

Single Strand Binding Proteins Increase the Processivity of DNA Unwinding by the Hepatitis C Virus Helicase

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Received 24 August 2007;
received in revised form
24 October 2007;
accepted 26 October 2007
Available online
1 November 2007

The nonstructural NS3 protein of the hepatitis C virus is a multifunctional enzyme with an N-terminal serine protease activity and a C-terminal helicase activity. The helicase is capable of unwinding both DNA and RNA duplexes; however, the overall processivity of the helicase is fairly low. We show here that single-strand binding (SSB) proteins enhance the unwinding processivity of both the NS3 helicase domain (NS3h) and the full-length protease–helicase NS3-4A. The detailed study of the effect of SSB on the DNA unwinding activity of NS3h indicates that the SSB stabilizes the helicase at the unwinding junction and prevents its dissociation. These results suggest a potential role for either cellular or virus-encoded SSB protein in improving the processivity of the NS3 *in vivo*.

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Edited by D. E. Draper

Keywords: HCV; unwinding; NS3; SSB; processivity

Introduction

Hepatitis C virus (HCV) is a single-stranded, plus-strand RNA virus and the causative agent of non-A, non-B hepatitis, a condition known to affect ~180 million individuals worldwide.^{1,2} The monocistronic message in the mRNA gets translated into a 9600-residue-long polypeptide, which then undergoes posttranslational cleavage to yield the structural (C, E1, and E2) and the nonstructural (NS2, 3, 4A, 4B, 5A, and 5B) proteins. The nonstructural proteins are important for viral replication and make up the viral replicative complex along with cellular host proteins whose roles are not well defined.^{2,3}

The NS3 protein of the HCV is a multidomain, multifunctional protein. Its N-terminal domain contains the serine protease activity, and the C-terminal domain contains the nucleoside 5'-triphosphatase/helicase activity. The two domains are loosely connected and can be independently expressed

while retaining their activities.^{3–5} The NS3 helicase belongs to the SF2 superfamily of helicases. This superfamily of proteins is characterized by the presence of seven conserved motifs.⁶ Conserved motifs I and II, referred to as Walker A and Walker B motifs, respectively, are important for ATP binding and hydrolysis.⁷ These helicases are defined by a conserved DexH/D sequence, which makes them members of the DEAD-box protein family that are involved in a variety of processes of RNA metabolism.^{8–10}

The HCV helicase is very unusual in that it is capable of binding single-stranded DNA (ssDNA) and RNA (ssRNA) and unwinding both DNA and RNA duplexes.^{11–13} Previous studies have shown that NS3h (helicase domain) and NS3 (protease–helicase) have poor DNA unwinding processivity relative to the processivity of NS3-4A (protease–helicase core peptide of 4A).^{14–16} Interestingly, the duplex RNA unwinding processivity is even poorer than the DNA unwinding processivity.¹⁶ Low processivity of unwinding is common to many SF1 and SF2 helicases (such as RecQ, Dda).^{17–19} One speculation for such poor unwinding processivity is that many processes of the RNA metabolism do not require helicases to travel along long stretches of the nucleic acid, and hence these helicases are evolved to function only for unwinding short stretches of RNA. Alternatively, since most of these helicases function as a part of large multiprotein assemblies, it

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Abbreviations used: ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; SSB, single-strand binding; HCV, hepatitis C virus; WT, wild type; DEAE, diethylaminoethyl.

is possible that accessory proteins are required to keep the helicase from dissociating by means of protein-protein interactions.

It has been shown in many helicases that the presence of single-strand binding (SSB) proteins improves the unwinding efficiency.^{17,20–23} The RecQ helicase from *Escherichia coli* has physical and functional interaction with the *E. coli* SSB protein.^{17,23,24} The *E. coli* PriA helicase requires the SSB protein to mediate interactions with PriB and to form the functional PriA–PriB complex.^{25–27} PriA shows a functional interaction with the Replicative protein A, the human homologue of *E. coli* SSB protein.²⁵ DNA helicases from *E. coli*, Helicase II (UvrD) and Helicase IV, have both been shown to have improved activity when supplemented with SSB.^{28,29} Similarly, many viral and archaeobacterial helicases show improved unwinding efficiencies in the presence of either homologous or heterologous SSB proteins.^{30,31} In this study, we have explored the effect of SSB proteins on the unwinding activity of the HCV helicases. We have used the bacterially expressed helicase domain (NS3h) and full-length protease-helicase (NS3-4A) covalently linked to a core peptide of NS4A to study the effect of SSB proteins on the DNA unwinding activity of these helicases. Our results show that SSB proteins increase the unwinding activity of HCV helicases. SSB proteins do not alter the rates of unwinding, but substantially improve the unwinding processivity of these helicases.

Results

NS3h has a low processivity of DNA unwinding

NS3h is a 3'–5' helicase that requires a ssDNA 3' tail for initial loading and for subsequent strand separation of the duplex region. Previous work has shown that the presence of an additional 5' tail in the unwinding substrate (fork substrate) does not hinder NS3h-catalyzed DNA unwinding.^{11,32} Therefore, most of the studies reported here have been carried out with fork substrates (Table 1) with a dT₃₅ 5' tail and a dT₁₅ 3' tail. As reported previously, when the strand separation of the 40-bp substrate was measured under single-turnover unwinding conditions, less than 1% ssDNA product was observed (Fig. 1a).¹⁵ The single-turnover unwinding conditions were achieved by preincubating the DNA substrate with NS3h and adding polyU (protein trap) with ATP during initiation of the reactions. When strand-separation reactions were carried out with a shorter 18-bp DNA substrate, ~85% of the double-stranded DNA (dsDNA) was unwound under single-turnover unwinding conditions. The average DNA unwinding rate of 3.7 bp/s at 22 °C (Fig. 1b) was similar to the previously reported rates by NS3h.¹⁵ When the strand separation of the 40-bp substrate was measured under conditions where NS3h can cycle between DNA-bound and free states (in the absence of polyU),

Table 1. DNA substrates

1. 3'15Tds40 (60%)

5'–T₃₅ G CAG AGC GGA AGG AGA AGA GAG CGA GAG CAG AGT GTA GAG–3'
3'–T₁₅ C GTC TCG CCT TCC TCT TCT CTC GCT CTC GTC TCA CAT CTC–5'

2. 3'35Tds40 (60%)

5'–T₃₅ G CAG AGC GGA AGG AGA AGA GAG CGA GAG CAG AGT GTA GAG–3'
3'–T₃₅ C GTC TCG CCT TCC TCT TCT CTC GCT CTC GTC TCA CAT CTC–5'

3. 3'15Tds40 (40%)

5'–T₃₅ G AGC GGA TTA CTA TAC TAC ATT AGA ATT CAG AGT GTA GAG–3'
3'–T₁₅ C TCG CCT AAT GAT ATG ATG TAA TCT TAA GTC TCA CAT CTC–5'

4. 1Tds40 (40%)

5'–G AGC GGA TTA CTA TAC TAC ATT AGA ATT CAG AGT GTA GAG–3'
3'–T₁₅ C TCG CCT AAT GAT ATG ATG TAA TCT TAA GTC TCA CAT CTC–5'

5. 3'5Tds30

5'–T₃₅ GAG CGG ATT ACT ATA CTA CAT TAG AAT TCA–3'
3'–T₅ CTC GCC TAA TGA TAT GAT GTA ATC TTA AGT–5'

6. 3'10Tds18

5'–T₃₅ TAG ACC ACA GTT ACA GCC–3'
3'–T₁₀ ATC TGG TGT CAA TGT CGG–5'

7. 25mer DNA

5'–A AAT TAA TAC GAC CA CTA TAG GGA–3'

~70% of the duplex was unwound (Fig. 1c). The rate of unwinding under these multiple-turnover reaction conditions, again in agreement with the previously published results,³³ was 10-fold slower (~0.4 bp/s) compared to the single-turnover rate of unwinding, indicating that strand separation rate is limited by NS3h cycling between DNA-bound and free states. These results demonstrate that NS3h has a low processivity of DNA unwinding.

SSB proteins enhance the unwinding processivity of NS3h

Many helicases such as PriA, Werner's syndrome helicase WRN, RecQ, and others have been shown to unwind duplex DNA efficiently only in the

presence of ssDNA binding proteins.^{23,25,26,34,35} To investigate if the unwinding efficiency of HCV NS3h protein would be enhanced by the presence of a heterologous SSB protein, helicase assays were performed in the presence of the *E. coli* SSB protein. The 40-bp forked substrate (3'15Tds40, 60%; Table 1) was preincubated with NS3h, and ATP was added to initiate the unwinding reaction. When the reaction was supplemented with SSB protein, robust unwinding of the 40-bp substrate was observed (Fig. 2a and b). Therefore, even if NS3h was initially bound to both the 3' and the 5' tail, addition of ATP likely results in the dissociation of the enzyme bound at the 5' tail (either because of reduced affinity or because of translocation to the end of the tail and falling off) allowing for SSB protein to bind. This effect was not peculiar to *E. coli* SSB, as similar results were observed with the bacteriophage T7 SSB protein, gp2.5 (Fig. 2b). SSB protein by itself was incapable of unwinding the duplex (data not shown). The unwinding rate in the presence of SSB (3.4 bp/s) was much faster than the unwinding rate under multiple turnover conditions (in the absence of polyU and SSB) and very close to the single-turnover unwinding rate (in the presence of polyU) of NS3h. Therefore, the 40-bp fork DNA was unwound to nearly 80% completion at the single-turnover unwinding reaction rate in the presence of SSB protein. These results indicate that the unwinding of the 40-bp DNA in the presence of SSB protein is not rate limited by NS3h cycling between DNA-bound and free states. Increasing NS3h concentration by fivefold did not increase the unwinding rate any further (data not shown). The results show that the SSB proteins dramatically increase the processivity of unwinding. In these reactions involving *E. coli* SSB, no PolyU (protein trap) was added. This was because it has been previously demonstrated that *E. coli* SSB binds to ssRNA as well.^{36,37} Thus,

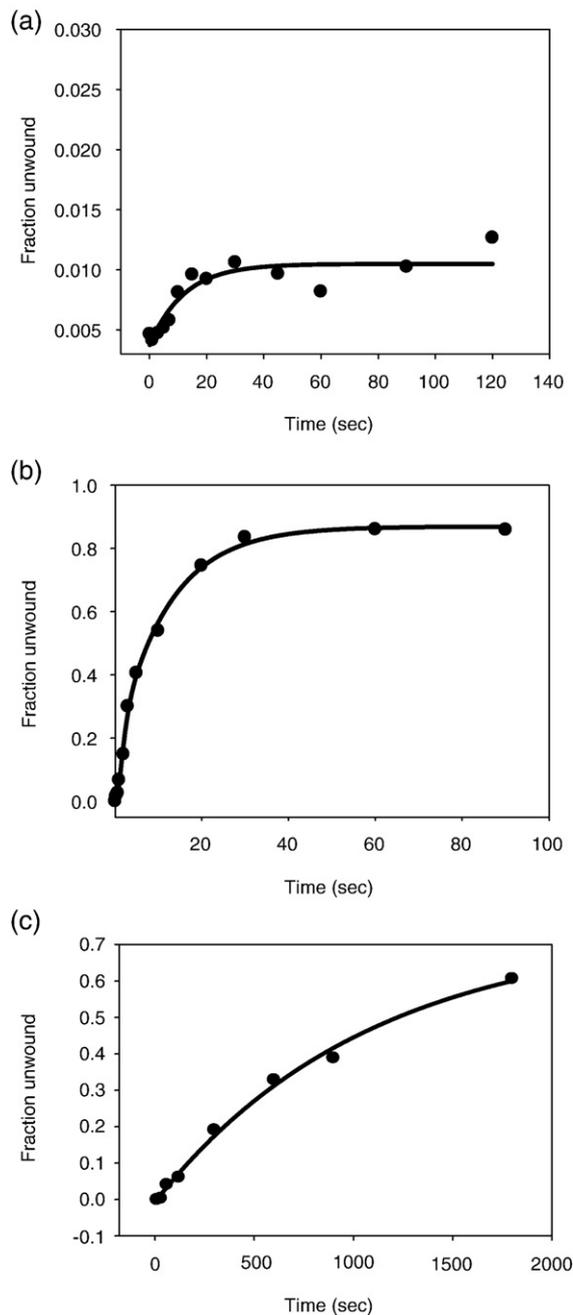


Fig. 1. HCV NS3h has a low intrinsic processivity of DNA unwinding. (a) Unwinding kinetics of a 40-bp forked DNA substrate (Table 1, 3) under single-turnover conditions. NS3h (200 nM) was incubated with 5 nM ss/dsDNA substrate in buffer in one syringe and mixed with equal volumes of 10 mM ATP, 1 μ M PolyU, and 400 nM DNA trap in the same buffer from the other syringe for the times indicated. The unwinding reaction was stopped by the addition of a 1.5-fold volume of quenching solution. The reactions were carried out in a rapid quenched-flow instrument. NS3h unwinds less than 1% of the total DNA. (b) Unwinding kinetics of the 18-bp forked DNA substrate (Table 1, 6) under single-turnover conditions. Reactions were performed as explained above, but an 18-bp forked DNA substrate was used instead of the 40-bp forked DNA. NS3h unwinds about 80% of the 18-bp substrate with a rate of 3.7 bp/s. (c) Unwinding kinetics of the 40-bp forked DNA substrate under multiple turnover conditions. Unwinding reactions were carried out by incubating 200 nM of NS3h with 5 nM ss/dsDNA substrate in buffer in one syringe and mixing with an equal volume of 10 mM ATP but no trap, in the same buffer from the other syringe for the times indicated. NS3h unwinds about 60% of the substrate at a rate of 0.4 bp/s.

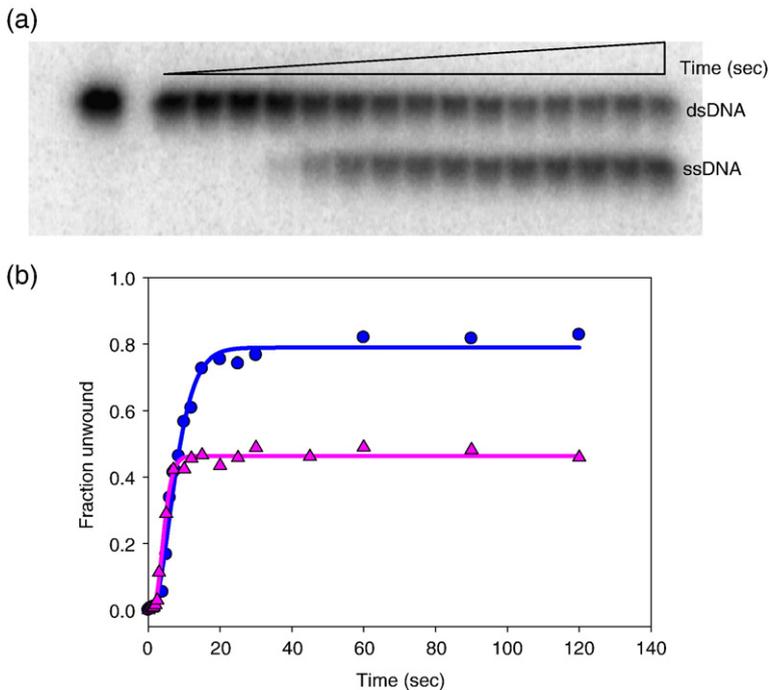


Fig. 2. DNA unwinding processivity of NS3h unwinding is enhanced by SSB proteins. (a) The 40-bp fork substrate and the unwound ssDNA (resolved by native PAGE) after various times of reaction with NS3h in the presence of *E. coli* SSB. NS3h (200 nM) was incubated with 5 nM ss/dsDNA substrate in buffer in one syringe and mixed with equal volumes of 10 mM ATP and 2 μ M *E. coli* SSB in the same buffer from the other syringe for the times indicated. (b) Fraction of the 40-bp DNA unwound in the presence of *E. coli* SSB (●) or T7 SSB, gp2.5 (▲), as a function of reaction time. NS3h (200 nM) was incubated with 5 nM ss/dsDNA substrate in buffer in one syringe and mixed with equal volumes of 10 mM ATP, 2 μ M *E. coli* SSB, or 10 μ M T7 SSB (gp2.5) in the same buffer from the other syringe, for the times indicated. The

unwinding rates range between 3.4 and 3.7 bp/s and the amount of products formed range between 83% and 87% in the presence of *E. coli* SSB. The unwinding rate in the presence of gp2.5 ranges between 5.7 and 5.9 bp/s and the amount of products formed range between 36% and 42%.

adding PolyU together with SSB would result in SSB getting trapped by PolyU and hence will not be available to bind to the substrate.

The 40-bp forked DNA substrate contains a 5'-dT₃₅ tail that can bind a molecule of *E. coli* SSB tetramer. To determine if the 5' tail was required for the stimulation of unwinding processivity by the

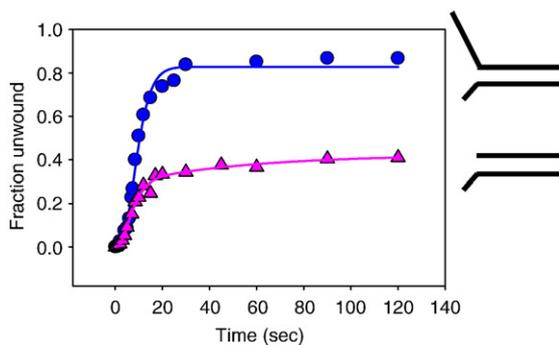


Fig. 3. Effect of the 5'-ssDNA tail on the unwinding efficiency of NS3h in the presence of SSB. The unwinding reaction by NS3h was carried out in the presence of SSB using substrates that either possessed the 5' tail (Table 1, 3) or lacked it (Table 1, 4). NS3h (200 nM) was incubated with 5 nM ss/dsDNA substrate in buffer in one syringe and mixed with equal volumes of 10 mM ATP and 2 μ M *E. coli* SSB in the same buffer from the other syringe for the times indicated. The fraction of DNA unwound in each case was plotted as a function of reaction time. The 40-bp substrate with the 5' tail was unwound to a greater extent (●) than the DNA without the 5' tail (▲), but with similar DNA unwinding rates in the range of 3.4–3.7 and 2.9–3.1 bp/s, respectively.

SSB, experiments were carried out with a 40-bp unwinding substrate lacking the 5' tail (1Tds40, 40%, Table 1). The fraction of products formed at the end of the reaction in the presence of SSB dropped by 50% when the 5' tail was removed (Fig. 3). These results indicate that the 35-nt-long 5' tail, although not a requirement, when present enhances the NS3h activity most likely by promoting efficient SSB binding to the displaced strand. Although SSB shows tightest binding to longer stretches of DNA (≥ 33 nt), the protein also binds to shorter DNAs albeit with reduced affinity.³⁸ In the experiment with the partial duplex substrate, although SSB does not bind initially, unwinding by the helicase would generate a single-stranded tail, which can be bound by SSB. Since the initial substrate-SSB interaction is not as strong as in the case of the forked substrate, the percentage of enzyme that does dissociate from the junction is higher in this case (as indicated by the decreased amplitude).

DNA reannealing trap does not mimic the action of SSB

The above-mentioned results demonstrate a dramatic effect of SSB on the unwinding processivity of NS3h. We therefore addressed the question of what SSB was doing that improved the unwinding processivity of NS3h. One reason for NS3h not being able to unwind long duplexes is that the initially separated strands reanneal behind the helicase during the unwinding time course, and SSB prevents DNA reannealing by binding to the displaced strand.

To test this hypothesis, we added a large excess of the 3' strand to the reaction along with the initiating ATP. The rationale behind doing this experiment was that, if all that SSB was doing was preventing reannealing behind the helicase during the unwinding time course, then the situation can be mimicked by the addition of excess amount of the strand that is complementary to the displaced strand (Fig. 4a). The excess of the 3'-loading strand (nonradioactive) would trap the displaced strand as it is generated by the action of NS3h, thereby preventing the displaced strand from reannealing to the loading strand. Addition of excess 3'-loading strand showed a marginal increase in the amount of products formed ($\sim 5\%$); however, this fold increase did not match that of SSB (Fig. 4b). The bimolecular rate constant of the two strands of DNA annealing is of the order of 10^5 s^{-1} . It is hence possible that in the time scale of the unwinding reaction, the externally added 3'-strand annealing to the displaced strand is not fast enough. One way to increase the rate of annealing is to increase the concentration of the DNA strand such that the lifetime of annealing of the displaced strand is now well within the time scale of the reaction under study. Thus, increasing the concentration of the 3'-loading strand to as high as $500 \mu\text{M}$ would mean that 50% of the DNA trap would have annealed to the displaced strand in 20 ms. These reaction conditions resulted in 10% product formation, which was still only one-eighth of the products formed with SSB (Fig. 4c). We conclude that

extensive reannealing of the strands does not occur during the unwinding reaction and that the mode of action of SSB is not merely to prevent reannealing of strands behind the helicase.

SSB promotes stable interactions of NS3h with the fork junction

Based on the above results, we propose that NS3h-catalyzed unwinding activity has a low processivity due to the tendency of the helicase to dissociate frequently from the unwinding substrate before reaching the end of a long stretch of dsDNA. One way SSB can increase the unwinding efficiency is by stabilizing the binding of NS3h to the fork junction, that is, keeping the helicase at the fork junction and preventing it from dissociating into solution. Another way SSB can increase the unwinding processivity is by stabilizing the partially unwound DNA intermediates and allowing NS3h to continue the unwinding process by undergoing multiple rounds of dissociation from and reassociation to these intermediates. Since we do not have a protein trap in the reaction, multiple NS3h molecules can bind to the loading strand in the intermediates, which can also increase the efficiency of unwinding by a functional cooperation mechanism,¹⁵ also observed in the T4 Dda helicase.¹⁹ To distinguish between the above scenarios, we designed the following mutant poisoning experiments.

We have shown previously that mutant D261A NS3h lacks ATPase activity and hence cannot

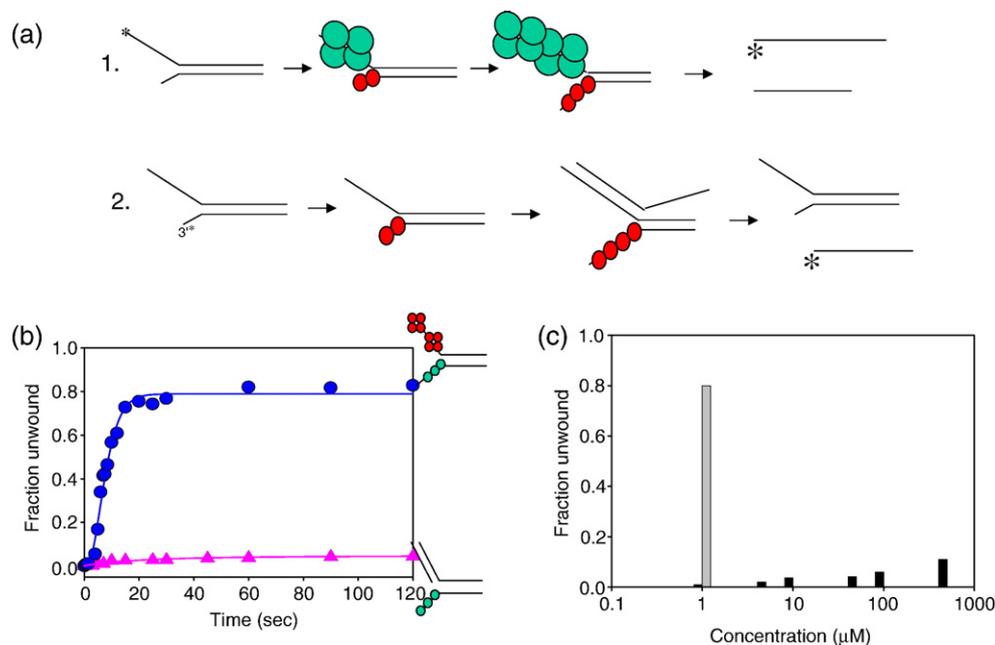


Fig. 4. Mechanism of SSB action–DNA reannealing trap. Experiments designed to investigate if SSB was preventing DNA reannealing behind the helicase. (a) Schematic representation of the unwinding reaction in (a-1) shows that the SSB tetramers (green) bind to the displaced strand and prevent DNA reannealing as NS3h molecules (red) unwind the DNA. The unwinding reaction in (a-2) shows that an excess of the DNA reannealing trap (strand complementary to the displaced strand) prevent DNA reannealing. (b) The kinetics of 40-bp substrate unwinding in the presence of SSB (●) and in the presence of the DNA reannealing trap (▲). (c) Fraction of DNA unwound in the presence of an increasing amount of the DNA reannealing trap, from 1 to $500 \mu\text{M}$ (black bar) compared to the reaction with SSB (grey bar).

unwind DNA. The mutant NS3h binds DNA with the same affinity as wild-type (WT) NS3h (Fig. 5a) both in the absence and in the presence of ATP.³³ WT NS3h was preincubated with the 40-bp forked substrate (2Tds40, 60%; Table 1) and an excess of mutant D261A NS3h (with or without SSB) was added with the initiating ATP. In the absence of SSB, unwinding is severely inhibited by the presence of excess mutant NS3h (Fig. 5b). This indicates that in the absence of SSB, NS3h cycles between DNA-bound and free states, and after the first round of unwinding and dissociation, the WT NS3h is replaced by the mutant NS3h that blocks DNA unwinding. This inhibition is quite severe and unwinding activity drops to almost zero when the ratio of the mutant to WT NS3h is 1:1. Interestingly, in the

presence of SSB, almost no decrease in unwinding was observed at 1:1 mutant-to-WT NS3h ratio. At very high ratios of mutant to WT NS3h (10:1) the activity decreased to only 50% (Fig. 5b). Measurement of the kinetics of unwinding (Fig. 5c) showed that the decrease in activity was due to the decrease in the unwinding amplitude, and the rate of unwinding by WT NS3h remained unchanged in the presence of the mutant NS3h. These results indicate that most of the initially DNA-bound WT NS3h does not freely exchange with the mutant NS3h present in solution when SSB is present. We conclude from these experiments that SSB increases the unwinding processivity by somehow preventing the NS3h bound at the junction from dissociating into solution.

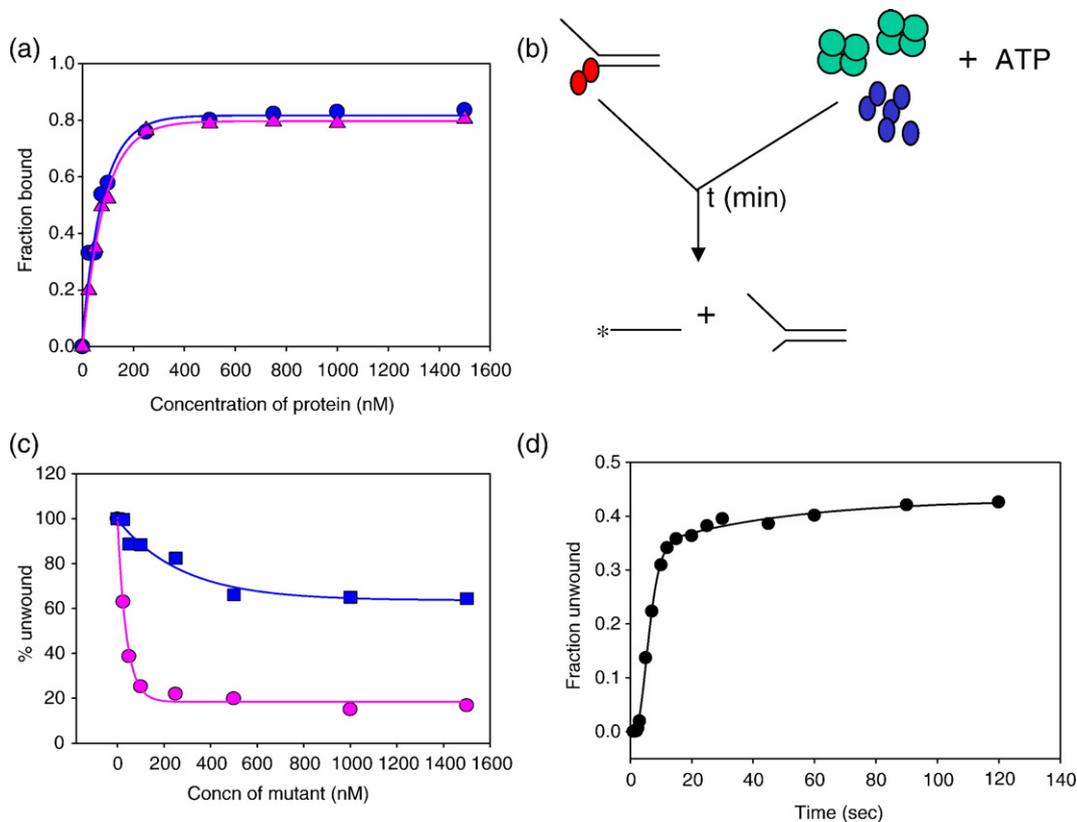


Fig. 5. Mechanism of SSB action: mutant poisoning experiments. (a) Wild-type NS3h (●) and mutant D261A NS3h (▲) bind to ssDNA (Table 1, 6) with equal affinity as assessed by the nitrocellulose filter binding assays. 25mer DNA (20 nM) was incubated with increasing concentrations of either WT NS3h or mutant D261A NS3h for 30 min at room temperature. The fraction of protein bound was plotted as a function of protein concentration. (b) Schematic representation of the unwinding experiments done in the presence of the mutant D261A. The red circles represent the WT NS3h, while the blue circles (on the right) represent the mutant D261A. The green tetramer represents the SSB protein. In the mutant poisoning experiments, the wild-type enzyme is preincubated with the DNA substrate and the reaction is initiated with the SSB–mutant NS3h–ATP mixture. The reaction is then quenched with SDS–ethylenediaminetetraacetic acid quenching solution after appropriate times. (c) The unwinding of the 40-bp substrate was measured using a constant amount of WT NS3h (100 nM) and increasing amounts of the mutant NS3h in the absence (●) or in the presence of SSB (■). Forked substrate (5 nM) was preincubated with 200 nM WT NS3h. The unwinding reaction was initiated by mixing with an equal volume of 10 mM ATP and increasing concentrations of mutant D261A, either in the presence or in the absence of 2 μ M *E. coli* SSB. The amount of products formed in each case was plotted as a function of increasing mutant concentration. (d) The kinetics of 40-bp substrate unwinding by NS3h in the presence of SSB and excess of the mutant D261A. WT NS3h (200 nM) was incubated with 5 nM ss/dsDNA substrate in buffer in one syringe and mixed with equal volumes of 10 mM ATP, 2 μ M *E. coli* SSB, and 1 μ M mutant NS3h D261A in the same buffer from the other syringe for the times indicated. About 40% of the substrate is unwound at a rate of 3.9 bp/s.

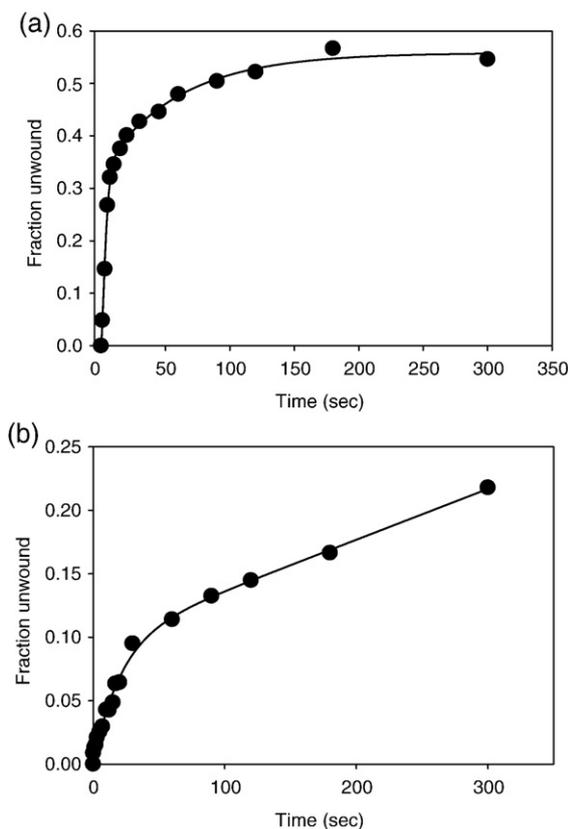


Fig. 6. Unwinding activity of the NS3h monomer. (a) Unwinding of the 30-bp substrate with a dT₅ 3'-loading tail and dT₃₅ 5' tail (Table 1, 5) by NS3h was carried out in the presence of SSB and excess of the mutant NS3h. The fraction of substrate unwound is plotted as a function of time. About 45% of the substrate is unwound by NS3h at an average rate 4 bp/s. (b) Unwinding reaction was carried out using 10 nM NS3h and 10 nM 30-bp forked DNA substrate. About 12% of the DNA is unwound (1.8 bp/s) in the burst phase, which is very close to the expected 16% (one-sixth of the total enzyme, since the average enzyme binding site on the DNA is seven bases).

NS3h monomer is capable of unwinding DNA duplexes

The forked DNA substrate that was used in the above experiments contained a dT₁₅ 3' tail that according to our previous studies would bind two molecules of NS3h at the start of the unwinding reaction.³⁹ To investigate if the same effect of SSB would be observed with a monomer of NS3h, we used a forked substrate with a 30 bp duplex region and a dT₅ 3'-loading tail (3'-5Tds30, Table 1). The short tail length ensures that only one molecule of NS3h is bound to the loading tail. The unwinding reactions were carried out by preincubating NS3h with the short-tail fork substrate and starting the reaction by adding ATP, SSB, and excess mutant D261A. Excess of the mutant added in the reaction would drastically reduce the possibility of another WT helicase from loading behind the prebound WT NS3h as the substrate gets progressively unwound. Given these stringently controlled conditions, we

observed substantial unwinding of the 30-bp substrate (Fig. 6a). The kinetics of unwinding were very similar to those with the 15-nt 3' tail, an unwinding rate of 4 bp/s, indicating that a single molecule of NS3h is sufficient for unwinding DNA with a high processivity in the presence of SSB. These results also demonstrate that a monomer of NS3h helicase is sufficient for DNA unwinding. Experiments were also done where the 30-bp substrate (3'-5Tds30, Table 1) was incubated with the DNA in a 1:1 ratio and the reaction was initiated using ATP from the other syringe in the presence of ATP. Burst-type kinetics is observed, with ~12% products formed at the end of the burst phase (Fig. 6b). This seems consistent with the fact that given equal proportion of enzyme and substrate, only one-sixth of the total enzyme population is favorably bound initially to generate the products. The rate of product formation is slightly slower (1.8 bp/s) than the earlier observed single-turnover rate of unwinding.

SSB increases the unwinding processivity of full-length HCV helicase

The unwinding kinetics of the full-length protease-helicase construct NS3-4A enzyme was studied under similar conditions. NS3-4A was preincubated with the 40-bp forked substrate (2Tds40, 40%; Table 1), and the reaction was initiated with ATP from the other syringe in the presence of either SSB or PolyU. Using PolyU in the reaction bound up any free NS3-4A that either did not form the enzyme-substrate complex or dissociated from the DNA during unwinding. This ensured single-turnover conditions for unwinding. As was the case with the helicase domain (NS3h), unwinding by NS3-4A in the presence of SSB resulted in nearly fourfold higher product formation than in the presence of PolyU (Fig. 7). Thus, SSB increases the processivity of DNA unwinding by the full-length NS3-4A construct as well. As was the case with NS3h, the unwinding rate was similar in the presence or absence of SSB.

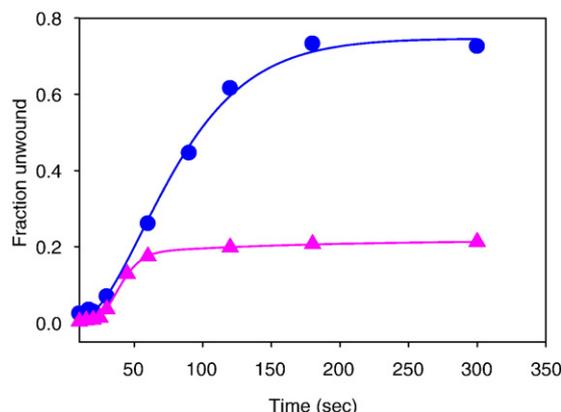


Fig. 7. SSB enhances the unwinding processivity of the full-length helicase-protease NS3-4A. The 40-bp forked substrate (Table 1, 3) is unwound by the full-length helicase-protease NS3-4A in the presence (●) and absence (▲) of SSB at average rates of 0.22 and 0.4 bp/s, respectively.

Discussion

RNA helicases are involved in various aspects of RNA metabolism such as transcription, splicing, translation, RNA degradation, etc.⁴⁰ The low unwinding processivity of these proteins is often correlated to their functions in the RNA manipulation. That, however, cannot explain the role of many of these RNA helicases in replication. Although HCV NS3 protein has not been established as the replicative helicase, knocking out NS3 inhibits viral replication *in vitro*.^{41,42} This enforces the fact that NS3 is essential for viral replication in HCV virions and also makes NS3 a strong candidate for the replicative helicase of HCV. In a similar scenario amongst the DNA helicases, where the replicative helicase has low unwinding processivity (e.g., DnaB, T7 gp4A, T4 Dda), the problem is alleviated when the unwinding is carried out in the presence of SSB proteins.^{43–45} Our results presented here indicate an identical situation where SSB proteins enhance the unwinding processivity by keeping the unwinding helicase longer on its track.

Under the conditions of enhanced processivity, we have been able to demonstrate that the NS3h monomer is active in unwinding DNA. It has already been established that NS3h has a binding site of about seven bases.³⁹ By using a forked substrate with a dT₅ loading tail, we could restrict the number of NS3h molecules bound initially to the 3' overhang to one. By carrying out the unwinding reaction in the presence of SSB and an excess of the NS3h helicase-deficient mutant, we ensured that the mutant NS3h rather than additional WT NS3h would bind to the intermediates. Since the mutant does not possess any unwinding activity, the observed DNA unwinding reaction is the outcome of activity of the NS3h monomer.

Although the exact mechanism by which SSB stabilizes the helicase at the fork junction is not known, we can speculate on two scenarios: (1) there is a physical interaction between the helicase and SSB, which prevents helicase dissociation from the fork junction, or (2) the binding of SSB alters the nucleic acid structure at the junction in such a way that the helicase now binds the junction with a higher affinity. Although the first situation sounds straightforward, in the context of *E. coli* SSB and HCV NS3h it does not hold any direct physiological relevance. The physical interaction between the HCV helicase and SSB can be nonspecific or electrostatic in nature, such as acidic regions of SSB interacting with basic regions of the HCV helicase. Our observations, however, open the possibility that there could be some contenders in the cell that play a role of SSB encoded either from within the HCV genome or from host proteins. One of the cellular RNA binding factors that was shown to be associated with the HCV genome and required for its replication was the polypyrimidine tract binding protein.^{46–48} This protein was also shown to interact with the proteins of the HCV replication complex, notably NS3 helicase and NS5B polymerase.⁴⁹

Another strong contender could be the HCV protein that has been identified to have RNA-binding properties, NS5A.⁵⁰ Within the cell, the replication complex is membrane bound. In such a situation, the presence of accessory proteins functionally homologous to SSB could ensure the required processivity by mediating protein–protein interactions with other proteins of the complex and preventing dissociation of the helicase from the replication complex and RNA.

Materials and Methods

Proteins

The helicase domain of genotype 1b HCV NS3 protein was overexpressed in *E. coli* carrying the pET21b-NS3HCV plasmid.⁵¹ NS3h protein was purified by metal-immobilization chromatography through its C-terminal hexa-His tag and stored as described previously.³³ The GenBank accession number of the protein is M62321. NS3-4A used in the experiments is a single-chain construct where the NS4A fragment is covalently attached to the N-terminus of the NS3 protein.⁵² The NS3-4A protein was purified as previously described.⁵² Purified NS3-4A was obtained from Dr. Madhura Gurjar (Robert Wood Johnson Medical School, NJ). The protein concentration was determined from absorbance at 280 nm in 8 M urea. The extinction coefficients of NS3h ($47,600 \text{ M}^{-1} \text{ cm}^{-1}$) and NS3-4A ($102,000 \text{ M}^{-1} \text{ cm}^{-1}$) were calculated by adding the molar extinction coefficients of tryptophan ($5690 \text{ M}^{-1} \text{ cm}^{-1}$) and tyrosine ($1280 \text{ M}^{-1} \text{ cm}^{-1}$) residues. The protein concentration was also checked using the Bradford method using bovine serum albumin as the standard.⁵³ NS3h D261A (previously referred to as D1316A based on the polyprotein reading frame) carried an Asp to Ala mutation at position 261 in the Walker B motif, which made the enzyme deficient in ATP hydrolysis.³³ The D261A mutant was purified and stored using the same procedure as for the wild-type NS3h.

The *E. coli* SSB protein (SSB) was purified as described by Lohman *et al.*⁵⁴ The bacteriophage T7 SSB protein, gp2.5, was overexpressed by induction with IPTG to a final concentration of 0.4 mM in *E. coli* BL21(DE3) containing the plasmid pAR3505. The cells were lysed using lysozyme/freeze–thaw treatment and the lysate was precipitated with 1 M ammonium sulfate. The protein pellet was resuspended in buffer and passed through three subsequent ion-exchange columns (DE-52, phosphocellulose-P11, and hydroxyapatite). The final eluent was concentrated on a diethylaminoethyl (DEAE) column to yield pure gp2.5, which was free of any detectable nucleases (Dr. Studier, personal correspondence).

Nucleic acids and other reagents

Oligodeoxynucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and purified by PAGE in 5 M urea, 50 °C. The oligodeoxynucleotide concentrations were determined from absorbance at 260 nm, using the extinction coefficient obtained by adding the extinction coefficients of the individual bases. Forked DNA substrates were generated by annealing together the 3' and the 5' strands. The unwinding substrates used in this study are listed in Table 1. PolyU, average length of 210

protein or NS3h D261A mutant protein, radiolabeled ssDNA substrate, 0.1 mg/ml bovine serum albumin, and reaction buffer were incubated for 30 min and were filtered through the wet membranes followed by washing with the reaction buffer.^{55,56} The amount of radioactivity retained by each membrane was measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Helicase unwinding assay

The kinetics of dsDNA unwinding was measured at 22 °C using RQF-3 Quench-Flow apparatus (KinTek Instruments, Austin, TX). NS3h (200 nM) was incubated with 5 nM ss/dsDNA substrate in buffer (50 mM Mops-NaOH, 5 mM MgCl₂, 5 mM DL-dithiothreitol, and 0.1% Tween 20, pH 7.0) in one syringe and mixed with 10 mM ATP and 2.0 μM SSB in the same buffer from the other syringe for the times indicated. The unwinding reaction was stopped by the addition of a 1.5-fold volume of quenching solution containing 100 mM ethylenediaminetetraacetic acid, 1% SDS, 500 nM PolyU, and 200 nM DNA trap. The duplex and unwound DNA substrates were resolved on a native 10% polyacrylamide gel, and the fraction of DNA unwound was determined using a PhosphorImager (Molecular Dynamics). The fraction of unwound substrate was calculated and corrected for the presence of ssDNA at time 0 using Eq. (1):

$$F = \frac{(SS \times DS_0) - (DS \times SS_0)}{DS \times (SS + DS)} \quad (1)$$

where F is the fraction of unwound substrate, DS and SS are intensities of duplex and unwound substrate bands at a given time, respectively, and DS_0 and SS_0 are intensities of duplex and unwound substrates at time 0, respectively.

Data analysis

The unwinding time courses were fit as described previously^{11,15,32} to the incomplete gamma function [Eq. (2)]:

$$F = \frac{A}{\int_0^\infty e^{-x} x^{n-1} dx} \int_0^{kt} e^{-x} x^{n-1} dx \quad (2)$$

where F is a fraction of unwound DNA substrate molecules, A is the amplitude of unwinding, k is the stepping rate, and t is reaction time. The number of steps, n , taken by the helicase to unwind the substrate was calculated as

$$n = \frac{L_{DS} - L_a}{s} \quad (3)$$

where L_{DS} is the number of base pairs in the DNA substrate duplex, L_a is the length of the shortest DNA duplex that can stay together under the experimental conditions (10 bp), and s is the step size. The average unwinding rate is $k \times s$. The software MATLAB with Optimization toolbox (MathWorks, Inc., Natick, MA) was used for all calculations.

technical advice on gp2.5 purification. This work was supported by NIH grant GM55310.

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Acknowledgements

We thank Dr. Madhura Gurjar for purified NS3-4A protein and Dr. William F. Studier for the

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