being required for the interaction.
Identification of the SSB Binding Site on RNase HI—To map the SSB binding site on RNase HI, we first used \(^1\)H-\(^15\)N HSQC two-dimensional NMR spectroscopy. Based on previous HSQC experiments that mapped the SSB-Ct peptide binding site on the RecQ DNA helicase (69), we anticipated that chemical shift positions for amides in RNase HI residues comprising the SSB-Ct binding site would be significantly altered by complex formation with an SSB-Ct peptide. This experiment was performed using RNase HI*, an RNase HI variant with known \(^1\)H-\(^15\)N chemical shift positions in which the three non-essential Cys residues are replaced with Ala (49). \(^1\)H-\(^15\)N HSQC spectra were collected as SSB-Ct peptide was titrated into a labeled RNase HI* sample. The amide chemical shift positions for 25 residues were altered significantly (>0.1 ppm) upon the addition of SSB-Ct peptide (Fig. 2A). These peaks either disappeared from the spectra (nine residues) or moved by >0.1 ppm (16 residues) (Fig. 2A). When mapped onto previously determined RNase HI crystal structures (55, 70), the SSB-Ct-sensitive residues cluster together to define a putative binding site on the protein surface that was on the opposite side of the enzyme relative to the active site (Fig. 2B).

To better visualize the RNase HI-SSB complex interface, we crystallized RNase HI in complex with the SSB-Ct peptide and determined its x-ray crystal structure to 2.0-Å resolution (Fig. 3 and Table 2). The crystals contained two RNase HI molecules per asymmetric unit (superimposed in Fig. 3C and referred to as chains A and B hereafter). Inspection of a \(F_o - F_c\) electron density map revealed clear density corresponding to four residues of the SSB-Ct (Asp-Ile-Pro-Phe) bound at the pocket identified by NMR for each RNase HI monomer (Fig. 3A). Electron density for the more N-terminal components of the SSB-Ct peptide was not observed, indicating that this element was not stably bound to RNase HI in the crystal. Examination of the structure revealed that the SSB-Ct binding pocket expanded relative to the apoRNase HI structure to accommodate the C-terminal Pro-Phe dipeptide (compare Figs. 2B and 3A). The surface areas buried within the RNase HI/SSB-Ct interfaces were 358 (A chain) and 385 Å\(^2\) (B chain).

The RNase HI SSB-Ct binding site shared structural similarities with previously characterized SSB binding sites (38, 51, 69, 71, 72). These similarities include a hydrophobic pocket that binds the Pro-Phe side chains and basic residues that interact with electronegative elements of the SSB-Ct (Fig. 3A). The hydrophobic pocket is comprised of RNase HI residues Val-5, Leu-26, Tyr-28, Ala-58, Leu-59, and Cys-63 as well as the hydrophobic portions of the side chains of Lys-3, Arg-31, Lys-33, and Glu-61 (Fig. 3, B and C). The basic regions in the SSB-Ct binding site include a "basic lip" at the edge of the hydrophobic pocket and an adjacent "basic ridge" surface (Fig. 3A). Within the basic lip region, the e-amino groups of Lys-33 and Lys-60 were localized near the SSB-Ct peptide \(\alpha\)-carboxyl group with distances of 2.6 Å for Lys-33 and 3.3 Å for Lys-60 (B chain complex) or 4.0 Å for both residues (A chain complex) (Fig. 3C). Despite their proximity to the \(\alpha\)-carboxyl group, the electron density for the amino groups of these Lys residues was poorly defined. However, similarly positioned basic residues in other SSB-interacting proteins have proven essential for stabilizing...
the interaction with SSB (38, 51, 69, 71, 72). In addition, the amide nitrogen of Lys-60 from RNase HI was 2.9 or 2.8 Å from the carbonyl oxygen of the SSB-Ct Pro in the A and B chain complexes, respectively (Fig. 3C). As a part of the basic ridge, the ε-amino group of Lys-3 from RNase HI was 3.1 Å away from the carboxyl group and 2.7 Å from the carbonyl oxygen of the SSB-Ct Asp in the B chain complex, whereas Lys-3 had distances of 3.6 and 2.6 Å from the same element in the A chain complex (Fig. 3C). Alignment of RNase HI sequences from 150 bacterial species highlighted the conservation of many of the residues that form the SSB binding site, particularly Val-5, Lys-33, Leu-59, and Cys-63 (Fig. 3B). This suggests that the interaction between RNase HI and SSB is likely to be conserved in most bacteria.

The RNase HI/SSB Interface Is Essential for Complex Formation in Vitro—Electropositive residues have been shown to play essential roles in stabilizing the interfaces between SSB and SSB-interacting proteins in vitro (38, 51, 69, 71, 72). Variants in which these residues are altered have proven to be important tools for assessing the impact of a loss of interaction with SSB in

FIGURE 2. Identification of the SSB-Ct binding site on RNase HI using 1H,15N HSQC two-dimensional NMR. A, a plot of chemical shift differences for RNase HI backbone amide atoms upon titration with an SSB-Ct peptide (SSB-Ct:RNase HI 2:1). Residues for which amide peaks disappeared upon SSB-Ct titration are colored magenta, whereas those that displayed significant chemical shift differences (Δ ppm > 0.1 ppm) are colored orange. B, structure of RNase HI (Protein Data Bank code 1F21) (55, 70) with the residues colored as in A to reflect SSB-Ct-dependent chemical shift differences. The RNase HI active site (gray) is on the opposite side of the protein relative to the apparent SSB-Ct binding surface.
reconstituted in vitro reactions and/or in cells. To assess the contributions of individual RNase HI residues observed bound to the SSB-Ct in the co-crystal structure, a panel of charge-neutralizing (Ala substitution) or charge reversal (Glu substitution) RNase HI variants were created. These variants were purified and tested for binding SSB using ITC (Table 1). RNase HI variants with Ala substitutions near the SSB-Ct carboxyl group (K60A and K33A) each formed weakened complexes with SSB (Kd of 21.0 ± 0.9 and 6.4 ± 1.2 μM, respectively). Variants with Glu substitutions at the same sites failed to interact with SSB (Table 1), consistent with important roles for both residues in interacting with the SSB-Ct. Sequence changes at RNase HI residues that bound directly to the SSB-Ct Asp main chain (Lys-3) or that were adjacent to the Asp side chain (Arg-29 and Arg-31) also produced variants with significantly weakened (8–30-fold) or eliminated SSB interactions (Table 1). These in vitro results are consistent with roles for Lys-3, Arg-29, and Arg-31 in coordinating the more N-terminal region of the SSB-Ct as predicted by our structural observations.

Interaction with SSB Stimulates RNase HI Activity in Vitro—We next examined whether the interaction between RNase HI and SSB influenced the kinetics of RNase HI-mediated RNA hydrolysis. An established RNase HI activity assay (73) was adapted to allow time-resolved measurement of nuclease activity using a RNA/DNA hybrid substrate that has an extended ssDNA region for SSB binding (Fig. 4A). The 3′ ssDNA region of the substrate ((dT)70) served as an SSB binding site, whereas the 5′ end contained a 14-base pair RNA/DNA hybrid. A quencher (IBHQ) was included at the 5′ end of the DNA to quench the fluorescence of a fluorescein on the 3′ end of the RNA. RNase HI activity was measured as the increase in observed fluorescence as short fluorescein-labeled RNA hydrolysis products dissociated from the DNA (73). The substrate was designed to mimic the structure of a lagging strand Okazaki fragment with an annealed RNA primer adjacent to 3′ ssDNA extension.

RNase HI kinetic data were collected at several substrate concentrations in the presence or absence of SSB. The data
were fit to the Michaelis-Menten equation to derive steady-state kinetic parameters $k_{\text{cat}}$ and $K_m$ (Table 3 and Fig. 4B). In the absence of SSB, the $k_{\text{cat}}$ for RNase HI-catalyzed hydrolysis of the substrate was $1.86 \pm 0.14 \text{ s}^{-1}$, and the $K_m$ was $5.99 \pm 0.90 \mu\text{M}$. However, when SSB was included in the reaction, the $K_m$ was reduced to $0.56 \pm 0.11 \mu\text{M}$, and $k_{\text{cat}}$ was decreased to $0.69 \pm 0.03 \text{ s}^{-1}$ relative to the reaction with the free substrate (Table 3). The inclusion of SSB also decreased $k_{\text{cat}}$ by $3.9 \pm 1.0$-fold for the substrate tested. This level of catalytic efficiency enhancement is similar to the $3$-fold enhancement in nuclease activity observed with another SSB protein partner, *E. coli* exonuclease I (74).

To determine whether SSB enhancement of RNase HI activity required direct interaction between the two proteins, SSBΔF was substituted for SSB in the activity assay. This variant maintains normal ssDNA binding properties but does not interact with RNase HI (Table 1). The $K_m$ and $k_{\text{cat}}$ values for RNase HI in the presence of SSBΔF more closely resembled those with the free substrate than with wild-type SSB ($K_m = 3.72 \pm 0.66 \mu\text{M}$ and $k_{\text{cat}} = 1.63 \pm 0.12 \text{ s}^{-1}$; Fig. 4C and Table 3). A modest $1.41 \pm 0.10$-fold enhancement in catalytic efficiency of RNase HI by SSBΔF is attributed to a $2$-fold decrease in $K_m$. Therefore, the decrease in RNase HI stimulation observed between wild-type SSB ($4$-fold) and SSBΔF ($1.4$-fold) is consistent with RNase HI-SSB complex formation being critical for stimulation of RNA/DNA hybrid hydrolysis.

Based on the above observation, we hypothesized that RNase HI variants with weakened SSB interactions would also be stimulated to a lesser extent by SSB than the wild-type enzyme. To test this hypothesis, we examined the effects of disrupting the interaction between SSB and RNase HI in the RNase HI activity assay with SSB-Ct binding site variants, specifically with sequence changes at the basic ridge and basic lip. We began with RNase HI variants that altered binding to either the Asp

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**TABLE 2**

| X-ray data collection and structure determination statistics |
|-------------------------------------------------------------|
| **Data collection**                                           |
| Wavelength (Å)                                               | 0.97856 |
| Resolution range (high resolution bin) (Å)                   | 50–2.0 (2.03–2.00) |
| Space group                                                  | P2221 |
| Unit cell                                                   |
| a, b, c (Å)                                                  | 60.498, 64.855, 79.726 |
| completeness (%)                                             | 99.9 (99.6) |
| Unique reflections/total collected                           | 21,970/88,106 |
| Reflections (5% of the total) were held aside throughout refinement. |
| Reflections/total collected                                   | 21,970/88,106 |
| Redundancy                                                   | 4.0 (3.9) |
| I( researcher)/I( sym ) (%)                                   | 27.4 (2.3) |
| $R_{\text{sym}}$ (%)                                          | 5.9 (57.5) |

**Refinement**

| Resolution (Å)                                               | 50–2.0 |
| $R_{\text{work}}/R_{\text{free}}$ (%)                        | 21.1/24.1 |
| r.m.s. deviations (Å)                                        | 0.006 |
| r.m.s. deviations (°)                                        | 1.07 |
| Ramachandran statistics (%)                                  | 98 |
| Favored                                                     | 2 |
| Allowed                                                     | 0 |
| Disallowed                                                  | 0 |
| No. atoms                                                   |
| Protein                                                     | 2,550 |
| Peptide                                                     | 70 |
| Water                                                       | 97 |
| B factor (Å$^2$)                                             | 42.1 |
| Protein                                                     | 51.9 |
| Water                                                       | 43.6 |

$R_{\text{work}} = \sum ||I_{\text{obs}}| - |I_{\text{c}}|| / \sum |I_{\text{obs}}|$, where $I_{\text{obs}}$ is the intensity measurement for reflection $j$ and $I_{\text{c}}$ is the mean intensity for multiply recorded reflections.

$R_{\text{free}} = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / |F_{\text{obs}}|$, where the working and free $R$ factors are calculated by using the working and free reflection sets, respectively. The free $R$ factors are attributed to a lesser extent by SSB than the wild-type enzyme. To test this hypothesis, we examined the effects of disrupting the interaction between SSB and RNase HI in the RNase HI activity assay with SSB-Ct binding site variants, specifically with sequence changes at the basic ridge and basic lip. We began with RNase HI variants that altered binding to either the Asp

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**FIGURE 4.** RNase HI-SSB complex formation stimulates RNA/DNA hybrid hydrolysis in vitro. A. Schematic diagram of molecular beacon-based RNase HI in vitro fluorescence activity assay. The substrate consists of a 14-nucleotide RNA/DNA hybrid with a 3’ fluorescein-labeled RNA and a 5’ IBHQ-labeled DNA. The DNA has a (dT)$_{10}$ tail for binding an SSB tetramer. RNA cleavage by RNase HI releases the fluorescein molecule, resulting in a measured increase in fluorescence. B. Michaelis-Menten kinetics plot depicting steady-state nuclease activity of wild-type RNase HI in the absence of SSB (black circles) and with wild-type SSB (blue squares) and SSBΔF (red triangles). C. Effects of SSB, SSBΔF, or RNase HI variants on RNase HI activity. Bars depict fold stimulation of RNase HI or RNase HI variant catalytic efficiency ($k_{\text{cat}}/K_m$) by either wild-type SSB or SSBΔF (Table 3). Data are the mean of at least three independent replicates with error bars depicting S.E.
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(Lys-3; basic ridge) or the α-carboxyl group of the Phe (Lys-60; basic lip) within the SSB-Ct. The K60A RNase HI variant, which retained weakened but measurable binding to SSB in ITC experiments (Table 1), had kinetic parameters and SSB stimulation similar to those observed for wild-type RNase HI (Fig. 4 and Table 3). In contrast, the kinetics of the K60E variant for which binding to SSB was not observed (Table 1) was stimulated only modestly (1.79 ± 0.50-fold) and at a level that matched that of SSBΔF stimulation of wild-type RNase HI (1.41 ± 0.10-fold) (Fig. 4C and Table 3). For the RNase HI Lys-3 variants, neither of which bound SSB in ITC experiments (Table 1), SSB stimulated both very weakly. SSB stimulated the K3A RNase HI variant 2.47 ± 0.38-fold and the K3E variant 2.11 ± 0.43-fold. In summary, the variants for which interaction with SSB was undetectable by ITC (K60E, K3A, and K3E RNases HI) had SSB–dependent changes in catalytic efficiencies closer to the level observed between wild-type RNase HI and SSBΔF. These data indicate that a functional interaction between SSB and RNase HI is required to significantly stimulate RNase HI activity in vitro.

**Discussion**

In this study, we identified a direct interaction between *E. coli* SSB and RNase HI and examined the consequences of disrupting this interaction for RNase HI nuclease activity in vitro. Calorimetric and structural analyses identified the elements from each protein that mediate RNase HI–SSB complex formation. Similar to other protein interactions with SSB, the SSB-Ct element is essential for interaction with RNase HI. An evolutionarily conserved set of basic and hydrophobic residues form a binding pocket in RNase HI that accommodates the C-terminal-most SSB residues. Steady-state kinetic experiments showed that SSB stimulates RNase HI nuclease activity by lowering the reaction $K_m$. This enhancement was diminished when SSB or RNase HI variants that were unable to interact with one another were substituted in the assay, indicating that SSB stimulation of RNase HI is dependent upon formation of the RNase HI–SSB complex. These results define a model in which RNase HI is recruited to and/or retained at RNA/DNA hybrid substrates by interacting with SSB near RNA/DNA hybrids.

The structures of complexes formed between the SSB-Ct and several SSB protein partners (exonuclease I, RecO recombinational mediator, RecQ DNA helicase, PriA DNA helicase, and the χ subunit of the bacterial replisome) have revealed shared electrostatic features that are essential to maintaining the SSB-Ct complex interface (38, 51, 69, 71, 72). These include a hydrophobic pocket that binds the Pro-Phe side chains of the SSB-Ct and basic lip/ridge residues that coordinate the acidic elements of the SSB-Ct. The basic lip binds to the α-carboxyl group of the C-terminal-most Phe, and the basic ridge binds to the Asp residues in the N-terminal half of the SSB-Ct. Comparing the SSB-Ct binding site for RNase HI with previously examined structures shows that although the overall electrostatic characteristics of the binding site are maintained there are also some notable differences. The first is that the buried surface area in the RNase HI–SSB-Ct complex, ~370 Å², is significantly less than those observed in other structures that range from 570 to 700 Å² (38, 51, 69, 71, 72). Despite this difference, the 2.0 ± 0.2 μM dissociation constant for the RNase HI–SSB complex is within the range that has been observed with other SSB/protein interactions (0.1–10 μM) (33). The second difference is that unlike other SSB-Ct interactions in which a single Arg side chain interacts with both α-carboxyl oxygen atoms in the SSB-Ct RNase HI utilizes a pair of Lys side chains, Lys-33 and Lys-60, to coordinate this group. Changing basic lip residues to Ala in other SSB-interacting proteins is sufficient to ablate the interaction, whereas Ala substitutions at Lys-33 or Lys-60 in RNase HI led to a weakened, but not complete, loss of RNase HI–SSB complex formation. This result could be due to the Lys residues sharing responsibility for binding to the α-carboxyl moiety. Nonetheless, charge reversal substitutions that alter either RNase HI Lys-33 or Lys-60 to Glu were sufficient to eliminate interactions with SSB.

SSB plays an important role in stimulating the activity of many of its interaction partners. Fitting with this trend, we found that interaction with SSB led to a 10-fold reduction in the $K_m$ and a 4-fold overall improvement in catalytic efficiency for RNase HI hydrolysis of RNA in RNA/DNA hybrids with an extended ssDNA binding site for SSB. The substrate used in our analysis, which positioned an SSB tetramer 5’ of the RNA strand, mimicked an RNA primer generated on the lagging strand template during DNA replication. Although the precise mechanism is not yet fully defined, one simple model supported by the data presented here is that the interaction between SSB and RNase HI could help to target RNase HI to its substrate. Accordingly, the SSB-dependent reduction in $K_m$ could reflect an increased substrate affinity. In addition, the SSB-dependent $k_{cat}$ reduction observed in our assay could arise from retention

**Table 3**

| RNase HI | SSB | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | Fold stimulation |
|---------|-----|-------|-----------|---------------|-----------------|
| Wild type None | 5.99 ± 0.90 | 1.86 ± 0.14 | 0.31 ± 0.05 | 3.91 ± 1.00 |
| Wild type Wild type | 0.56 ± 0.11 | 0.69 ± 0.03 | 1.22 ± 0.23 | 3.12 ± 0.10 |
| Wild type SSBΔF | 3.72 ± 0.66 | 1.63 ± 0.12 | 0.44 ± 0.08 | 1.41 ± 0.10 |
| K3A None | 4.04 ± 0.52 | 0.64 ± 0.03 | 0.16 ± 0.02 | 2.47 ± 0.38 |
| K3A Wild type | 2.03 ± 0.13 | 0.79 ± 0.02 | 0.39 ± 0.03 | 1.03 ± 0.03 |
| K3E None | 5.42 ± 0.57 | 1.50 ± 0.07 | 0.28 ± 0.03 | 0.85 ± 0.03 |
| K3E Wild type | 3.46 ± 0.54 | 2.03 ± 0.11 | 0.59 ± 0.10 | 2.11 ± 0.43 |
| K60A None | 5.67 ± 0.89 | 1.76 ± 0.12 | 0.31 ± 0.05 | 2.11 ± 0.43 |
| K60A Wild type | 0.94 ± 0.10 | 1.47 ± 0.05 | 1.56 ± 0.17 | 5.05 ± 1.03 |
| K60E None | 2.39 ± 0.48 | 1.08 ± 0.08 | 0.45 ± 0.10 | 1.79 ± 0.50 |
| K60E Wild type | 1.20 ± 0.21 | 0.98 ± 0.04 | 0.81 ± 0.15 | 1.79 ± 0.50 |
of RNase HI on SSB/ssDNA reaction products that would cause the enzyme to non-productively dwell on SSB-bound ssDNA for longer periods of time than with the SSB-free substrate. Because the concentrations of RNase HI and SSB used in the activity assay were well below the $K_d$ for the RNase HI-SSB complex in the absence of nucleic acids, the catalytic improvement by SSB is most likely due to an interaction between RNase HI and SSB that is prebound to the substrate. Interestingly, RNase HI variants for which interactions with SSB could not be detected by ITC appeared to retain slightly elevated SSB-dependent activity. This effect may be due to retention of a modest residual affinity between the proteins that can allow a weakened interface to be formed when the two proteins are bound to a common substrate.

The experiments described here have defined several in vitro characteristics of the RNase HI/SSB interaction. Given the evolutionary conservation of the SSB binding site on RNase HI and the prevalence of SSB at DNA replication forks in vivo, SSB-mediated localization of RNase HI to the replication fork could be important for targeting RNase HI to active sites of replication in bacterial cells. In E. coli, lagging strand Okazaki fragments are typically 0.5–2.0 kb in length and require a 10–12-nucleotide RNA primer for initiation (1). This arrangement produces nucleoprotein structures in which long stretches of SSB-coated ssDNA are directly adjacent to RNA/DNA hybrids that are used for Okazaki fragment initiation. Formation of the RNase HI-SSB complex could function to recruit RNase HI to the lagging strand to facilitate primer degradation, potentially targeting the nuclease to the 5’ portion of the primer. RNase HI shares its RNA primer removal activity with the 5’–3’-exonuclease activity of DNA polymerase I, which removes the 3’ end of the primer at the RNA-DNA junction (23). Processing of RNA primers at the replication fork helps to prevent the incorporation of reactive RNA within the genome and the accumulation of DNA strand breaks.

A second possible role for RNase HI-SSB complex formation is to facilitate degradation of R-loops that can act as obstacles to replication fork progression. R-loops formed by nascent RNA strand invasion of recently transcribed duplex DNA “behind” RNA polymerase can create very long (~1-kb) RNA/DNA hybrids (75). The detrimental effects of persistent R-loops are well established in both prokaryotes and eukaryotes, and it has become clear that R-loop removal is essential to prevent fork stalling and/or collapse, recombination errors, and subsequent accumulation of DNA breaks (6, 10, 12, 13, 18, 19, 76–80). Accordingly, cells have evolved multiple R-loop removal pathways, many of which rely on factors that directly associate with RNA polymerase (5, 81–86). RNase HI is also important for R-loops removal, and it is possible that SSB-mediated localization of RNase HI serves as a replication fork-specific strategy that helps to target its R-loop removal activity to sites of replication (5, 82, 85, 87). As mentioned previously, SSB is concentrated at the lagging strands of replication forks, forming a structurally dynamic SSB-ssDNA nucleoprotein complex. Co-localization of RNase HI with SSB could position the enzyme to act rapidly to degrade R-loops that are proximal to advancing replication forks and in doing so could help to resolve replication-transcription collisions. Alternatively, SSB could bind to displaced ssDNA within extended R-loops and directly recruit RNase HI to transcription-dependent RNA/DNA hybrids independently of the position of replication forks. Additional research is needed to determine the cellular relevance of these possible roles for RNase HI-SSB complex formation. Interestingly, human RNase HI has also been identified as an interaction partner of the eukaryotic SSB, replication protein A (88). The RNase HI/replisome protein A interface is presumed to differ from the bacterial interface defined here because there is no SSB-Ct equivalent outside of bacteria. However, the observation that both bacterial and eukaryotic SSBs interact with type 1 RNases H suggests that maintenance of this complex could be broadly important for RNase HI/1 localization and removal of RNA/DNA hybrids. Our identification of the RNase HI-SSB complex in E. coli and mapping of the interface that mediates the interaction provide new avenues for future studies aimed at determining the roles of SSB in regulating the cellular activities of RNase HI.

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