Diffusion of human Replication Protein A along single stranded DNA

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Abstract

Replication Protein A (RPA) is a eukaryotic single stranded (ss) DNA binding protein that plays critical roles in most aspects of genome maintenance, including replication, recombination and repair. RPA binds ssDNA with high affinity, destabilizes DNA secondary structure and facilitates binding of other proteins to ssDNA. However, RPA must be removed from or redistributed along ssDNA during these processes. To probe the dynamics of RPA-DNA interactions, we combined ensemble and single molecule fluorescence approaches to examine human RPA diffusion along ssDNA and find that an hRPA hetero-trimer can diffuse rapidly along ssDNA. Diffusion of hRPA is functional in that it provides the mechanism by which hRPA can transiently disrupt DNA hairpins by diffusing in from ssDNA regions adjacent to the DNA hairpin. hRPA diffusion was also monitored by the fluctuations in fluorescence intensity of a Cy3 fluorophore attached to the end of ssDNA. Using a novel method to calibrate the Cy3 fluorescence intensity as a function of hRPA position on the ssDNA, we estimate a one-dimensional diffusion coefficient of hRPA on ssDNA of $D_1 \sim 5000$ nucleotide$^2$s$^{-1}$ at 37°C. Diffusion of hRPA while bound to ssDNA enables it to be readily repositioned to allow other proteins access to ssDNA.

Keywords: RPA, single molecule fluorescence, FRET, dynamics, DNA hairpin melting, diffusion coefficient
Introduction

Replication protein A (RPA) is the major eukaryotic single stranded (ss) DNA binding (SSB) protein that plays a central role in genome maintenance by binding tightly to ssDNA[1-4]. Similarly to bacterial SSB proteins[5], RPA binds with high affinity to ssDNA that is formed transiently during DNA replication, recombination and repair, protecting it from nucleases, and destabilizing unwanted secondary structures (e.g., hairpins[6] and G-quadruplexes[7]). Additionally, through direct protein-protein interactions, RPA coordinates the targeting of other DNA processing proteins to their sites of action on DNA[1-3]. RPA also serves as an important intermediate in DNA damage checkpoint signaling[8]. In all eukaryotes, RPA is a hetero-trimer consisting of Rpa1 (~70 kDa), Rpa2 (~32 kDa) and Rpa3 (~14 kDa). All three subunits contain oligonucleotide/oligosaccharide binding (OB) folds[9], with Rpa1 containing 4 OB-folds (F, A, B and C), while one additional OB-fold is contained in each of Rpa2 (D) and Rpa1 (E) (see Figure 1A). RPA2 also contains a winged helix domain that is primarily involved in protein interactions[1-4]. OB-folds A, B, C and D function in ssDNA binding[3, 10] as shown in a crystal structure of Ustilago maydis RPA bound to ssDNA (dT32) (Figure 1B) in which 25 of the DNA nucleotides are observable[11]. OB-fold F is primarily involved in protein interactions[12] but it also seems to bind weakly to ssDNA[13] and affects interactions with partial duplex DNA[14]. OB-fold E shows weak affinity for telomeric DNA[15].

Whereas RPA must bind with high affinity to ssDNA to carry out its functions, it must also be displaced from ssDNA or be redistributed along ssDNA to make room for other DNA processing proteins to carry out their functions. Yet, there is little known about the dynamics of RPA while bound to ssDNA. Using a combination of ensemble and single molecule fluorescence approaches, we show in this report that human RPA (hRPA) protein diffuses rapidly along ssDNA, while remaining bound to ssDNA. We further show that hRPA diffusion along ssDNA is functional in that it enables RPA to transiently invade and destabilize (melt) a DNA hairpin
structure, which is an essential property of hRPA. We propose that this ability to diffuse on ssDNA provides RPA with a simple mechanism by which it can coordinate assembly and disassembly of other proteins during its multiple functions in genome maintenance.

**Results**

**Equilibrium binding affinity and kinetics of hRPA binding to ssDNA.**

Human RPA is a hetero-trimer composed of a 70 kDa subunit, Rpa1, a 32 kDa subunit, Rpa2, and a 14 kDa subunit, Rpa3 (Figure 1A). A recent structure of a truncated version of the *Ustilago maydis* RPA bound to (dT)$_{32}$ (Figure 1B) shows three OB-folds within Rpa1 (A, B and C) interacting with ~20 nts, while OB-folds A, B, C and D from Rpa2 interact with 25 nts[11]. Our previous studies showed that *S. cerevisiae* RPA (scRPA) undergoes a [NaCl]-dependent transition between two ssDNA (poly(dT)) binding modes[16]. Figure 1C indicates that hRPA displays the same behavior. At [NaCl] < 50 mM, hRPA binds poly(dT) with an occluded site size of 22±1 nucleotides (nts), whereas at [NaCl] > 1 M, hRPA binds poly(dT) with an occluded site size of 28-30 nts[16]. This suggests that below 50 mM NaCl, RPA interacts with ssDNA using primarily the three OB-folds (A, B, and C) within Rpa1, whereas at higher [NaCl] RPA binds with a larger site size that involves additional interactions with OB-fold (D) within Rpa2[10, 16].

The majority of the experiments reported here were performed in Buffer T plus 500 mM NaCl at 25°C so that the DNA hairpins that we investigate have high stability in the absence of hRPA. hRPA binds to ssDNA with high affinity even at such high [NaCl] concentrations[16]. To determine the equilibrium constant, $K_{\text{obs}}$, at 500 mM NaCl for hRPA binding to (dT)$_{30}$, a ssDNA long enough to maintain all contacts, we performed equilibrium titrations monitoring the quenching of hRPA tryptophan fluorescence at 1.0, 0.9 and 0.8 M NaCl. The values of $K_{\text{obs}}$, obtained from fitting the hRPA-(dT)$_{30}$ binding isotherms to a 1:1 binding model are plotted as log$K_{\text{obs}}$ vs. log[NaCl] in Figure 1D. Linear extrapolation of these data yields $K_{\text{obs}} \sim 10^{10}$ M$^{-1}$ for
hRPA binding to (dT)$_{30}$ at 500 mM NaCl. Figure 1D also shows that $K_{\text{obs}}$ at 1M NaCl for hRPA binding to 3'-Cy3-(dT)$_{29}$ and (dT)$_{30}$ are the same thus indicating little effect of the Cy3 fluorophore label on $K_{\text{obs}}$. We also examined the association and dissociation kinetics of hRPA binding to 3'-Cy3-(dT)$_{30}$, monitoring the change in Cy3 fluorescence that accompanies binding/dissociation (data not shown). In Buffer T, 500 mM NaCl at 25.0˚C, stopped-flow kinetic measurements (data not shown) indicate a bimolecular association rate constant, $k_a = 1.8 \times 10^8$ M$^{-1}$ s$^{-1}$, and a dissociation rate constant, $k_d=0.018$ s$^{-1}$, consistent with our estimate of $K_{\text{obs}}$. Hence, under these conditions, hRPA has an average lifetime of ~55 sec on (dT)$_{30}$.

**Diffusion of hRPA along ssDNA probed by single molecule fluorescence.**

Single molecule total internal reflectance fluorescence (smTIRF) studies were carried out as described[17] and shown schematically in Figure S1. DNA with a biotinylated 18 bp duplex “handle” and a ssDNA flanking region (sequences of all oligodeoxynucleotides are given in Table S1) was immobilized on a glass slide using neutravidin-biotin chemistry as described in Methods. As an initial qualitative test of whether hRPA can diffuse along ssDNA, we performed a smTIRF experiment by binding hRPA labeled with an average of one Cy5 fluorophore (see Methods) to a surface bound 3'-Cy3-(dT)$_{60}$ and monitored the FRET (Förster Resonance Energy Transfer) efficiency between the Cy3 (donor) and Cy5 (acceptor) (Figure 2A). The Cy3 and Cy5 fluorescence signals show anti-correlated fluctuations, consistent with movement of Cy5-hRPA relative to the Cy3 at the 3’ end of the ssDNA. These FRET fluctuations are not due to multiple hRPA binding and dissociation events since the average lifetime of hRPA on (dT)$_{60}$ under these identical conditions is at least 55 sec (see above). An alternative is that hRPA is stably bound to a position on the DNA and that the FRET fluctuations arise due to the dynamics of the flexible Cy3-labeled ssDNA enabling contact with the acceptor labeled protein. If this were the case similar FRET fluctuations should be observed when the Cy5 acceptor is placed directly on the DNA substrate. A control experiment (Figure S2) with a DNA containing Cy3 and Cy5
separated by (dT)$_{60}$ shows a stable FRET signal near 0.15, indicating that the large fluctuations observed in Figure 2A are not due solely to the conformational dynamics of the ssDNA, but require the presence of bound hRPA. These and previous experiments show that the conformational dynamics of the ssDNA are very fast and averaged out within the 32 ms time resolution of these experiments[18]. We suggest that the FRET fluctuations reflect diffusion of hRPA toward and away from the Cy3 at the 3’ end of the DNA. A second test of hRPA diffusion was performed using a DNA in which Cy5 and Cy3 are both on the DNA and separated by (dT)$_{30}$, followed by an additional 18 nt of mixed sequence ssDNA containing all four bases (Figure 2B). The DNA alone shows a stable FRET signal with a value near 0.30 (Figure 2B). Upon hRPA binding, large anti-correlated Cy3 and Cy5 FRET fluctuations are observed (Figure 2C). These are expected if hRPA moves along the DNA due in part to the bending of the DNA as seen in the crystal structure (Figure 1B). However, when a complementary ssDNA is annealed to the 18 nt sequence to form a duplex, a stable FRET signal at 0.20 is observed due to confinement of hRPA to the (dT)$_{30}$ ssDNA (Figure 2D). The experiments in Figure 2 provide qualitative evidence that hRPA can diffuse along ssDNA. We do not know the precise position of the Cy5 label on the hRPA used in the experiments in Figure 2A since labeling could occur at the N-termini of any of the three hRPA subunits (see Methods), hence we are unable to analyze the fluctuations observed in Figure 2A more quantitatively. An alternative method to obtain quantitative information about hRPA diffusion is discussed below.

**hRPA can transiently melt DNA hairpins using its ability to diffuse on ssDNA.**

One important function of hRPA is to destabilize secondary structures, such as hairpins and G-quadruplexes, which can inhibit the functions of other proteins that act on ssDNA[1, 5, 19]. To determine if hRPA diffusion plays a role in destabilizing DNA secondary structures, we examined the ability of hRPA to destabilize a seven bp DNA hairpin possessing a stretch of (dT)$_{30}$ on the 5’ side of the hairpin. Cy3 and Cy5 labels were incorporated at the base of the
hairpin so that a decrease in FRET would accompany hairpin melting. The (dT)\textsubscript{30} stretch can accommodate one hRPA. In the absence of hRPA, a stable high FRET signal (0.91) is observed, consistent with a stable, closed hairpin (Figure 3A). Upon addition of hRPA large anti-correlated Cy3/Cy5 fluctuations occur between a high FRET state (0.83) and a low FRET state (~0.26) (Figure 3B). To assess the extent to which the DNA hairpin is melted, we examined a DNA in which the hairpin was replaced by (dT)\textsubscript{20} so that the Cy3 and Cy5 were separated by the same DNA contour length. In the absence of hRPA a stable FRET efficiency (0.66) was observed (Figure S3A), but when hRPA was added, FRET fluctuations between 0.21 and 0.66 were observed (Figure S3B). The 0.21 FRET efficiency is the same as the low FRET efficiency observed in Figure 3B and likely occurs when the hRPA moves fully toward the 3’ end. These results suggest that hRPA is able to fully melt the seven bp hairpin, albeit transiently. We also examined whether hRPA can invade a longer 18 bp hairpin on the 5’ side of a (dT)\textsubscript{30} as monitored by Cy3/Cy5 probes placed internally in the hairpin, 9 bp from the base (Figure S3C). However, we observed no effect of hRPA on the FRET signal from these probes indicating that a single hRPA is not able to move this far into the hairpin.

As a further test of whether hRPA melts the hairpin by diffusing in from the (dT)\textsubscript{30} binding site, we examined a DNA containing a (dT)\textsubscript{30} stretch and the same 7 bp DNA hairpin, but these two regions are connected by a 3’-3’ phosphodiester linkage (marked by a red X in the DNA in Figure 3C). Since the ssDNA binding site of RPA is polar[11, 20] (Figure 1B), if RPA melts the hairpin by a diffusional mechanism then both DNA segments ((dT)\textsubscript{30} and the hairpin) must have the same continuous backbone polarity. In fact, a stable FRET signal (0.91) is observed for this 3’-3’-linked DNA in the presence of hRPA indicating that reversal of the phosphodiester backbone blocks RPA diffusion and its access to the DNA hairpin. Ensemble fluorescence experiments monitoring hRPA Trp fluorescence quenching demonstrates that hRPA can bind to this DNA. Therefore, on the normal hairpin DNA, hRPA binds to the (dT)\textsubscript{30} region and transiently
melts the hairpin DNA by diffusing in from the (dT)$_{30}$ region. A control DNA containing a hairpin, but no (dT)$_{30}$ loading site shows no FRET changes even in the presence of 300 nM hRPA (Figure S3D) indicating that the (dT)$_{30}$ binding site is required for hRPA to melt the hairpin. The rates of hRPA-induced opening ($k_{op}$) and closing ($k_{cl}$) of the DNA hairpin were estimated by fitting the dwell times of the closed and open states, as identified by a Hidden Markov analysis[21], to a single exponential decay (details in Methods and Figures S4B and S5). From these we estimate $k_{op} = 3.6 \pm 0.2$ s$^{-1}$ and $k_{cl} = 6.1 \pm 0.5$ s$^{-1}$ at 500 mM NaCl (Table 1). We note that hRPA is also able to transiently melt the hairpin in the presence of Mg$^{2+}$ (Buffer T containing 100 mM NaCl, 5 mM MgCl$_2$) (Figure S4A), conditions often used in enzymatic studies of replication, recombination and repair, with similar rates (Table 1), as well as at lower [NaCl] (20 mM (Figure S4B, Table 1). hRPA is also able to melt out the hairpin with similar rates regardless of whether the Cy3 is located on the thymidine base or as part of the DNA backbone (Table 1).

We next examined the ability of hRPA to melt out the same seven bp DNA hairpin, but with a (dT)$_{30}$ on the 3' side of the hairpin (Figure 3D). Although hRPA is able to melt the hairpin with this opposite orientation, it does so much less efficiently. The population of melted hairpins is very low with rates, $k_{op} = 0.54 \pm 0.8$ s$^{-1}$ and $k_{cl} = 6.3 \pm 0.8$ s$^{-1}$ at 500 mM NaCl. Interestingly, it is the rate of hairpin opening that is affected by having the (dT)$_{30}$ on the 3' side of the DNA hairpin. This suggests the interesting possibility that the hRPA does not invade the hairpin by simply capturing the ssDNA that forms transiently due to fluctuations in the hairpin, but may facilitate the melting process. Otherwise it should not matter whether the hairpin is on the 3' or 5' side of the (dT)$_{30}$. This is a distinct possibility since the region of hRPA that faces the hairpin differs when it is bound to these two DNA molecules (see cartoon in Table 1).

We also examined two variants of hRPA in which one or more of the OB-folds were deleted. We used two truncations of the Rpa1 subunit, FAB, in which OB-fold C, Rpa2 and
Rpa3 were removed, and ABC-D-E in which OB-fold F was removed. Both variants retain the ability to transiently melt the DNA hairpin possessing a \( (dT)_{30} \) on the 5’ side of the hairpin (Figure 3E and F). Hence, both variants must also be able to diffuse along ssDNA. The rates for hRPA-induced hairpin opening and closing (Table 1) differ somewhat from the values for the full length hRPA, again suggesting that hRPA may play some role in facilitating hairpin DNA melting. Since OB-folds A and B are sufficient for high affinity binding of RPA[22, 23], these data suggest that OB-folds A and B are sufficient to support diffusion.

**Use of Cy3 fluorescence on ssDNA to monitor diffusion of unlabeled hRPA.**

Cy3 fluorescence is enhanced when in the vicinity of a protein as first noted in studies of UvrD[24] and this has been used to study the binding of many proteins to nucleic acids[25-36]. This is also the case for hRPA (Figure 4). Equilibrium titrations of hRPA binding to a series of DNA molecules differing in ssDNA length, \( L (3’\text{-Cy3-}(dT)_{L}) \), were analyzed using the Binding Density Function Method[37] to determine the average Cy3 fluorescence enhancement when one hRPA is bound (Figure S6). The Cy3 fluorescence enhancement per one hRPA bound decreases with increasing ssDNA length (Figure 4E). This indicates that the average Cy3 fluorescence intensity is dependent on its distance from hRPA along the ssDNA contour length (Figure 4E). As we show quantitatively below, Cy3 fluorescence intensity is sensitive to the ssDNA contour length between the Cy3 and a single hRPA bound to the DNA. Thus, the Cy3 fluorescence can be used to monitor diffusion of hRPA on ssDNA due to the change in the DNA contour length that separates hRPA from the Cy3 end of the DNA.

Figure 4A shows a Cy3 smTIRF time trace for a single DNA molecule with a ssDNA region \((3’\text{-Cy3-}(dT)_{60})\). Upon addition of 37 pM hRPA, the average Cy3 fluorescence intensity increases (Figure 4B). Histograms of hRPA binding events (Figure 4D) indicate that the average Cy3 fluorescence enhancement (34%) observed in the smTIRF experiment is the same as for a
single hRPA binding to the same DNA in ensemble studies (Figure 4E). Notably, in addition to the increase in the average Cy3 fluorescence intensity, we also observe an increase in the fluctuations in the Cy3 fluorescence intensity (Figure 4B). At much higher hRPA concentration (370 nM), transient binding of a second hRPA to a single (dT)₆₀ can be observed as an additional Cy3 fluorescence enhancement (60%) due to the fact that one of the hRPA molecules is forced to bind closer to the Cy3 end (Figure 4C), consistent with ensemble titrations (Figure S6). However, this second hRPA dissociates quickly, consistent with RPA lacking cooperative binding[16, 38, 39]. The binding of a second hRPA is also marked by smaller Cy3 fluorescence fluctuations (compared to 1 hRPA bound). As we show below, the increased Cy3 fluorescence fluctuations shown in Figure 4B are due to diffusion of a single hRPA hetero-trimer along the ss DNA.

The same experiment performed for a DNA with a longer ssDNA region (3'-Cy3-(dT)₁₄₀) showed a similar result (Figure 5A and B). Binding of hRPA (10 pM) results in both an increase in Cy3 fluorescence intensity and fluctuations. The time trace in Figure 5B also shows an example of hRPA dissociation and rebinding events that clearly differ from the Cy3 fluctuations observed when hRPA remains bound to the DNA. To gain insight into the increased Cy3 fluorescence intensity fluctuations due to binding of a single hRPA, we performed auto-correlation analyses on the Cy3 fluctuations associated with the DNA alone and the DNA in the presence of hRPA (10 pM). Low hRPA concentrations were used to minimize the probability that more than one hRPA is bound per DNA. For 3'-Cy3-(dT)₁₄₀ alone, the average auto-correlation function, G(τ), is flat (red symbols in Figure 5C) indicating that those fluctuations are random. However, a measureable averaged auto-correlation function was obtained upon analysis of (3'-Cy3-(dT)₁₄₀) bound with a single hRPA (blue symbols in Figure 5C). The auto-correlation function was best described by a two exponential decay, with τₑ,₁ = 46±4 msec and τₑ,₂ = 298±18 msec. We examined and performed auto-correlation analyses on data for DNA
molecules with ssDNA lengths of L= 60, 90, 120 and 140 nucleotides at low [hRPA] (1-50 pM) at two temperatures (10˚C and 25˚C). For each DNA length, the resulting G(τ) was fit to a two exponential decay (Figure S7). The values of τc,1 ranged from 20 to 50 msec, are near the time resolution of the instrument (32 msec) and are due to photophysical or detection noise as noted previously[40] and thus were not considered further. For ssDNA molecules with a single hRPA bound, τc,2 increases with increasing ssDNA length, with a steeper increase observed for the data at 10˚C (Figure 5D). As shown below, Monte Carlo simulations of a protein undergoing a one-dimensional random walk along DNA indicate that τc should increase nearly linearly with increasing DNA length and that τc varies inversely with the one-dimensional diffusion coefficient of the protein on the DNA. Figure 5E shows an Arrhenius plot of ln(1/τc,2) vs. inverse temperature for hRPA bound to 3’-Cy3-(dT)120 from which we calculate an activation energy for hRPA diffusion (Ea = 10.1±0.5 kcal/mole). The dependence of τc,2 on ssDNA length and temperature supports our conclusion that the hRPA-induced Cy3 fluorescence intensity fluctuations reflect hRPA diffusion along the ssDNA.

**Calibration of the ssDNA contour length dependence of the hRPA-induced Cy3 fluorescence enhancement.**

Since the values of τc in Figure 5D appear to be sensitive to hRPA diffusion along ssDNA, we sought to use these to obtain a quantitative estimate of the one-dimensional diffusion coefficient, D1, for hRPA on ssDNA. Our approach was to perform Monte Carlo simulations of a random walk of hRPA on ssDNA for an input value of D1 to simulate the time-dependent fluctuations of the position of hRPA on the DNA. These could then be analyzed in the same way that we analyzed the Cy3 fluorescence fluctuations to obtain simulated values of τc (τsim) to compare with the experimental values. However, in order to use this approach we need to know how the Cy3 fluorescence changes as a function of the DNA contour length between the hRPA and the Cy3 labeled 3’-end of a DNA (dT)L. For this we synthesized a series
of chimeric DNA molecules containing two distinct single stranded segments with widely
different affinities for hRPA (Figure 6A). These contain an 18 bp duplex DNA “handle” with a
stretch of (dT)\textsubscript{30} followed by an additional “N” dT nucleotides in which the phosphodiester bonds
between each successive nucleotide alternate between 3′-3′ phosphodiester linkages and 5′-5′
phosphodiester linkages (referred to as (dT\textsuperscript{alt})\textsubscript{N}, and represented by the red saw tooth in Figure
6A). Such a DNA molecule has a high affinity site for hRPA binding ((dT)\textsubscript{30}) that is short enough
to limit hRPA diffusion, followed by a stretch of (dT\textsuperscript{alt})\textsubscript{N} to which hRPA has much lower affinity
(~450-fold) as shown by the equilibrium isotherms for hRPA binding to (dT)\textsubscript{30} vs. (dT\textsuperscript{alt})\textsubscript{30} (Figure
6C). This affinity difference ensures that in a 1:1 hRPA complex with a chimeric (dT)\textsubscript{30}-(dT\textsuperscript{alt})\textsubscript{30}
more than 99% of the hRPA will be bound to the (dT)\textsubscript{30} stretch. Importantly, the alternating
ssDNA (dT\textsuperscript{alt})\textsubscript{N} has the same flexibility as normal (dT)\textsubscript{N} since the same average FRET efficiency
value is observed for (Cy5-(dT)\textsubscript{60}-Cy3) and (Cy5-(dT)\textsubscript{30}-(dT\textsuperscript{alt})\textsubscript{30}-Cy3) (Figure S2).

Ensemble titrations at two different DNA concentrations were performed for the series of
DNA chimeras ((dT)\textsubscript{30}-(dT\textsuperscript{alt})\textsubscript{N}–Cy3-3’), with N= 2, 5, 10, 20, 30 and 40, monitoring the
enhancement of Cy3 fluorescence upon binding of hRPA. These were analyzed using the
Binding Density Function Analysis (Figure S8)[37] to obtain the Cy3 fluorescence enhancement
for binding one hRPA to the high affinity (dT)\textsubscript{30} site (Figure 6D). The Cy3 fluorescence enhancement displays an exponential decrease with increasing N. For comparison, the binding
of one hRPA to the (dT)\textsubscript{30} site of (dT)\textsubscript{30}-(dT\textsuperscript{alt})\textsubscript{30}–Cy3-3′ induces only a ~10% increase in Cy3
fluorescence compared to a ~32% increase upon binding to a normal DNA of the same length
((dT)\textsubscript{60}). The lower enhancement results from the fact that in the chimeric DNA containing the
(dT\textsuperscript{alt})\textsubscript{30}, hRPA binds only to the normal polarity (dT)\textsubscript{30} site and thus is constrained to be further
away from the Cy3 (in nucleotides along the contour length). If we assume the absence of end
effects, then when one hRPA is bound to a (dT)\textsubscript{L} with normal polarity, its average position will be
in the middle of the ssDNA region. Then the Cy3 enhancement for one hRPA bound to a
chimeric DNA with N alternating polarity nucleotides \(((dT)_{30}(dT_{alt})^NCy3-3')\) should be the same as the Cy3 enhancement for a normal polarity DNA \((dT)_L-Cy3-3'\) with \(L= (30+2N)\) (see Figure 6B). Using this relationship, Figure 6D shows that the Cy3 fluorescence enhancement from ensemble studies with \((dT)_{30}(dT_{alt})^NCy3-3'\), and \((dT)_LCy3-3'\), as well as single molecule experiments with normal \((dT)_LCy3-3'\) \((L = 30, 50 \text{ and } 60)\) all fall on the same exponential curve given by \(E = E_0 + E_m \exp(-N/N_C)\) (where \(E_0 = 4\pm7, \ E_m = 65\pm7\) and \(N_C = 15\pm4\)) or equivalently \(E = E_0 + E_m \exp(-(L-30)/L_C)\), where \(L_C = 30\pm7\). This indicates that hRPA binding to the normal polarity ssDNA does not show significant end effects and that the hRPA enhancement of Cy3 fluorescence upon binding to these ssDNA molecules decays exponentially with the ssDNA contour length that separates them. These results also provide further support to the conclusion that \((dT_{alt})^N\) has the same flexibility as normal \((dT)^N\). Studies performed at \(10^\circ C\) indicate that the same dependence of Cy3 fluorescence enhancement on \(N\) applies at both \(10^\circ C\) and \(25^\circ C\) (data not shown). These results enable us to use the Cy3 fluorescence enhancement upon hRPA binding to calculate the average number of nucleotides that separate the hRPA from the Cy3-3' end for each \((dT)_L\).

We also performed smTIRF experiments for hRPA binding to the chimeric \(((dT)_{30}(dT_{alt})^N-Cy3-3')\) molecules to examine the Cy3 fluctuations and the auto-correlation times obtained when hRPA cannot diffuse along the ssDNA. In this case, the only mechanism to bring Cy3 close to the hRPA would be due to the flexibility of the \((dT_{alt})^N\). When hRPA binds to the high affinity \((dT)_{30}\) site a Cy3 fluorescence enhancement is observed, however, the Cy3 fluctuations are much smaller and an autocorrelation function with a much smaller amplitude is obtained (Figure S9). This suggests that although the increase in average Cy3 fluorescence intensity upon hRPA binding to the normal polarity Cy3-(dT)_L is due to Cy3 interactions with hRPA due to DNA flexibility, the increase in non-random Cy3 fluorescence fluctuations is due to hRPA diffusion along the ssDNA.
One-dimensional diffusion coefficients of hRPA on ssDNA.

We performed Monte Carlo simulations in order to estimate one-dimensional diffusion coefficients \((D_1)\) of hRPA on ssDNA from the auto-correlation times. We simulated one-dimensional random walks for a protein with a contact size on the DNA of 30 nucleotides, for different input values of \(D_1\) and ssDNA length, \(L\), as outlined in Figure 7A and B (see Methods). We used the empirically determined relationship between Cy3 fluorescence enhancement and \(N\) (number of nucleotides along the ssDNA contour length between the Cy3 and the edge of hRPA (Figure 7C) to convert RPA position into Cy3 fluorescence intensity. Use of this calibration assumes that the conformational dynamics of the ssDNA are much more rapid than the movement of hRPA along the DNA so that the average Cy3 fluorescence equilibrates as hRPA moves from one position to the next along the ssDNA, thus providing a measure of the number of nucleotides between Cy3 and the protein edge. This assumption is consistent with smTIRF studies[18] that show no time-dependent FRET changes for the same types of ssDNA molecules used here when labeled with Cy3 and Cy5 that are separated by \((dT)_L\) (see Figure S2). We then averaged the Cy3 signal in 32 ms bins to match the time resolution of the experiment. Auto-correlation functions were then performed on 100 sets of simulated Cy3 time traces for each input value of \(L\) and \(D_1\) and averaged in the same manner as for the experimental data. These averaged auto-correlation functions were then fit to a single exponential decay function to obtain \(\tau_{\text{sim}}\). We note that it is important to know the quantitative relationship between Cy3 fluorescence intensity and hRPA position, since this will affect the quantitative results of the simulations, emphasizing the need to calibrate this effect for the particular protein and DNA under study.

Figures 7C and D show the dependence of the simulated auto-correlation times, \(\tau_{\text{sim}}\), on \(D_1\) for the four ssDNA lengths used in our experiments (\(L=60, 90, 120\) and 140 nucleotides).
The values of \( \tau_{\text{sim}} \) are inversely proportional to \( D_1 \) for each length (Figure 7C) and increase with increasing ssDNA length (Figure 7D) for a given value of \( D_1 \). This is the same behavior observed for the experimental \( \tau_C \) (Figure 5D). The simulated data in Figure 7C indicate that \( \tau_{\text{sim}} \) is more sensitive to \( D_1 \) for the longer lengths of ssDNA, with \( L=60 \) nts showing the least sensitivity. This suggests that the data obtained for the longer ssDNA lengths should provide a more accurate estimate of \( D_1 \).

Figure 7D shows \( \tau_{\text{sim}} \) as a function of ssDNA length for values of \( D_1 \) from 900 to 4000 nt\(^2\)s\(^{-1}\). The \( \tau_{\text{sim}} \) values (small black circles) increase linearly with \( L \), for \( L=90 \) to 140 nt, but deviate from linearity for \( L \leq 60 \) nt. Both the individual \( \tau_{\text{sim}} \) values and the slopes of the \( \tau_{\text{sim}} \) vs. \( L \) plots decrease with increasing \( D_1 \). The experimental \( \tau_C \) values determined at 10\(^{\circ}\)C and 25\(^{\circ}\)C are also shown in Figure 7D. The \( \tau_C \) values determined at 10\(^{\circ}\)C show the predicted linear dependence on \( L \), and agree well with the \( \tau_{\text{sim}} \) for \( D_1=1000 \) nt\(^2\)/s. The experimental values of \( \tau_C \) determined at 25\(^{\circ}\)C increase with \( L \) for \( L=90,120 \) and 140 nt, but the value for \( L=60 \) nt is indistinguishable from the value for \( L=90 \) nt. This suggests that at 25\(^{\circ}\)C, the hRPA diffusion is too fast to be accurately assessed for the shorter DNA lengths. Comparison of the experimental \( \tau_C \) and \( \tau_{\text{sim}} \) values for \( L=120 \) and 140 nt indicates a diffusion coefficient of 2800\(\pm\)200 nt\(^2\)/s at 25\(^{\circ}\)C and 1050\(\pm\)10 nt\(^2\)/s at 10\(^{\circ}\)C. Based on these estimates and the activation energy of 10.1\(\pm\)0.5 kcal/mol determined from \( \tau_C \) for experiments with \( L=120 \) nt (Figure 5E), we extrapolate to a value of \( D_1 \sim 5100\pm400 \) nt\(^2\)/s for hRPA at 37\(^{\circ}\)C.

**Discussion**

Single molecule fluorescence has proved invaluable for studies of the dynamics of proteins on single stranded DNA[41]. We present evidence from single molecule TIRF
experiments that a single hRPA hetero-trimer can diffuse along ssDNA, while bound with high affinity. Furthermore, this ability to diffuse along ssDNA is important functionally since it provides the mechanism by which hRPA is able to transiently melt DNA hairpins. We also designed a novel set of chimeric DNA molecules to calibrate the dependence of the Cy3 fluorescence enhancement on the distance (along the contour length) between the bound hRPA and the Cy3 fluorophore on 3’-Cy3-(dT)ₖ. The origin of the protein-induced Cy3 fluorescence enhancement has been proposed to be due to an effect on the photo-induced cis-trans isomerization within the Cy3 fluorophore[34, 36, 42]. The ssDNA molecules used in our study are very flexible, hence transient loop formation can bring the Cy3 in direct contact with the hRPA. Our calibration studies show that the magnitude of the Cy3 fluorescence enhancement decreases exponentially with the number of nucleotides along the contour length between the edge of the hRPA and the Cy3 end of the DNA. This is consistent with the expectation for the end-to-end distance of a semi-flexible or worm-like chain[18, 43] whose flexibility can be described by a single parameter, its persistence length. Previous studies of duplex DNA binding proteins concluded that the protein-induced Cy3 enhancement effect decreases linearly with protein distance from Cy3[34]; however, those data are also well described as an exponential decrease. More studies on other systems will be needed to determine whether the particular protein under study affects the magnitude and/or distance dependence of the Cy3 fluorescence enhancement.

Although ssDNA flexibility can explain the distance dependence of the average Cy3 fluorescence enhancement, the observation that the amplitude of the Cy3 fluorescence fluctuations increases upon hRPA binding reflects movement of hRPA along the ssDNA. Our smTIRF studies with the ssDNA chimeras that restrict hRPA diffusion show that ssDNA flexibility is not responsible for the increase in Cy3 fluctuations upon hRPA binding and the finite values of τᵥ obtained from auto-correlation analysis of those fluctuations. The conformational fluctuations of the ssDNA are fast and equilibrate within the 32 msec resolution of our CCD.
camera (see Figure S2 and ref[18]). Hence, the average Cy3 fluorescence intensity enhancement likely equilibrates for each position of hRPA as it diffuses along the ssDNA. Using the Cy3 fluorescence calibration data and Monte Carlo simulations, we show that the ssDNA length dependence of the experimental $\tau_C$ values is consistent with $\tau_C$ being sensitive to one-dimensional diffusion of hRPA on ssDNA. Based on these simulations, we estimate $D_1$ for hRPA on poly(dT) at several temperatures. The agreement between simulations and experiment is excellent at 10°C, yielding $D_1 = 1050 \pm 10 \text{ nt}^2\text{s}^{-1}$. At 25°C, we estimate $D_1 = 2800 \pm 200 \text{ nt}^2\text{s}^{-1}$, although we observe deviations of the experimental values of $\tau_C$ from the $\tau_{\text{sim}}$ for the shorter ssDNA lengths (60 and 90 nts). Those deviations may be due to the ability of RPA to partially melt the 18 bp duplex handle, which would yield a higher than expected value for the experimental $\tau_C$. Such deviations are expected to be more significant at 25°C than at 10°C and contribute more for shorter DNA lengths, as we observe. The advantage of the approach presented here to quantitatively examine diffusion of proteins bound to ssDNA is that unlabeled protein can be used and longer DNA lengths can be studied as compared to FRET-based approaches[40, 44, 45]. The extension of this method to other proteins should be straightforward, requiring only calibration of the distance dependence of the protein-induced Cy3 fluorescence enhancement.

We also demonstrate that hRPA diffusion provides the mechanism for hRPA to transiently disrupt DNA secondary structure. As shown for E. coli SSB[40], this ability to diffuse also allows hRPA to be pushed along the ssDNA, either by a directionally polymerizing protein, such as a Rad51 filament, or by a DNA polymerase. Our observation that transient melting of a DNA hairpin is more efficient when hRPA invades the hairpin from ssDNA on the 5’ side of the hairpin is intriguing and suggests a role for hRPA in facilitating the DNA hairpin disruption. Interestingly, scRPA has been shown to selectively promote the nuclease activity of Dna2 on a
5’ flap DNA substrate, but inhibit it on a 3’ flap DNA substrate[46]. It is possible that the preference that we observe here for hairpin melting may play a role in that selectivity.

Our demonstration that hRPA can diffuse on ssDNA and use this to disrupt DNA secondary structures adds these features to the growing list of similarities with the generally homo-tetrameric bacterial SSB proteins, such as E. coli SSB. E. coli SSB is a tetramer with 4 OB-folds that interact with ssDNA in its (SSB)₆₅ mode[47, 48] and hRPA has four OB-folds that primarily interact with ssDNA[10, 49, 50]. Both display salt-dependent transitions between ssDNA binding modes that involve switches in the numbers of OB-folds used to interact with ssDNA[16, 47, 48]. Although RPA and E. coli SSB bind with high affinity with very long lifetimes, they both can undergo a reasonably rapid protein concentration dependent exchange reaction with free protein[51, 52]. RPA has yet to be shown to undergo a direct or intersegment transfer reaction between ssDNA sites as has been shown for E. coli SSB[28, 53].

Although both hRPA and E. coli SSB can diffuse along ssDNA, the diffusion coefficient on poly(dT) for an hRPA hetero-trimer at 37°C (~5,000 nt²s⁻¹) is ~20-fold larger than that estimated for an E. coli SSB tetramer at 37°C (270 nt²s⁻¹)[40]. An additional difference is that the activation energy for hRPA diffusion (10.1±0.5 kcal/mol) is half of that estimated for E. coli SSB (19.6±1.7 kcal/mol)[40]. These differences suggest that different mechanisms are used for diffusion of these two proteins. In its fully wrapped (SSB)₆₅ binding mode, ~65 nucleotides of ssDNA interact with all four subunits[47, 48, 54, 55], whereas RPA interacts with only 30 nts in its largest mode[11]. Hence it is not surprising that the more extensively wrapped SSB diffuses more slowly along ssDNA than does RPA. The mechanism of E. coli SSB diffusion seems to involve a reptation mechanism in which a small ssDNA bulge forms where the ssDNA enters the SSB protein[56]. In addition to this possibility, the mechanism of RPA diffusion might involve sequential local dissociation and rebinding of two or more of its OB-folds. Our observation that the hRPA truncation, FAB, can diffuse along ssDNA indicates that diffusion requires a minimum
of only two DNA binding OB-folds. Recent experiments have shown that RPA bound to ssDNA can undergo rapid exchange with unbound RPA and the rate of exchange increases with increasing free RPA concentration[51]. This implies a transient intermediate in which both RPA molecules undergoing the exchange are bound simultaneously to the ssDNA. The ability of RPA to diffuse along ssDNA would facilitate this exchange.

There are now several examples of proteins that can diffuse along double stranded (ds) DNA (see [57] for a review). The TRBP family of RNA binding proteins have also been shown to diffuse along dsRNA[45]. Estimates of one-dimensional diffusion coefficients have been made for some of the dsDNA binding proteins, starting with the classic lac repressor (D ~ 10^6 bp^2 s^{-1})[58-61], DNA glycosylase 1 (D = 4.8±1.1x10^6 bp^2 s^{-1})[62], p53 tumor suppressor protein (D = (2.60 ± 2.17) x 10^6 bp^2/s)[63], Msh2-Msh6 DNA repair complex (D = 2.2 x10^6 bp^2 s^{-1})[64], T. aquaticus MutS (D = 3.0 to 5.0 x10^5 bp^2 s^{-1})[65], and a Type III restriction enzyme (D = 8.0±0.5 x 10^6 bp^2 s^{-1}) [66]. These are considerably larger than the diffusion coefficient that we estimate for hRPA on ssDNA and much larger than the estimate for E. coli SSB on ssDNA[40], suggesting different mechanisms of diffusion for these two classes of proteins. One difference between the dsDNA and ssDNA binding proteins is that the affinities of E. coli SSB and hRPA are much higher than the affinities of the dsDNA binding proteins in their non-specific DNA binding modes. The function of diffusion for proteins on dsDNA is generally thought to increase the rate of location of a target[67]; fast diffusion in this case would be essential. However, for hRPA and SSB, the rate of diffusion may be less important than the fact that they can diffuse at all and in doing so destabilize hairpins and be moved to avoid inhibiting other proteins. Ensemble kinetic studies also inferred that the phage T4 gene 32 protein can diffuse along ssDNA[68]. Two other E. coli-like SSB proteins from Plasmodium falciparum[17] and T. thermophiles[69] can also diffuse on ssDNA, as can cytidine deaminase[70], although no estimates of diffusion coefficients have been made. Finally, the POT1-TPP1 complex has also been shown to diffuse on a
telomeric ssDNA overhang, although POT1, which contains only two OB-folds does not show this ability[44], indicating that the ability to diffuse along ssDNA is not a general property of all ssDNA binding proteins.

Materials and Methods

Buffers. Buffer T is 10 mM Tris, pH 8.1, 0.1 mM Na₂EDTA, 1 mM 2-mercaptoethanol. For experiments at different temperatures, the buffer was prepared so that pH 8.1 was maintained at each temperature. Buffer I[71] is 20 mM Tris-HCl pH 8.1, 0.1 mM Na₂EDTA, 1 mM DTT, 0.8% (w/v) dextrose, 2.5 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma Aldrich, MO), 20 units/ml glucose oxidase and 20 units/ml catalase with 0.5 M NaCl.

Proteins. hRPA was expressed from plasmid p11d–tRPA in BL21(DE3) cells (Novagen) by auto-induction[72] in one liter of ZYP5052 medium shaken at 300 rpm at 37°C for 24h. RPA was purified as described[73, 74] with modifications (see SI Materials and Methods). The hRPA concentrations were determined by absorbance at 277 nm using an extinction coefficient of 8.57 x 10^4 M⁻¹ cm⁻¹ determined from the hRPA amino acid sequence and the absorbance spectrum of hRPA denatured in 6 M guanidinium HCl[75, 76]. The hRPA variants, ABC-D-E and FAB, were purified as described[77, 78]. The amino termini of the three subunits of hRPA were targeted for labeling with Cy5 as described[79] (for details see SI Materials and Methods). The hRPA was 92% labeled (overall efficiency for the heterotrimer) with a 4:3:1 labeling ratio for the 70:32:14 kDa subunits (as determined by denaturing PAGE).

DNA. Poly(dT) was from Midland Certified Reagent Co. (Midland, TX) and had an average length of 850 nucleotides and its concentration was determined using, ε₂₆₀ = 8140 M⁻¹ cm⁻¹ (nucleotides)[80]. Oligodeoxynucleotides were either synthesized using a Mermaid 4
synthesizer (Plano, TX) with reagents from Glen Research (Sterling, VA) or purchased from Integrated DNA Technologies (IDT), Inc. (Coralville, IA). After purification by polyacrylamide gel electrophoresis[81], the oligodeoxynucleotides were further purified by reverse phase HPLC using an Xterra MS C18 Column (Waters, Milford, MA). The oligodeoxynucleotides used in this study are given in Table S1. DNA and proteins were dialyzed into the indicated buffers. Extinction coefficients for the oligodeoxynucleotides were calculated using the nearest neighbor assumption[82].

**Ensemble Fluorescence experiments.** Fluorescence titrations were conducted in Buffer T using a PTI QM-4 fluorometer (Photon Technology International, Birmingham, NJ, USA) as described[16] (for details see *SI Materials and Methods*). Titrations of Cy3 labeled DNA with hRPA monitored the Cy3 fluorescence enhancement ($\lambda_{ex} = 515$ nm, $\lambda_{em}$= 570 nm). Excitation and emission slit widths were set at 0.50 mm (2 nm bandpass).

Occluded site sizes for hRPA binding to poly(dT) and equilibrium constants, $K_{obs}$, for hRPA binding to (dT)$_{30}$ and (dT)$_{30}$-Cy3 were measured in Buffer T, pH 8.1, 25.0°C at different [NaCl] as described[16]. The average Cy3 fluorescence enhancements upon binding one hRPA molecule to the series of (dT)$_L$-3'-Cy3 and the chimeric, (dT)$_L$-(dT$_{alt}$)$_N$-3'-Cy3 were obtained by analysis of two titrations performed at two [DNA] (10 - 40 nM) in Buffer T, pH 8.1, 0.5 M NaCl, monitoring Cy3 fluorescence using the binding density function method[37] (for details see *SI Materials and Methods*).

**Single molecule total internal reflectance fluorescence (smTIRF).** The smTIRF experiments were performed with an objective type total internal reflectance microscope (Olympus IX71, model IX2_MPITIRTL) equipped with a 3-laser system (488nm, 532nm and 635nm) and an oil-immersed, high numerical aperture TIRFM objective (PlanApo N, 60X/1.45 N.A., Olympus) as described[17] (see Figure S1). The slide holder was temperature controlled.
by a BC-110 Bionomic controller (20/20 Technology Inc., Wilmington, NC) and the objective was also connected to an objective heater (Bioptechs Inc., Butler, PA). Data were collected and analyzed using software packages generously provided by Taekjip Ha (University of Illinois, Urbana) (for details see SI Materials and Methods).

**Random Walk Simulations.** Monte Carlo simulations of a random walk of a protein, with contact size of 30 nucleotides along a ssDNA of length, L, were performed using MatLab (Mathworks, Natick, MA) as depicted in Figure 7A (for details see SI Materials and Methods).

**Acknowledgements**

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Figure Legends

**Figure 1. Binding of hRPA to ssDNA.** (A) Subunit composition of the hetero-trimeric hRPA showing the six OB-folds. (B) Structure of *Ustilago maydis* RPA bound to (dT)$_{32}$ showing OB-folds A, B, and C of Rpa1 and OB-fold D of Rpa2 interacting with 25 nucleotides (PDB ID 4GOP)[11]. (C) The occluded site-size of hRPA bound to poly(dT) is dependent on [NaCl] (Buffer T, 25.0°C). (D) Dependence on [NaCl] of the equilibrium association constant ($K_{\text{obs}}$) for hRPA binding to (dT)$_{30}$ (log-log plot) (Buffer T, 25.0°C). The dashed line shows a linear extrapolation to obtain an estimate of $K_{\text{obs}} = 1 \times 10^{10}$ M$^{-1}$ at 0.50 M NaCl (open circle). A measurement of $K_{\text{obs}}$ for hRPA binding to (dT)$_{29}$-Cy3-T-3' (red circle) is also shown at 1.0 M NaCl indicating that the presence of Cy3 on the DNA has little effect on $K_{\text{obs}}$.

**Figure 2. hRPA can diffuse along single stranded DNA.**
(A) Representative smTIRF time trace of a surface bound 3'-Cy3-(dT)$_{60}$ bound with a single Cy5-labeled hRPA (250 nM, unbound RPA removed by buffer flow; n=37 complexes analyzed containing Cy3-labeled DNA and one Cy5-labeled hRPA, N=2204 DNA molecules in field). (B) smTIRF time trace of a DNA containing a 49 nt ssDNA region in which Cy5 and Cy3 are separated by (dT)$_{30}$, followed by an additional 18 nt of mixed sequence ssDNA (n=70, N=251). (C) smTIRF time trace of the DNA in panel (B) bound with unlabeled hRPA showing large FRET fluctuations due to hRPA diffusion (100 nM hRPA, unbound RPA removed by buffer flow; n=63, N=637). (D) smTIRF time trace of the DNA molecule in panels (B) and (C) to which a complementary 18 nt ssDNA was annealed to the 18 nt mixed sequence region at the 3' end of the DNA, thus constraining hRPA to remain bound to the (dT)$_{30}$ region, thus reducing the FRET fluctuations (500 nM hRPA, unbound RPA removed by buffer flow; n=47, N=624).

**Figure 3. Transient melting of a DNA hairpin by a diffusing hRPA.**
(A) A 7 bp DNA hairpin with Cy3 and Cy5 at the base of the hairpin and a (dT)\textsubscript{30} ssDNA on the 5' side of the hairpin is stable in the absence of hRPA as indicated by the stable high FRET state (0.91) and the single Gaussian FRET probability distribution (n=64, N=795). (B) In the presence of hRPA, anti-correlated Cy3/Cy5 fluctuations are observed between high FRET (0.83 - closed hairpin) and low FRET state (0.26-melted hairpin). The FRET distribution is well described by a two Gaussian fit (n=100, N=2417). (C) hRPA melting of the DNA hairpin is blocked for a DNA in which the phosphodiester backbone polarity linking (dT)\textsubscript{30} and the hairpin is reversed via a 3'-3' linkage (red X) as indicated by the absence of FRET fluctuations and the stable FRET signal at 0.91 (n=41, N=424). (D) The frequency of hRPA-induced hairpin opening decreases when the (dT)\textsubscript{30} is placed on the 3' of the DNA hairpin, indicating a preference for hRPA melting of the hairpin when it invades from the 5' side. The low FRET state (0.20-melted hairpin) is much less populated than the high FRET state (0.89-closed hairpin) (n=29, N=341). (E) An hRPA truncation possessing only the FAB OB-folds of the Rpa1 subunit can also melt the DNA hairpin. The FRET distribution is two state (n=143, N=3435). (F) An hRPA in which OB-fold F has been deleted can melt the DNA hairpin. The FRET distribution is two state (n=130, N=4480). The concentration of hRPA or its variants was 500 nM, and the unbound proteins were removed before data collection.

**Figure 4.** hRPA binding to 3'-Cy3-(dT)\textsubscript{L} increases the average Cy3 fluorescence intensity and the Cy3 fluorescence fluctuations. (A) Representative single molecule TIRF time trace of the Cy3 fluorescence from a single DNA molecule (3'-Cy3-(dT)\textsubscript{60}) attached to the surface via a biotin-neutravidin-biotin-18 bp handle. (B) Upon addition of hRPA (37 pM), an increase in average Cy3 fluorescence and fluorescence fluctuations occurs due to binding of a single hRPA. (C) At much higher hRPA concentrations (370 nM), transient binding of a second hRPA molecule can be observed. (D) The Cy3 fluorescence intensity distributions for (3'-Cy3-(dT)\textsubscript{60}) (n=29) and upon one hRPA bound to the same 3'-Cy3-(dT)\textsubscript{60} molecules shows the increase in
both Cy3 fluorescence and Cy3 fluorescence fluctuations. The 34% increase in Cy3 intensity indicates a single hRPA bound as shown by ensemble studies (Figure S6). (E) The average Cy3 fluorescence enhancement per one hRPA bound decreases with increasing ssDNA length, L. This was observed in both ensemble (blue circles) and smTIRF studies (green triangles).

**Figure 5.** hRPA diffusion monitored by fluorescence fluctuations of Cy3-labeled ssDNA. (A) smTIR Cy3 fluorescence intensity time trace of a single DNA 3'-Cy3-(dT)$_{140}$ without hRPA. (B) An increase in Cy3 fluorescence intensity and fluctuations upon hRPA (10 pM) addition to 3'-Cy3-(dT)$_{140}$ ; a dissociation/rebinding event is also shown. (C) Auto-correlation functions of the Cy3 fluorescence traces from DNA (3'-Cy3-(dT)$_{140}$) alone (red squares-average of 105 molecules) and DNA with one hRPA bound (3'-Cy3-(dT)$_{140}$ with one RPA bound (blue circles-average of 98 molecules). In the absence of hRPA, the Cy3 fluctuations are random whereas upon binding one hRPA a finite G($\tau$) is observed that can be fit by a two exponential function with $\tau_{C,1} = 46\pm4$ msec (green dashed curve) and $\tau_{C,2} = 298\pm18$ msec (red dashed curve). (D) $\tau_{C,2}$ increases with increasing ssDNA length at 10ºC and 25ºC (see Figure S7). (E) Arrhenius plot of $1/\tau_{C,2}$ for one hRPA bound to 3'-Cy3-(dT)$_{120}$ yields $E_a = 10.1\pm0.5$ kcal/mole.

**Figure 6.** Calibration of the effect of ssDNA contour length on the hRPA-induced Cy3 fluorescence enhancement. (A) Schematic of the chimeric DNA molecules containing a normal polarity (dT)$_{30}$ linked to an alternating polarity (dT$^{alt}$)$_N$ with a Cy3 at the 3' end. (B) Schematic of a normal polarity DNA with ssDNA of length, L ((dT)$_L$). If hRPA binds, on average to the middle of the ssDNA, then the relationship between L and N is given by L=30 + 2N. (C) hRPA binds with 450-fold lower affinity to (dT$^{alt}$)$_{30}$ in which the phosphodiester backbone polarity is reversed after each nucleotide ($K_{obs}=(4.2\pm0.2)x10^5$ M$^{-1}$) than to normal polarity (dT)$_{30}$ ($K_{obs}=(1.9\pm0.1)x10^8$ M$^{-1}$) in (Buffer T, 1.0 M NaCl, 25ºC). (D) Dependence on N of the Cy3 fluorescence enhancement induced by binding a single hRPA to a series of DNA chimeras (with varying N) obtained from analysis of ensemble equilibrium titrations of DNA with hRPA (red
circles). Cy3 fluorescence enhancement for a single hRPA bound to normal polarity (dT)\textsubscript{L} from ensemble titrations (open blue circles) and from smTIRF studies (open green triangles). The data were fit to a single exponential function, \(E = E_0 + E_m\exp(-N/N_C),\) with \(E_0 = 4\pm7, E_m = 65\pm7\) and \(N_C = 15\pm4\) or equivalently \(E = E_0 + E_m\exp(-(L-30)/L_C),\) with \(L_C = 30\pm7\).

**Figure 7. Estimation of one-dimensional diffusion coefficient of hRPA on ssDNA.** (A) Cartoon describing the model used to simulate a one-dimensional random walk along ssDNA of hRPA with a contact size of 30 nucleotides and a 1 nt step size. (B) A simulated auto-correlation function for \(L = 90\) nucleotides and \(D_1 = 3000\) nt\(^2\)s\(^{-1}\), was obtained in four steps. (1)-The position (in nucleotides) of the 3'-Cy3 on the DNA relative to the edge of a diffusing hRPA was generated; (2)-The Cy3 fluorescence calibration curve was used to obtain the hRPA position dependence into a Cy3 fluorescence trace; (3)-The Cy3 signal was averaged in 32 msec bins to reflect the time resolution of the CCD camera, and (4)- Auto-correlation analyses were performed on 100 simulations and averaged to obtain \(G(\tau)\). (C) \(1/\tau_{sim}\) values from single exponential fits to the simulated \(G(\tau)\) obtained for hRPA diffusion along ssDNA of three lengths \((L = 90 (■), 120 (▲), \) and 140 (♦) nucleotides) as a function of diffusion coefficient. Black lines represent linear fits to the data. (D) \(\tau_{sim}\) values (●) plotted as a function of ssDNA length, \(L\), for different values of \(D_1\). Error bars are smaller than the data symbols and ranged from 1 to 4 ms. Black lines represent linear fits excluding the points for \(L = 60\) nt. Experimental values of \(\tau_c\) for hRPA on ssDNA at 10°C (red circles) and 25°C (blue squares) are overlaid on the plot.
References


[72] Studier FW. Protein production by auto-induction in high density shaking cultures. Protein expression and purification. 2005;41:207-34.
Table 1. Rates of DNA hairpin opening and closing induced by hRPA.

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<th>k_{close} (s^{-1})</th>
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^{(a)} Unless indicated, experiments were conducted in Buffer I (20 mM Tris-HCl pH 8.1, 0.1 mM Na$_2$EDTA, 1 mM DTT, 0.8% (w/v) dextrose, 2.5 mM Trolox, 20 units/ml glucose oxidase, 20 units/ml catalase) plus 0.5 M NaCl, 25 °C. The flow channel was washed with 200 μl of the imaging buffer to remove free protein before data collection.

^{(b)} Buffer I plus 0.10 M NaCl, 5 mM MgCl$_2$, no EDTA, 25 °C.

^{(c)} Buffer I plus 0.020 M NaCl, 25 °C.
Figure 2

A

B

C

D

DNA + hRPA

DNA

Figure 2
Figure 3

Graphical representations showing time-dependent changes in FRET ( Förster resonance energy transfer) across different conditions. The graphs display intensity (a.u.) and normalized counts as functions of time (sec) and FRET values. The images depict various experimental setups involving RPA (replication protein A), FAB, and different DNA sequences marked with Cy3 and Cy5 fluorescence dyes. The FRET values are indicated at specific positions: 0.91, 0.83, 0.26, 0.89, 0.20, 0.83, 0.84, and 0.19.
Figure 5

A. 

B. 

C. 

D. 

E. 

$E_a = 10.1 \pm 0.5 \text{ kcal/mole}$
Figure 6

A

B

C

D

Figure 6

A

B

C

D

Figure 6
Figure 7

A

N = Edge Distance to Cy3

x = 0

Contact Size = 30 nt

x = L

B

(1) hRPA Random Walk on ssDNA

(2) Cy3 Fluorescence

(3) TIRF Signal
(32 ms binning)

(4) Autocorrelation of the TIRF Signal

C

\[ \frac{1}{\tau_{sim}} (\text{s}^{-1}) \]

L = 90 nt

L = 120 nt

L = 140 nt

D

\[ \tau_{c, sim} (\text{s}) \]

D in nt²/s

60 80 100 120 140

ssDNA Length (nt)
Supplemental Information

Materials and Methods

Buffers. Buffer T is 10 mM Tris, pH 8.1, 0.1 mM Na₂EDTA, 1 mM 2-mercaptoethanol. For experiments at different temperatures, the buffer was prepared so that pH 8.1 was maintained at each temperature. Imaging buffer, Buffer I is 20 mM Tris-HCl pH 8.1, 0.1 mM Na₂EDTA, 1 mM DTT, 0.8% (w/v) dextrose, 2.5 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma Aldrich, MO), 20 units/ml glucose oxidase and 20 units/ml catalase with 0.5 M NaCl.

Proteins. hRPA was expressed from plasmid p11d-tRPA in BL21(DE3) cells (Novagen) by auto-induction in one liter of ZYP5052 medium shaken at 300 rpm at 37°C for 24h. About 10 to 15 g of cell paste was obtained per liter of culture. RPA was purified as described, but with the addition of a single-stranded DNA cellulose column where RPA was loaded in Buffer T, 100 mM NaCl and eluted with a linear [NaCl] gradient (0.10 to 2 M NaCl) to ensure that all of the hRPA has ssDNA binding activity. The protein solution was also passed through a double-stranded DNA column at 100 mM NaCl to remove any trace nucleases. The purified hRPA showed no detectable nuclease contamination as assayed with 5'-32P-(dT)30 even after 3 hours. The hRPA concentrations were determined by absorbance at 277 nm using an extinction coefficient of 8.57 x 10⁴ M⁻¹ cm⁻¹ determined from the hRPA amino acid sequence and the absorbance spectrum of hRPA denatured in 6 M guanidinium HCl. The hRPA variants, ABC-D-E and FAB, were purified as described.

The amino termini of the three subunits of hRPA were targeted for labeling with Cy5 as described. 3.5 nmol of hRPA (8.8 μM stock concentration) was dialyzed for 48 hours with 2 buffer changes vs. labeling buffer (50 mM potassium phosphate pH 7.0, 0.5 M NaCl, 10% glycerol, and 1 mM DTT). A 5-fold molar excess of Cy5 Mono NHS Ester (9.3 mM stock concentration) (Amersham, Piscataway, NJ), dissolved in anhydrous dimethyl sulfoxide (Molecular Probes, Grand Island, NY), was added to the hRPA and incubated for 3 hours at 4°C with gentle oscillation. At pH 7.0, this reagent will react predominantly with the N-termini of proteins. The labeling reaction was quenched by addition of 100-fold molar excess of 1M TRIS pH 8.1 and hRPA was separated from unincorporated dye by passing it over a BioGel P6 polyacrylamide gel column (35 cm length, 1 cm diameter) (BioRad, Hercules, CA). The hRPA was 92% labeled (overall efficiency for the heterotrimer) with a 4:3:1 labeling ratio for the 70:32:14 kDa subunits (as determined by denaturing PAGE).

DNA. Poly(dT) was from Midland Certified Reagent Co. (Midland, TX) and had an average length of 850 nucleotides and its concentration was determined using, ε₂₆₀ = 8140 M⁻¹ cm⁻¹ (nucleotides). Oligodeoxynucleotides were either synthesized using a Mermaid 4 synthesizer (Plano, TX) with reagents from Glen Research (Sterling, VA) or purchased from Integrated DNA Technologies (IDT), Inc. (Coralville, IA). After purification by polyacrylamide gel electrophoresis, the oligodeoxynucleotides were further purified by reverse phase HPLC using an Xterra MS C18 Column (Waters, Milford, MA). The oligodeoxynucleotides used in this study are given in Table S1. DNA and proteins were dialyzed into the indicated buffers. Extinction coefficients for the oligodeoxynucleotides were calculated using the nearest neighbor assumption.
Table S1. Sequences of Oligodeoxynucleotides

<table>
<thead>
<tr>
<th>DNA Sequence(^{(1)})</th>
<th>Annealed with the biotin containing strand, BX, and used in the experiments in the indicated Figure (e.g., F2A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3 5'-GCC TCG CTG CCG TCG CCA-biotin-3'</td>
<td></td>
</tr>
<tr>
<td>B5 5'-biotin-TGG CGA CGG CAG CGA GGC-3'</td>
<td></td>
</tr>
<tr>
<td>B3Cy5 5'-Cy5-GCC TCG CTG CCG TCG CCA-biotin-3'</td>
<td></td>
</tr>
<tr>
<td>S1 5'-TGG CGA CGG CAG CGA GGC (dT)(_{L}) Cy3-T-3' (L = 30, 50, 60, 90, 120,140)</td>
<td>B3 in F2A (L=60); B3 in F4A-E, FS6A-C (L=30, 50, and 60); B3 in F5A-E (L=60 to 140); B3Cy5 in FS2A (L=60);</td>
</tr>
<tr>
<td>S2(^{(2)}) 5'-TGG CGA CGG CAG CGA GGC (dT)(_{N}) Cy3-T-3' (N = 2, 5, 10, 20, 30, or 40)</td>
<td>B3 in F6D; B3Cy5 in FS2B (N=30)</td>
</tr>
<tr>
<td>S3(^{(3)}) 5'-TGG CGA CGG CAG CGA GGC (dT)(_{30}) Cy3-T GTG ACT GAG ACA GTC ACT T-Cy5-T-3'</td>
<td>B3 in F3A, F3B, F3E, F3F; B3 in FS4A, FS4B</td>
</tr>
<tr>
<td>S4 5'-TGG CGA CGG CAG CGA GGC (dT); Cy3-T GTG ACT GAG ACA GTC ACT T-Cy5-T-3'</td>
<td>B3 in FS3D</td>
</tr>
<tr>
<td>S5 5'-Cy5-TT CAC TGA CAG AGT CAG TGT Cy3 (dT)(_{30}) GCC TCG CTG CCG TCG CCA-3'</td>
<td>B5 in F3D</td>
</tr>
<tr>
<td>S6 5'-T-Cy5-TT CAC TGA CAG AGT CAG TGT Cy3-3'-3'- (dT)(_{30}) CGG AGC GAC GGC AGC GGT-5'</td>
<td>B3 in F3C</td>
</tr>
<tr>
<td>S7 5'-TGG CGA CGG CAG CGA GGC (dT)(<em>{30}) Cy3-(dT)(</em>{20})-Cy5-T-3'</td>
<td>B3 in FS3A, FS3B</td>
</tr>
<tr>
<td>S8 5'-TGG CGA CGG CAG CGA GGC (dT)(_{30}) Cy3 T CTA GCT CAG GAC CCA TGG -3'</td>
<td>B3Cy5 in F2B, F2C; B3Cy5+S9 in F2D</td>
</tr>
<tr>
<td>S9 5'-CCA TGG CTC CGT AGC TAG-3'</td>
<td></td>
</tr>
<tr>
<td>S10 5'-TGG CGA CGG CAG CGA GGC (dT)(_{30}) GTG ACT GCC-Cy3-TG GTG TGC GAG ACC ACC A-Cy5-G CCA GTC AC-3'</td>
<td>B3 in FS3C</td>
</tr>
<tr>
<td>S11 5'-(dT)(_{30})-3'</td>
<td>F1D</td>
</tr>
<tr>
<td>S12 5'-(dT)(_{29})-Cy3-T-3'</td>
<td>F1D; F6C</td>
</tr>
<tr>
<td>S13(^{(2)}) 5'-(dT(<em>{all}))(</em>{30})-Cy3-3'</td>
<td>F6C</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Cy3 and Cy5 were incorporated into the DNA backbone for these fluorophore-labeled DNAs (Glen Research Cat. 10-5913 for Cy3 and 10-5915 for Cy5). \(^{(2)}\) (dT\(_{all}\))\(_{N}\) indicates a DNA in which the phosphodiester linkage was reversed after each nucleotide. These were synthesized by alternating the normal 5'T3' phosphoramidite (Glen Research Cat. 10-1030) and the reverse 3'T5' phosphoramidite (Cat. 10-0301). \(^{(3)}\) We made two versions of S3 DNA. In one, the Cy3 was incorporated in the phosphodiester linkage and in the other the Cy3 was coupled to the C-6 of the thymine base. For the latter DNA, an Amino-C6-dT (Glen Research Cat. 10-1039) was incorporated into the DNA and a Cy3-Mono NHS ester (GE Healthcare) was coupled to the amino-C6-dT after synthesis. The position of the Cy3 did not affect the ability of hRPA to melt out the DNA hairpin.
**Ensemble Fluorescence experiments.** Fluorescence titrations were conducted in Buffer T using a PTI QM-4 fluorometer (Photon Technology International, Birmingham, NJ, USA) as described\textsuperscript{13}. The cuvettes were coated with Sigmacote\textsuperscript{®} solution (Sigma Aldrich, St Louis, MO). Titrations of hRPA with poly(dT) or (dT)\textsubscript{30} monitored the quenching of hRPA Trp fluorescence ($\lambda_{\text{ex}} = 295$ nm, $\lambda_{\text{em}} = 343$ nm). Excitation and emission slit widths were set at 0.50 mm (2 nm bandpass) and 1.00 mm (4 nm bandpass), respectively. The shutters were opened for 16 seconds during which eight data points were collected. The average of the 8 data points constituted one measurement. The data from 3 measurements were averaged to yield the fluorescent intensity at a particular DNA to protein ratio. Corrections for inner filter effects were performed as described\textsuperscript{13}. Titrations of Cy3 labeled DNA with hRPA monitored the Cy3 fluorescence enhancement ($\lambda_{\text{ex}} = 515$ nm, $\lambda_{\text{em}} = 570$ nm). Excitation and emission slit widths were set at 0.50 mm (2 nm bandpass).

Occluded site sizes for the hRPA binding to poly(dT) were determined in Buffer T (25.0°C) at different [NaCl] as described\textsuperscript{13}. In brief, poly(dT) was titrated into hRPA solution under stoichiometric conditions while monitoring the Trp fluorescence quenching. The occluded site-size is defined as the ratio of ([poly(dT)(nucleotide) to [hRPA]) at the stoichiometric break point. Titrations were conducted for at least two hRPA concentrations that showed no shift in the stoichiometric break point.

Equilibrium constants, $K_{\text{obs}}$, for hRPA binding to (dT)\textsubscript{30} and (dT)\textsubscript{30}-Cy3 were measured in Buffer T, pH 8.1, 25.0°C at 0.80, 0.90, and 1.0 M NaCl, since $K_{\text{obs}}$ is too high to measure at lower [NaCl]. Solutions of hRPA (15 nM) were titrated with (dT)\textsubscript{30} while monitoring quenching of hRPA Trp fluorescence. The binding isotherm was fit to a single binding site model as described\textsuperscript{13}. The binding constant at 0.5 M NaCl was estimated by linear extrapolation of a log$K_{\text{obs}}$-$\log$[NaCl] plot. The average Cy3 fluorescence enhancements upon binding one hRPA molecule to the series of (dT)$_L$-3'-Cy3 and the chimeric, (dT)$_L$-(dT$^{\text{alt}}$)$_N$-3'-Cy3 were obtained by analysis of two titrations performed at two [DNA] (10 - 40 nM) in Buffer T, pH 8.1, 0.5 M NaCl, monitoring Cy3 fluorescence using the Binding Density Function method\textsuperscript{39}.

**Single molecule total internal reflectance fluorescence (smTIRF).** The smTIRF experiments were performed with an objective type total internal reflectance microscope (Olympus IX71, model IX2_MPIITIRTL) equipped with a 3-laser system (488nm, 532nm and 635nm) and an oil-immersed, high numerical aperture TIRFM objective (PlanApo N, 60X/1.45 N.A., Olympus) as described previously\textsuperscript{14} (see Figure S1). The slide holder was temperature controlled by a BC-110 Bionomic controller (20/20 Technology Inc., Wilmington, NC) and the objective was also connected to an objective heater (Bioptechs Inc., Butler, PA). Data were collected and analyzed using software packages generously provided by Taekjip Ha (University of Illinois, Urbana-Champaign). The alignment and mapping of donor and acceptor channels were carried out using 0.2 µm crimson beads (Thermo Fisher Scientific, Inc., Waltham, MA) as described\textsuperscript{14}. A two channel flow cell assembly was prepared as described\textsuperscript{15} and both channels were coated with a freshly prepared solution containing 1.5% (w/v) biotin-polyethylene glycol-succinimidyl valerate (SVA) (average MW 5000) and 25% (w/v) mPEG-SVA (average MW 5000) (Laysan Bio, Arab, AL) in 0.1M NaHCO$_3$ (pH 8.1) for at least 3 hours. A typical slide has two flow cells with approximate 10 µl/channel. The flow channels were washed with water and, if needed, were subjected to photobleaching by a high intensity laser (532nm) to remove background signals. A 0.2 mg/mL solution of NeutrAvidin (Thermo Scientific, Waltham, MA) in Buffer T, 0.1 M NaCl was flowed into the channels and incubated for 5 minutes. Excess NeutrAvidin was washed out, and the surface background was photobleached before biotinylated DNA (5 – 10 pM in imaging buffer 0.1M NaCl) was immobilized onto the flow cell.
DNA was attached to the slide, by flowing 5-10 pM of biotinylated DNA over the surface followed by incubation for 1-5 minutes. The excess DNA was washed away when a few hundred molecules were immobilized. The data was collected using an Andor iXon EMCCD 897 camera. Every frame has a 30.27 ms exposure time (32.02 ms cycles) and 2000 to 14000 frames were recorded at a position. Corrections were applied for leakage of donor into the acceptor channels and instrumental detection efficiencies as described\textsuperscript{15-17}. For experiments with Cy3 and Cy5, an approximate FRET efficiency was calculated\textsuperscript{15} from the ratio \( E = I_a/(\gamma I_a + I_d) \) where \( \gamma \) represents detection efficiencies and \( I_a \) and \( I_d \) are intensities of acceptor and donor, respectively. For smTIRF experiments of hRPA bound to single stranded DNA labeled with Cy3, a solution of hRPA (10 µl of 1-50 pM) was flowed into the flow cell and the data were recorded without washing away the free protein. For the DNA hairpin experiments (DNA labeled with Cy3 and Cy5), unbound hRPA was washed out with image buffer (20 flow-cell volumes (200 µl)) before data were collected.

**Analysis of smTIRF data.** smTIRF time traces with a 32 ms resolution were processed without smoothing. Normalized FRET or Cy3 fluorescence intensity histograms were constructed by pooling time traces from multiple molecules. The data were assigned to bins in 0.02 FRET increments and the counts in each bin were divided by the total count. These normalized counts were then divided by the bin width such that the total area of the histogram was normalized to one. The histogram was then fit to either a single or two-Gaussian distribution. In experiments measuring FRET between Cy5-labeled hRPA and Cy3-labeled DNA, we designate “n” as the number of DNA molecules that were included in the analysis. These had a Cy3 label and a single hRPA bound with non-zero intensities. We designate “N” as the total number of Cy3-labeled DNA molecules identified by the analysis program. In experiments measuring FRET with both Cy3 and Cy5 labels on the DNA, we designate “n” as the number of DNA molecules that were included in the analysis. These were labeled with both Cy3 and Cy5 with non-zero intensities. We designate “N” as the total number of Cy3-labeled DNA molecules identified by the analysis program. These include only single Cy3-labeled DNA as well as DNA in which the Cy5 has been photobleached.

Hidden Markov analysis was used to identify the FRET states and determine the rates of conversion between states as described\textsuperscript{18}. The FRET time traces resulting from hRPA-induced melting of the DNA hairpin showed predominately two-state behavior. Less than 1% of the states showed a FRET efficiency value of \(~0.5\) and the dwell time in this state was very short (1 to 3 frames) suggesting that the average lifetime of this state is very short (equal to or less than the 32 ms time resolution). Cross-correlation analysis methods\textsuperscript{19} were used to demonstrate that the Cy3 and Cy5 fluorescence intensities are truly anti-correlated in Figures 2A, 2C, 3B, 3C, 3D and 3F.

Auto-correlation analyses were performed on individual Cy3 time traces using eq. (S1) and the average auto-correlation function from multiple time traces was fit to a two-component exponential decay to obtain the characteristic auto-correlation lifetimes, where \( \tau \) is the lag time,

\[
G(\tau) = \frac{\sum_{i=1}^N (I_i - \bar{I})(I_{i+\tau} - \bar{I})}{\sum_{i=1}^N (I_i - \bar{I})^2}
\]

\[ (S1) \]

\( I_i \) is the fluorescence intensity at time \( i \), \( \bar{I} \) is the average fluorescence intensity, and \( I_{i+\tau} \) is the intensity at time \( i+\tau \).

**Random Walk Simulations.** Monte Carlo simulations of a random walk of a protein, with contact size of 30 nucleotides along a ssDNA of length, \( L \), were performed using MatLab (Mathworks, Natick, MA) as depicted in Figure 7A. The protein was initially bound at random to
a binding site between x =0 and x = L where L is the length of the ssDNA in nucleotides and x=0 is the position of the Cy3 labeled 3’ end of the ssDNA. The x position is the position of the center of mass of the protein; the edges are located at x±14 nucleotides. A uniform random number generator was used to assign the initial site of the protein with corrections then being made for the site size of the protein so that the edge positions do not extend beyond x= 0 or L. The random walk was initiated as follows. A random number was generated between 0 and 1; a value < 0.5 was assigned as a step backwards (towards x = 0), while a value > 0.5 was considered as a step forward (towards x = L). This process was then repeated for the desired number of steps. The time between steps was constant as defined in eq. (S2),

$$\Delta t_{\text{steps}} = 1 \, \text{step}^2/(2D_1) \quad \text{(S2)}$$

where $$D_1$$ is the one dimensional diffusion coefficient in units of step$$^2$/s ($$D_1$$ is converted to units of nt$$^2$/s by multiplying by ($$\Delta x$$)$^2$, where $$\Delta x$$ is the step size in nucleotides). The protein was constrained to remain bound to the DNA by using reflective boundaries so that when the edge of the simulated protein reached either x = 0 or x = L, the next step was restricted to be in the direction away from this barrier. To ensure that the number of steps used was large enough to obtain appropriate statistical coverage of the random walk, the number of steps was constrained using eq. (S3)$^{20}$. Using this guideline, the number of steps ranged from 20,000 to 250,000 depending on L. These step number guidelines generally led to simulations of 10 to 40 s (depending on $$D_1$$, L, and $$\Delta x$$), which are experimentally feasible timescales. When the number of steps used is less than half this value, the autocorrelation $$\tau_{\text{sim}}$$ for a given set of simulation conditions is underestimated compared to its value for large $$N_{\text{steps}}$$.

The time trajectories of the protein position, relative to the Cy3 DNA end in nucleotides, N, along the contour length, (Figure 7B-panel 1) were converted to Cy3 fluorescence intensity (I) time traces (Figure 7B-panel 2) using Eq. S4,

$$I = I_0(1 + (0.01)E_m \exp(-N/N_C)) \quad \text{(S4)}$$

where $$I_0 = 200$$, is the Cy3 intensity for DNA alone, $$E_m = 65$$ and $$N_C = 15$$ (obtained from the calibration data in Figure 6D). The critical length parameter in the calibration equation affected the $$\tau_{\text{sim}}$$, while the maximum enhancement parameter minimally affected $$\tau_{\text{sim}}$$ and the baseline simulation intensity had no effect on $$\tau_{\text{sim}}$$. The Cy3 data were then averaged over a 32 ms bin size to reflect the time resolution of the experiment (Figure 7B-panel 3). Randomly generated white noise was added to the TIRF signal at each binned data point to simulate the instrumental noise (Figure 7B-panel 4). The noise was either a positive or negative integer and the distribution of the noise magnitude was centered on a value of 22 a.u.; an estimate of instrumental noise was obtained from analyzing the spread of the residual signal after photobleaching of a Cy3 fluorophore in various experiments. The signal to noise ratio in the simulation affected only the amplitude of the autocorrelation curve, $$G(t=0)$$. An auto-correlation analysis was performed on each time trace in the same manner as was done with the experimental time traces. 100 individual autocorrelation functions were averaged and the average auto-correlogram was then fit to a first order exponential decay to obtain $$\tau_{\text{sim}}$$. As shown in the Results section, $$\tau_{\text{sim}}$$ decreased with increasing $$D_1$$ (Figure 7C) and increased with increasing L (Figure 7D). Increasing the site size of the protein by “n” nucleotides in the simulation had the same effect as increasing L by the same number of nucleotides. Using a contact size of 28 vs. 30 nucleotides for hRPA did not affect the resulting estimate of $$D_1$$. The
The value of $D_1$ was estimated from the experimental value of $\tau_C$ using the relationship, $1/\tau = CD_1$, obtained from the simulated data in Figure 7C, where $C = (11.1L - 690)^{-1}$. The dependence of $C$ on $L$ was derived empirically from linear fits of the simulated data. The reported values of $D_1$ at $10^\circ C$ and $25^\circ C$ were calculated from the average of the values of $D_1$ obtained from the data of $L = 120$ nt and $L = 140$ nt experiments. Errors in the individual values of $D_1$ were estimated from the experimental error of the $\tau_C$ value and the average $D_1$ error was calculated using standard uncertainty propagation analysis. The value of $D_1$ at $37^\circ C$ was obtained by extrapolation from the value of $D_1$ at $10^\circ C$ and the experimentally determined activation energy assuming an Arrhenius relationship.

**Supplementary Figure Legends**

**Figure S1.** Schematic of the smTIR instrument showing a typical setup for excitation of Cy3 fluorophore. Biotinylated-fluorophore-labeled DNA molecules are immobilized on the polyethylene glycol (PEG) coated surface of a slide by NeutrAvidin binding to the biotinylated DNA end and to a biotinylated Polyethylene glycol (PEG) on the surface. Only a fraction of the PEG molecules are biotinylated.

**Figure S2.** Normal and alternating backbone ssDNA have the same flexibility. smTIRF experiments were performed on two DNA molecules of the same length (60 nucleotides) labeled with a Cy3 (donor) and Cy5 (acceptor) as indicated. (A) A histogram of 163 DNA molecules with possessing a normal 5’ to 3’ phosphodiester backbone with Cy5 and Cy3 separated by a 60-nucleotides. An average FRET efficiency of 0.14 was observed (shown with its fitting error). (B) A histogram of 78 DNA molecules containing a normal 5’ to 3’ (dT)$_{30}$ segment followed by a (dT$^{alt}$)$_{30}$ segment in which the phosphodiester backbone alternates between a 5’-5’ linkage and a 3’-3’ linkage between each nucleotide. The average FRET efficiency for each DNA is the same under the experimental conditions (Buffer I, 0.5 M NaCl, 25.0°C), consistent with both ssDNA molecules having the same flexibility. Representative time traces show a stable FRET signal with no large fluctuations indicating that the dynamics of the ssDNA chain are fast and thus averaged within the 32 ms time resolution of the smTIRF instrument. Autocorrelation analyses performed on the FRET signal fluctuations are flat indicating the absence of correlated motions.

**Figure S3.** (A) This DNA substrate was designed as a control for the behavior of the DNA hairpin substrate shown in Figure 3A and B in its fully open (melted) conformation. In the absence of hRPA, an average FRET efficiency of 0.66 is observed ($n=112$, $N=414$). (B) When 100nM hRPA was added to the DNA substrate in (A) and excess (unbound) hRPA removed by flow, transitions between FRET efficiencies of 0.64 and 0.21 are observed, suggesting that the DNA hairpin in Figure 3B is fully melted by hRPA ($n=58$, $N=659$). (C) A DNA hairpin substrate in which the Cy3/Cy5 FRET pair was moved 9 base pairs into an 18-base pair hairpin. No FRET transition is observed in the presence of hRPA (500 nM, unbound hRPA removed by buffer flow) indicating that hRPA is unable to move that far into the DNA hairpin ($n=54$, $N=190$). (D) When the binding site (dT)$_{30}$ is replaced by a single (dT)$_{1}$, hRPA is unable to melt the 7 base-pair hairpin even at high hRPA concentrations (300 nM). This indicates that hRPA does not bind directly to the hairpin, but requires a ssDNA binding site to load and melts the hairpin by diffusing in from this ssDNA binding site (Buffer I, 0.5 M NaCl, 25.0°C) ($n=158$, $N=782$).

**Figure S4.** (A) hRPA can melt out a DNA hairpin in the presence of 5 mM MgCl$_2$, 100 mM NaCl (Buffer I, 25.0°C) (incubated with 100 nM hRPA, unbound hRPA removed by buffer flow; $n=43$, step size of the protein used in the simulations had no effect on the $\tau_{sim}$ behavior when $D_1$ is reported in units of nt$^2$/s.
N=1290). (B) At 20 mM NaCl where the average occluded site size is 22 nucleotides, hRPA can also melt out a short DNA hairpin (incubated with 500 pM hRPA, unbound hRPA removed by buffer flow; n=122, N=2256). The transition between opening and closing is mainly a two-state process as identified using a Hidden Markov analysis method (magenta line).

Figure S5. Determination of the rates of protein induced opening and closing of DNA hairpins by hRPA and hRPA variants. Dwell times obtained from Hidden Markov analyses of Cy3/Cy5 FRET fluctuation time traces of the type shown in Figures 3, S3 and S4 are shown as histograms. Open events are the dwell times associated with the hairpin being closed (high FRET of 0.8 - 0.9). Open events are the dwell times associated with protein induced hairpin melting (low FRET of 0.2 - 0.3). The curves are single exponential fits to the data from which we obtain the values for the rate constants, \( k_{\text{open}} \) and \( k_{\text{close}} \), shown in Table 1 (Buffer T, 25.0 °C). Panels (A) and (B)- hRPA on DNA with (dT)\(_{30}\) on the 5' side of the hairpin (0.5 M NaCl). Panels (C) and (D)- hRPA on DNA with (dT)\(_{30}\) on the 5' side of the hairpin (0.1 M NaCl, 5 mM MgCl\(_2\)). Panels (E) and (F)- truncated hRPA (ABCDE) on DNA with (dT)\(_{30}\) on the 5' side of the hairpin (0.5 M NaCl). Panels (G) and (H)- truncated hRPA (FAB) on DNA with (dT)\(_{30}\) on the 5' side of the hairpin (0.5 M NaCl). Panels (I) and (J)- hRPA on a DNA with no hairpin (\( k_{0.64-0.21} = 0.75 \pm 0.03 \text{ s}^{-1}; k_{0.21-0.64} = 3.85 \pm 0.26 \text{ s}^{-1} \)). Panels (K) and (L)- hRPA on DNA with (dT)\(_{30}\) on the 3' side of the hairpin (0.5 M NaCl).

Figure S6. The average Cy3 fluorescence enhancement upon binding one hRPA is dependent on DNA length. Ensemble titrations monitoring Cy3 fluorescence of three DNA molecules with ssDNA regions, (dT)\(_{30}\), (dT)\(_{50}\) and (dT)\(_{60}\) labeled at the 3' end with Cy3 (green dot) with hRPA were performed at two DNA concentrations (20 and 40 nM) (buffer T, pH 8.1, 0.5 M NaCl, 25 °C). Each set of titrations (panels A, B and C) was analyzed using the Binding Density Function method\(^{21}\) to determine the average Cy3 fluorescence enhancement as a function of the average number of bound hRPA molecules (lower panels). A) A 72% increase in Cy3 fluorescence was observed when one hRPA binds to (dT)\(_{30}\)-Cy3-T. B) A 38% increase in Cy3 fluorescence was observed when one hRPA binds to (dT)\(_{50}\)-Cy3-T. C) A 32% increase in Cy3 fluorescence was observed when one hRPA binds to (dT)\(_{60}\)-Cy3-T.

Figure S7. smTIRF experiments were performed on a series of 3'-Cy3 labeled DNA molecules of the type shown schematically in Figure S6 with different ssDNA lengths (dT)\(_{L}\), as indicated, with one hRPA bound per DNA molecule for four DNA lengths (L= 60, 90, 120, 140 nucleotides) at 25.0°C (A (n=65), B (n=35), C (n=23), and D (n=153)), three DNA lengths (L=90, 120, 140 nucleotides) at 10.0°C (E (n=23), F (n=25), and G (n=41)), and one DNA length (L=120 nucleotides) at 6.0°C (H (n=33)), and 17.0°C (L (n=24)) (Buffer I, pH 8.1, 0.5 M NaCl). Autocorrelation analyses of the Cy3 fluorescence fluctuations were performed yielding the autocorrelation functions shown. The auto-correlation functions were fit to a two exponential decay yielding the indicated values of \( \tau_{c.1} \) and \( \tau_{c.2} \). The values of \( \tau_{c.2} \) are shown in Figures 5D, 5E and 7D and were used to estimate the one-dimensional diffusion coefficient for hRPA on ssDNA.

Figure S8. Ensemble calibration experiments were performed with the chimeric DNA molecules shown schematically to determine the hRPA-induced Cy3 fluorescence enhancement as a function of the distance in nucleotides between the edge of hRPA ad the Cy3 label on the DNA end. Each DNA contains a high affinity site for hRPA (dT)\(_{30}\), followed by an alternating phosphodiester backbone region (red sawtooth), N nucleotides in length ((dT\(_{30}\))\(_{N}\)). Two ensemble titrations (at 10 and 20 nM DNA) were performed for each DNA by titrating DNA with hRPA and monitoring the increase in Cy3 fluorescence (buffer T, 0.5 M NaCl, 25 °C). Each set of two titrations (top panels) were analyzed using the Binding Density Function method\(^{21}\) to determine the average Cy3 fluorescence enhancement upon binding the first hRPA to the high
affinity site (lower panels). These data are plotted in Figure 6D (red circles) and show that the average Cy3 fluorescence enhancement per one hRPA bound decreases exponentially as the distance along the ssDNA contour length between hRPA and Cy3 increases.

**Figure S9:** In the absence of hRPA diffusion, a Cy3 enhancement is observed, but with lower Cy3 fluorescence fluctuations and a small auto-correlation function. (A) Representative smTIRF Cy3 fluorescence time trace for a chimeric DNA molecule (see schematic) with (dT)₃₀-(dT)ₐₐₙ₉₂₀ and one hRPA bound showing an increase in Cy3 fluorescence. (B) Averaged auto-correlation functions of the Cy3 fluorescence fluctuations were obtained for three chimeric DNA molecules with N=20 (average of 32 molecules), N=30 (average of 25 molecules) and 40 nucleotides (average of 41 molecules) with one hRPA bound. A small auto-correlation function is observed that decreases with increasing N when hRPA is constrained to remain bound to the (dT)₃₀ binding site.

**Supplementary References**


Figure S1
Figure S2

(A) Cy5

(\text{dT})_{60}

Cy3

Normalized Counts

0.14 ± 0.01

FRET

Intensity (a.u.)

(B) Cy5

(\text{dT})_{30}

(\text{dT}^{alt})_{30}

Cy3

Normalized Counts

0.15 ± 0.01

FRET

Intensity (a.u.)

Time (sec)

G(\tau)

Time (sec)
Figure S3

A) DNA + hRPA

B) DNA + hRPA

C) DNA + hRPA

D) DNA + hRPA

FRET (a.u.)

Normalized Counts

Time (sec)

0.66
0.64
0.21
0.89
0.93
Figure S4

(A) 0.1 M NaCl, 5 mM MgCl₂

(B) 0.02 M NaCl
Figure S5

0.1M NaCl, 5 mM MgCl₂
$\tau_{c,1} = 18\pm6 \text{ ms}$
$\tau_{c,2} = 176\pm20 \text{ ms}$

$\tau_{c,1} = 25\pm5 \text{ ms}$
$\tau_{c,2} = 187\pm16 \text{ ms}$

$\tau_{c,1} = 29\pm6 \text{ ms}$
$\tau_{c,2} = 245\pm31 \text{ ms}$

$\tau_{c,1} = 46\pm4 \text{ ms}$
$\tau_{c,2} = 298\pm8 \text{ ms}$

$\tau_{c,1} = 38\pm5 \text{ ms}$
$\tau_{c,2} = 297\pm34 \text{ ms}$

$\tau_{c,1} = 40 \text{ ms}$
$\tau_{c,2} = 628\pm14 \text{ ms}$

$\tau_{c,1} = 50 \text{ ms}$
$\tau_{c,2} = 812\pm12 \text{ ms}$

$\tau_{c,1} = 108\pm17 \text{ ms}$
$\tau_{c,2} = 789\pm29 \text{ ms}$

$\tau_{c,1} = 29\pm6 \text{ ms}$
$\tau_{c,2} = 426\pm12 \text{ ms}$

$\tau_{c,1} = 17 \text{ oC}$
$\tau_{c,2} = 17 \text{ oC}$
Figure S8
Figure S9