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Ligand induced stabilization of the melting temperature of the HSV-1 single-strand DNA binding protein using the thermal shift assay

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ABSTRACT

We have adapted the thermal shift assay to measure the ligand binding properties of the herpes simplex virus-1 single-strand DNA binding protein, ICP8. By measuring SYPRO Orange fluorescence in microtiter plates using a fluorescence-enabled thermal cycler, we have quantified the effects of oligonucleotide ligands on the melting temperature of ICP8. We found that single-stranded oligomers raise the melting temperature of ICP8 in a length- and concentration-dependent manner, ranging from 1 °C for $(dT)_5$ to a maximum of 9 °C with oligomers $\geqslant$10 nucleotides, with an apparent K_d of <1 μM for $(dT)_{20}$. Specifically, the results indicate that ICP8 is capable of interacting with oligomers as short as 5 nucleotides. Moreover, the observed increases in melting temperature of up to 9 °C, indicates that single-strand DNA binding significantly stabilizes the structure of ICP8. This assay may be applied to investigate the ligand binding proteins of other single-strand DNA binding proteins and used as a high-throughput screen to identify compounds with therapeutic potential that inhibit single-strand DNA binding. As proof of concept, the single-strand DNA binding agent ciprofloxacin reduces the ligand induced stabilization of the melting temperature of ICP8 in a dose-dependent manner.

1. Introduction

SYPRO Orange was developed as a sensitive protein stain, allowing the detection of as little as 1 ng of protein following SDS–PAGE as well as in native and two-dimensional gels [1,2]. It is a fluorescent stain with excitation maxima of 280 and 470 nm, and an emission maximum of 569 nm, exhibiting a ~500-fold increase in fluorescence intensity upon protein binding [1]. More recently, SYPRO Orange has found an intriguing application in measuring thermal denaturation of proteins [3]. Essentially, thermal unfolding of proteins exposes hydrophobic regions that are bound by SYPRO Orange with a concomitant increase in fluorescence emission. This permits the determination of the melting transition point or melting temperature[1] (T_m) of a given protein as a measure of its thermal stability. One significant application of the assay has been in comparing the thermal stability of mutant proteins to gain insight into the impact on protein folding [4–7], and to evaluate the quality of proteins by distinguishing between folded and unfolded preparations [8]. Importantly, this method may also be used to investigate how ligands affect protein stability by shifting the T_m [3,9]. Indeed, the thermal shift assay has found application as a screen to identify protein ligands, with the potential to facilitate hit identification in drug discovery, and to aid in protein structure determination [10–13]. For instance, the thermal shift assay has been used to demonstrate that divalent cations can stabilize the gp2 nuclease subunit of the bacteriophage SPP1 terminase, with Mn^{2+} increasing the T_m by ~8 °C [14]. In another study with aspartyl-tRNA synthetase, of the various ligands, only the sulfamoyl-adenylated-asp analogue was shown to significantly increase the thermal stability of the enzyme, while neither tRNA, adenosine triphosphate or aspartic acid had a significant effect on T_m [15]. Regarding macromolecular ligands, in a study of the regulatory DNA binding protein ExsD from *Pseudomonas aeruginosa*, it was demonstrated that DNA binding reduced the T_m by 12 °C, consistent with a reduction in protein stability as a result of the dissociation of the ExsD trimer [16]. In fact, to our knowledge, this is the only prior study using the thermal shift assay to have examined the effect of DNA ligands on protein stability. Here we have adapted the assay to investigate how single-stranded (ss) DNA ligands affect the stability of a ssDNA binding protein (SSB), specifically that of herpes simplex virus-1 (HSV-1) Infected Cell Polypeptide 8 (ICP8).

ICP8 is a 1196 amino acid, 128,342 Da polypeptide and one of seven essential viral DNA replication proteins [17]. Biochemically it functions as a SSB, binding to ssDNA with moderate cooperativity

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[1] *Abbreviations used:* EMSA, electrophoretic mobility shift assay; HSV, herpes simplex virus; ICP8, Infected Cell Polypeptide 8; SSB, single-strand DNA binding protein; ss, single-stranded; T_m, melting temperature.

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(ω = 15), an association constant $K = 0.55 \times 10^{-6}\ M^{-1}$, a site size of 10 ± 1 nucleotides, and stretching the DNA by 150% [18]. ICP8 also binds to RNA albeit with reduced affinity compared to its ssDNA binding activity [19]. Functionally, ICP8 performs a critical role at the viral DNA replication fork by maintaining ssDNA regions and by interacting with various other replisome components via specific protein–protein interactions [reviewed in [20–24]]. In addition, ICP8 has been demonstrated to function as a recombinase, capable of promoting strand annealing and strand invasion as well as mediating strand exchange reactions in conjunction with the HSV-1 helicase-primase or UL12 nuclease [25–28].

Here we have used the thermal shift assay to measure the ssDNA binding parameters of ICP8. To our knowledge, this is the only report that demonstrates the application of the thermal shift assay to study ssDNA-SSB interactions. We believe that the thermal shift assay may be used to easily gain insight into the ssDNA binding properties of other SSB. Importantly, since SSBs perform critical and invariably essential functions, they represent excellent antibacterial and antiviral drug targets. Therefore, the thermal shift assay may be used as a rapid screen to identify compounds that inhibit the ssDNA binding activity of SSB. Indeed, using ICP8, we provide proof of concept that the thermal shift assay may be used to identify compounds that disrupt ssDNA-SSB interactions and have application as antiviral or antibacterial agents.

2. Materials and methods

2.1. Oligonucleotides, proteins and reagents

Oligothymidylate of various sizes (Operon Biotechnologies) were as follows: $(dT)_5$, $(dT)_7$, $(dT)_9$, $(dT)_{10}$, $(dT)_{11}$, $(dT)_{13}$, $(dT)_{15}$, $(dT)_{17}$, $(dT)_{20}$, $(dT)_{40}$, and $(dT)_{60}$. Oligonucleotides PBAZ48 (24-mer, 5′ CGAGGCTGGATGGCCTTCCCCATT) and PBAZ49 (20-mer, 5′ TGGCCTTTGGCCTTTGGCCT) were synthesized by Operon Biotechnologies. Oligomers were purified by the manufacturer, either by QuickLC™ for those between 10 and 40 nucleotides or from polyacrylamide gels for $(dT)_{60}$, PBAZ48 and PBAZ49. Oligonucleotide concentrations are expressed in moles of molecules. Where indicated, oligonucleotides were 5′-^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Perkin Elmer, 3000 Ci/mmol) followed by removal of unincorporated nucleotides using Microspin G-25 columns (GE Healthcare). ICP8 was purified as previously described [29]. Its concentration, expressed in moles of monomeric protein, was determined using an extinction coefficient of 82,720 $M^{-1}\ cm^{-1}$ at 280 nm. SYPRO Orange and ciprofloxacin were purchased from Invitrogen and Sigma, respectively.

2.2. Thermal shift assay

The thermal shift assay was performed using an Applied Biosystems ABI Fast 7500 system. Unless otherwise stated, reactions (25 µl) in fast optical 96-well reaction plates (Applied Biosystems) contained 20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, l µM ICP8, SYPRO Orange (500-fold dilution of the commercial stock), and oligonucleotide, as indicated. The plates were covered with optical foil during the reaction in the thermal cycler. The instrument was programmed in the *Melt Curve* mode with *SYBR Green Reagent* detection and the *Standard* speed run. The reporter was selected as *FAM* and *None* for the quencher. Each melt curve was programmed as follows: 25 °C for 5 min, followed by a 1 °C increase per min from 25 to 95 °C, and finally 25 °C for 5 min. The raw data was extracted in MS-Excel format. Arbitrary fluorescence was plotted as a function of temperature. In our studies, T_m is defined as the temperature with the highest SYPRO Orange fluorescence, coinciding with the maximum number of SYPRO Orange binding sites exposed by thermal denaturation. No significant background fluorescence was observed in the absence of protein. Upon reaching the T_m, the fluorescence slowly decreased due to aggregation of the protein at higher temperatures [10]. It should also be noted that while this method reproducibly reveals the temperature at which the maximum fluorescence occurs, the magnitude of the signal exhibits a certain degree of variance. Given that the temperature increment for each melt curve was 1 °C, the resolution of the T_m reported herein is 1 °C. The data for each experiment is based on a number of replicates as indicated in the relevant figure legends. In all cases the deviation from the average was less than 1 °C.

2.3. Electrophoretic mobility shift assay

ICP8 was incubated with 5′-^{32}P-labeled oligothymidylate as indicated in 10 µl containing 20 mM HEPES-NaOH, pH 7.5 and 150 mM NaCl for 10 min on ice. The reactions were supplemented with 3 µl loading buffer (final concentration: 40 mM Tris–acetate pH 7.6, 1 mM EDTA, 10% glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol) and immediately subjected to electrophoresis through 4% polyacrylamide (bis:acrylamide ratio 29:1)-TBE gels at 100 V for two hours at room temperature. Following electrophoresis, the gels were dried onto DE81 chromatography paper (Whatman), analyzed and quantitated by storage phosphor analysis with a Molecular Dynamics Storm 820. DNA binding was calculated as a percentage of the bound probe over total radioactivity.

3. Results

3.1. ssDNA ligand increases the thermal stability of ICP8

In this study we adapted the thermal shift assay as described under Section 2, to determine the T_m of ICP8 and to evaluate how its thermal stability is impacted by ssDNA ligand. Analysis of the melting curves shows an initial increase in SYPRO Orange fluorescence starting at 36 °C that gradually increases with temperature (see Fig. 4 for representative curves). In our studies, T_m is defined as the temperature with the highest fluorescence, coinciding with the maximum number of SYPRO Orange binding sites exposed by thermal denaturation. Under our experimental conditions, ICP8 exhibits a T_m of 45 °C.

We wanted to examine whether ssDNA ligands can affect the thermal stability of ICP8. Given that ICP8 forms nucleoprotein filaments on ssDNA, we predicted that ssDNA oligomers capable of interacting with ICP8 would stabilize it and raise the T_m. Fig. 1 shows the effect on T_m of (dT) oligomers ranging from 5 to 60 nucleotides at 10 µM. Under the specified experimental conditions, the first detectable shift in T_m was with $(dT)_{10}$ which raised the T_m by 1 to 54 °C. One interpretation of this finding is that a 10-mer oligomer represents the minimal length to which ICP8 can bind. This would be consistent with previous determinations of the binding site size of ICP8 [18,20,30]. The most quantitative of these studies using fluorescence anisotropy found that ICP8 has a binding site size of 10 ± 1 nucleotides [18]. Interestingly, oligomers between 11 and 20 nucleotides elicited progressive increases in the T_m, with a maximum shift of 9 to 54 °C. Oligomers of 40 and 60 nucleotides had no further effect on the thermal stability of ICP8. Overall, these results show that ssDNA ligands can significantly change the thermal stability of ICP8 in a length-dependent manner, increasing its T_m by a maximum of 9 °C.

3.2. Characterization of the ssDNA binding properties of ICP8 using the thermal shift assay

In order to determine the dependence of the increase in T_m on the concentration of ssDNA ligand we performed titrations with

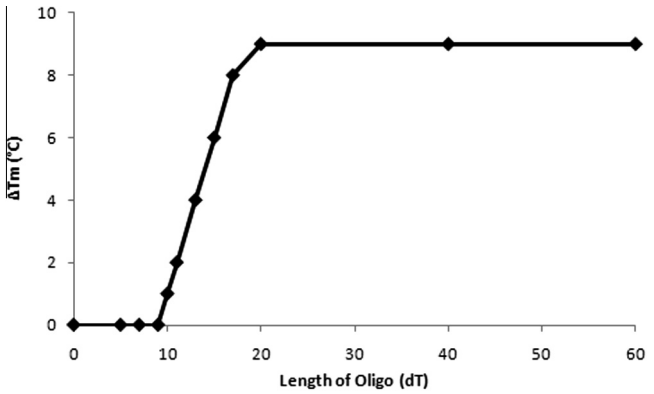

Fig. 1. Oligonucleotide length-dependent stabilization of the T_m of ICP8. Reactions were performed as described in Section 2 with 10 μM of the (dT) oligomers as indicated. ΔT_m = change in T_m relative to that in the absence of ligand (45 °C). The data are the average of 9 separate acquisitions.

the various (dT) oligomers ranging from $(dT)_5$ to $(dT)_{20}$. As shown in Fig. 2, each of the oligomers tested increased the T_m of ICP8 in a concentration-dependent manner, with oligomers $\geqslant 10$ increasing T_m by 9 °C. Using these data, we estimate an apparent K_d for $(dT)_{20}$ of <1 μM. Notably, using this approach we were able to demonstrate an interaction between ICP8 and oligomers less than 10 nucleotides. Thus, $(dT)_7$ and $(dT)_9$ were able to increase the T_m of ICP8 by 2 and 3 °C, respectively. In fact, even $(dT)_5$ was capable of increasing the T_m of ICP8 by 1 °C, albeit at high concentrations (>50 μM). Consequently, these data indicate that ICP8 can indeed interact with ssDNA as short as 5 nucleotides in length, albeit with lower apparent affinity. To confirm this observation, the interaction of ICP8 with short oligomers was also examined using electrophoretic mobility shift assays (EMSA). Fig. 3A shows that in addition to the complexes formed with $(dT)_{\geqslant 10}$, ICP8 was capable of forming weak complexes with both $(dT)_5$ and $(dT)_7$ and more so with $(dT)_9$. Moreover, to validate the interaction with $(dT)_5$, Fig. 3B and C show that it was capable of challenging complexes of ICP8 formed with $(dT)_{20}$ when present at a 1000-fold excess, albeit significantly more weakly than the competition achieved with $(dT)_{10}$ and $(dT)_{20}$, indicating that the affinity for $(dT)_5$ must be several orders of magnitude less.

3.3. Application of the thermal shift assay to identify compounds that inhibit ssDNA binding

We wanted to investigate the utility and provide proof of principle that the thermal shift assay could be used as a screening tool

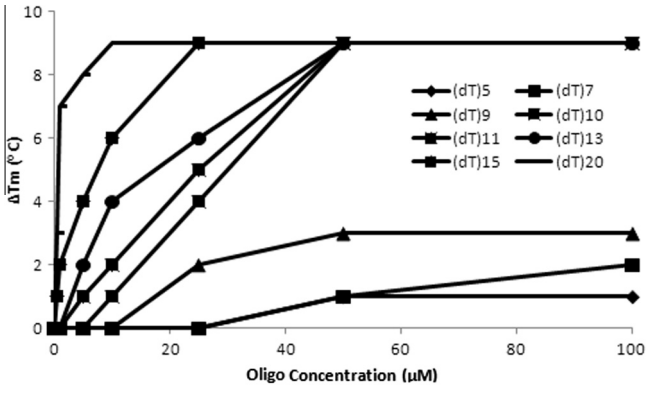

Fig. 2. Oligonucleotide concentration-dependent stabilization of the T_m of ICP8. Reactions were performed as described in Section 2 with the indicated concentrations of the various (dT) oligomers. ΔT_m = change in T_m relative to that in the absence of ligand (45 °C). The data are the average of 2 separate acquisitions.

to identify inhibitors of the ssDNA binding activity of ICP8. In this assay such compounds would prevent ligand induced thermal stabilization. In lieu of an inhibitor of the ssDNA binding activity of ICP8, which has thus far not been developed and in fact remains a long term goal that may be realized using this assay, we used ciprofloxacin as a compound that is known to specifically bind to ssDNA [31]. Hence, ciprofloxacin should prevent the interaction between ssDNA and ICP8. Therefore, two synthetic oligonucleotides (PBAZ48, 24-mer and PBAZ49, 20-mer) that contain a preferred ciprofloxacin binding site (5′ TGGCCT) [31], were used as ligands to evaluate their effect on the T_m of ICP8 in the absence and presence of ciprofloxacin. The data show that the concentration of ciprofloxacin used in this experiment (200 μM) had no effect on the T_m of ICP8 (Fig. 4A and B). Consistent with the (dT) oligomers used in the earlier experiments, both ciprofloxacin-target containing oligonucleotides increased the T_m by the maximum amount of 9 °C (Fig. 4A and B). Significantly, in the presence of 200 μM ciprofloxacin, both oligonucleotide ligands were only able to increase the T_m by 2 °C, indicating that the drug prevented ligand induced stabilization of ICP8. Further analysis using the 24-mer PBAZ48 revealed a dose-dependent reduction in ligand stabilization with increasing ciprofloxacin concentration, stabilizing T_m by only 1 °C at 500 μM, with an apparent IC_{50} of ~80 μM (Fig. 4C). These findings support our notion that the thermal shift assay may be developed into a screen to identify compounds that prevent the ssDNA binding activity of ICP8 and likely other SSB.

4. Discussion

In this study we explored the utilization of the thermal shift assay as a platform to investigate the interaction of SSB with ssDNA, with a view of applying the assay to readily define the basic ssDNA binding properties of this class of protein, and notably as a high-throughput screen to identify ssDNA binding inhibitors with therapeutic potential. Our study focused on the SSB from HSV-1, the essential DNA replication protein ICP8. Thus, we developed the assay in multi-well microtiter plates using a fluorescence enabled thermal cycler to permit the rapid and quantitative evaluation of ssDNA ligands on the T_m of ICP8 by measuring SYPRO Orange fluorescence.

The thermal shift assay allowed us to determine several basic parameters that define the interaction of ICP8 with ssDNA. In summary, we found that ICP8 exhibits a T_m of 45 °C. Importantly, oligonucleotide ligands stabilized its T_m by a maximum of 9 °C in a length- and concentration-dependent manner. This increase in T_m indicates that ligand binding significantly stabilizes the structure of ICP8. Our data show that $(dT)_{10}$ was the shortest oligonucleotide that provided the maximum increase in T_m. This observation indicates that 10 nucleotides represents the minimum length of ssDNA on which an ICP8 protomer can effectively assemble and is consistent with prior analysis that defined the site size of ICP8 as 10 nucleotides [18]. Interestingly, oligomers that contained multiple site size equivalents (20- and 60-mers) and therefore represent a platform on which ICP8 can form a nucleoprotein filament, did not increase the T_m beyond 9 °C. This suggests that the thermal shift assay may not be suitable for detecting stabilization induced as a consequence of cooperative protein–protein interactions as would occur in the context of the ICP8 nucleoprotein filament. Alternatively, the assay may be limited by the overall thermal stability of ICP8 such that it is functionally denatured at temperatures >54 °C. Notably, we observed increases in T_m in the presence of oligomers shorter than 10 nucleotides, with $(dT)_9$, $(dT)_7$ and $(dT)_5$ increasing T_m by 3, 2 and 1 °C, respectively. This finding was confirmed by direct protein-DNA interaction measurements using EMSA, thereby demonstrating that ICP8 can in fact interact with

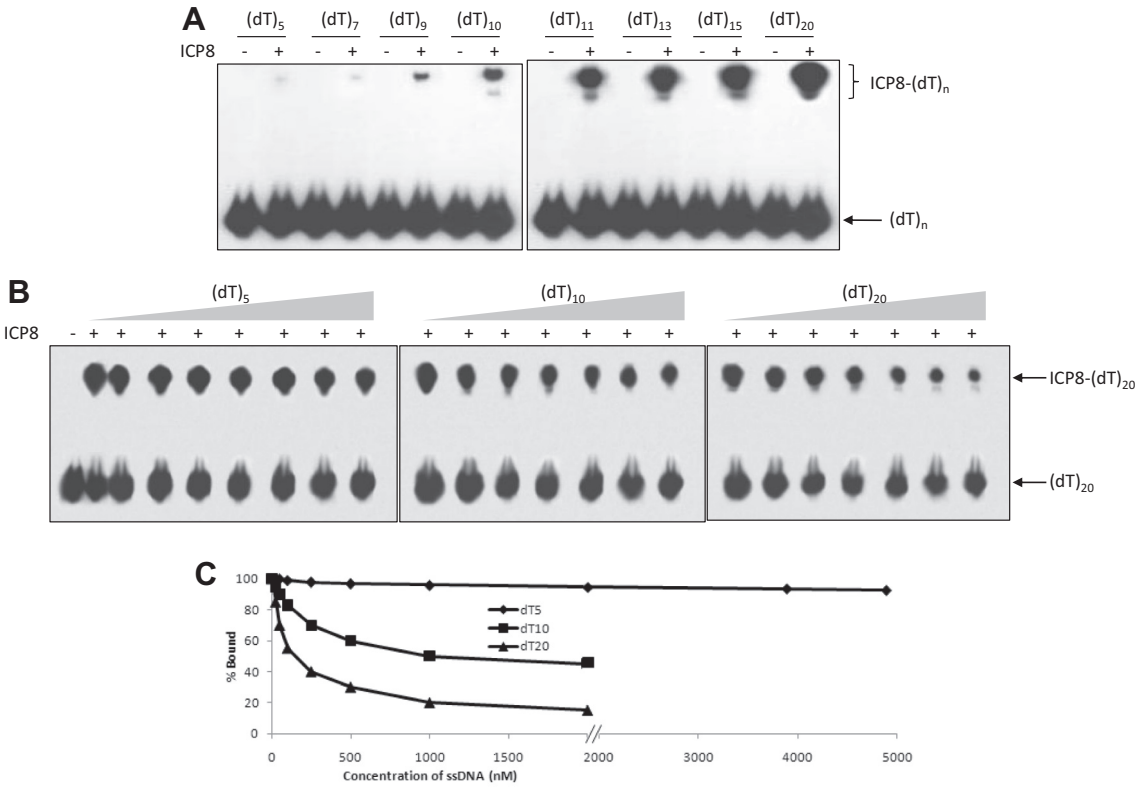

Fig. 3. Interaction of ICP8 with (dT) oligomers by gel mobility shift. (A) EMSA was performed as described in Section 2 with 1 nM of the various (dT) oligomers and 500 nM ICP8 as indicated. The positions of free $(dT)_n$ and of the ICP8-$(dT)_n$ complexes are as indicated. (B) ICP8-$(dT)_{20}$ complexes formed with 1 μM ICP8 and 500 nM 5′-^{32}P-labeled $(dT)_{20}$ were competed with unlabeled $(dT)_5$ (100, 250, 500, 1000, 2000, 4000 and 5000 nM), $(dT)_{10}$ (25, 50, 100, 250, 500, 1000 and 2000 nM) and $(dT)_{20}$ (25, 50, 100, 250, 500, 1000 and 2000 nM), as indicated and analyzed by EMSA as described in Section 2. The positions of free $(dT)_{20}$ and of the ICP8-$(dT)_{20}$ complexes are as indicated. (C) Quantitation of the data in (B).

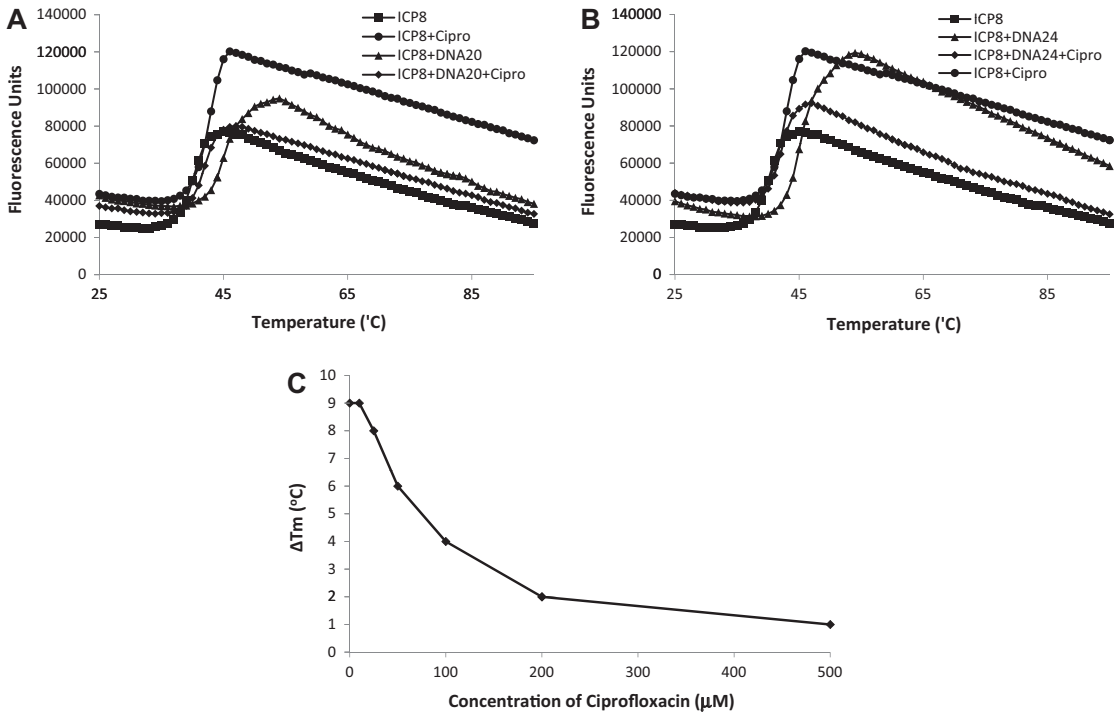

Fig. 4. Effect of ciprofloxacin on ligand induced stabilization of the T_m of ICP8. Reactions were performed as described in Section 2 with 10 μM DNA20 (PBAZ49) (A) and DNA24 (PBAZ48) (B), in the absence or presence of 200 μM ciprofloxacin (Cipro) as indicated. (C) Dose-dependent reduction of ligand stabilization by ciprofloxacin. Reactions were performed as described in Section 2 with 10 μM PBAZ48 (24-mer) and the indicated concentrations of ciprofloxacin. ΔT_m = change in T_m relative to that in the absence of ligand (45 °C). The data are the average of 2 separate acquisitions.

ssDNA shorter than 10 nucleotides albeit with reduced apparent affinity. To our knowledge this is the first demonstration that ICP8 can bind oligonucleotides as short as 5 nucleotides. The thermal shift assay can also be used to extract information pertaining to the binding equilibrium. Hence, the apparent K_d of <1 μM for $(dT)_{20}$ determined herein is comparable to the association constant determined by fluorescence anisotropy measurements ($K = 0.55 \times 10^6\ M^{-1}$) [18]. Overall, the results obtained in this study using the thermal shift assay compare well to previously determined parameters using other biochemical and biophysical techniques. Consequently, this validates the application of the thermal shift assay as a technique to easily determine the basic ssDNA binding parameters of SSB including their site size and binding affinity with the advantage of being a rapid and potentially high-throughput method, utilizing SYPRO Orange as an environmentally sensitive dye.

A further objective of this study was to validate the use of the thermal shift assay as a screening tool to identify compounds that disrupt the ssDNA-SSB interaction. Particularly in the case of microbial SSB, such compounds could potentially be developed into novel antibacterial and antiviral agents. In our case using ICP8, the magnitude of ligand induced stabilization (9 °C increase in T_m) creates an excellent starting point to identify compounds that reduce or eliminate a ligand-dependent shift in T_m. Indeed, in lieu of a compound that blocks the ssDNA binding activity of ICP8, we took advantage of the sequence-dependent ssDNA binding activity of ciprofloxacin to prevent ICP8 from interacting with its ligand [31]. Our finding of a dose-dependent reduction in T_m stabilization by ciprofloxacin provides proof of concept that the thermal shift assay may be used as a screen to identify compounds that disrupt ssDNA-SSB interactions with potential therapeutic effect.

In closing, our findings using ICP8 support the proposed applications of the thermal shift assay, successfully meeting the objectives of this study. To our knowledge this is the first study to utilize the thermal shift assay to measure ligand induced changes in the T_m of a SSB.

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