Modulation of the Herpes Simplex Virus Type-1 UL9 DNA Helicase by Its Cognate Single-strand DNA-binding Protein, ICP8*

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The mechanism of stimulation of a DNA helicase by its cognate single-strand DNA-binding protein was examined using herpes simplex virus type-1 UL9 DNA helicase and ICP8. UL9 and ICP8 are two essential components of the viral replisome that associate into a complex to unwind the origins of replication. The helicase and DNA-stimulated ATPase activities of UL9 are greatly elevated as a consequence of this association. Given that ICP8 acts as a single-strand DNA-binding protein, the simplest model that can account for its stimulatory effect predicts that it tethers UL9 to the DNA template, thereby increasing its processivity. In contrast to the prediction, data presented here show that the stimulatory activity of ICP8 does not depend on its single-strand DNA binding activity. Our data support an alternative hypothesis in which ICP8 modulates the activity of UL9. Accordingly, the data show that the ICP8-binding site of UL9 constitutes an inhibitory region that maintains the helicase in an inefficient ground state. ICP8 acts as a positive regulator by neutralizing this region. ICP8 does not affect substrate binding, ATP hydrolysis, or the efficiency of translocation/DNA unwinding. Rather, we propose that ICP8 increases the efficiency with which substrate binding and ATP hydrolysis are coupled to translocation/DNA unwinding.

We are using the UL9 DNA helicase of herpes simplex virus type-1 (HSV-1) and its cognate single-strand DNA-binding protein (SSB), ICP8, to study the mechanism by which SSBS stimulate DNA helicase-mediated DNA unwinding. Stimulation of helicase activity due to specific association with an SSB has been documented in numerous cases, but little is known about the manner in which it occurs (for example see Refs. 1–8). It should be noted, however, that, in some cases, stimulation of helicase activity may not necessitate physical association of the SSB with the helicase. UL9 and ICP8 are two HSV-1 gene products that are essential for viral origin-dependent DNA replication (reviewed in Refs. 9 and 10). UL9 is a sequence-specific DNA-binding protein of ~94 kDa that recognizes elements within the viral origins of replication (11, 12). In addition, it possesses 3’ to 5’ helicase activity and associated ATPase and single-stranded DNA (ssDNA) binding activities (13–16). The minimum length of ssDNA required to stimulate the ATPase activity of UL9 is 14 nucleotides (15). ICP8 is a ~128-kDa protein that, relative to other known SSBS, binds ssDNA with low affinity and low cooperativity (17). The site size of ssDNA binding is 10 ± 1 nucleotides (17).

ICP8 forms a specific and stoichiometric complex with UL9 by interacting with its extreme C terminus (8, 18, 19). A complex of UL9 and ICP8 has been implicated in unwinding the viral origins of replication (20, 21). In addition, ICP8 has been shown to greatly and specifically increase the ssDNA-stimulated ATPase and helicase activities of UL9, enhancing its processivity (8, 16). In contrast, ICP8 has no effect on ATP hydrolysis in the absence of ssDNA cofactor, indicating that it does not alter the chemical step of ATP hydrolysis (8).

We previously hypothesized that ICP8 stimulates UL9 by tethering it to the DNA template, thereby increasing its processivity (8). Here we show that, in contrast to our hypothesis, the stimulatory effect of ICP8 is not dependent on its ssDNA binding activity. To gain further insight into the mechanism of stimulation, we examined the properties of a UL9 mutant that mimics ICP8-stimulated UL9. Moreover, we further characterized the effects of ICP8 on the ATPase and helicase activities of UL9 and two additional UL9 mutants that possess defects in DNA unwinding. Our results indicate that ICP8 acts as a positive regulator by neutralizing an inhibitory region of UL9 to allow more efficient coupling between substrate binding and ATP hydrolysis and translocation/DNA unwinding.

EXPERIMENTAL PROCEDURES

Materials—ATP (disodium salt), heparin-aragase, phosphoenolpyruvate (potassium salt), and NADH were purchased from Sigma. [γ-32P]ATP (4,500 Ci/mmol) was from ICN.

Bovine serum albumin (DNase-free) and T4 polynucleotide kinase were obtained from Amersham Pharmacia Biotech and New England Biolabs, respectively. Rabbit muscle L-tart dehydrogenase and pyruvate kinase, as solutions in 50% glycerol, were obtained from Sigma. Proteinase K was purchased from Roche Molecular Biochemicals. Escherichia coli SSB and Sequenase version 2.0 DNA polymerase were purchased from U.S. Biochemical Corp. UL9 (22), UL9DM27 (19), UL9C111A (22), UL9C301A,2 and ICP8 (18) were purified as described previously. Protein concentrations, expressed in moles of monomeric protein, were determined using extinction coefficients of 89,220 (UL9), 89,100 (UL9C111A and UL9C301A), 83,530 (UL9DM27), and 82,720 (ICP8) or 1 cm−1 at 280 nm, or by the method of Bradford (23) using bovine serum albumin as a standard.

M13 mp18 virion DNA was purchased from New England Biolabs. Oligodeoxyribonucleotides PB-11 (100-mer) (16), 60-mer (16), 20-mer right (16), PB-9 (22-mer) (16), (dT)15 (8), (dT)20 (8), (dT)60 (8), and 60-mer hairpin (8) were as described. The 11-mer (PB-66, 5′-d(CCATGATTACGG)) was obtained from Operon Technologies. The helicase substrates consisting of 5′-32P-labeled 100-mer annealed to M13 ssDNA, and that consisting of a 5′-32P-labeled 20-mer annealed to the 5′ side of

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1 The abbreviations used are: HSV-1, herpes simplex virus type-1; EPPS, N-(2-hydroxyethyl)piperazine-N’-(3-propanesulfonic acid); SSB, single-strand DNA-binding protein; ssDNA, single-stranded DNA.
a 60-mer, producing a partial DNA duplex with a 3’ ssDNA extension, were constructed as described previously (16). The helicase substrate with strands of increasing length was produced by extending M13 ssDNA primed with the -40 sequencing primer in the presence of dideoxynucleoside triphosphates and Sequenase version 2.0 DNA polymerase as described previously (16).

**UV Cross-linking—**Two nmol of ICP8 in 2 ml of 20 mM HEPES-NaOH, pH 7.6, 0.1 M NaCl, 10% glycerol, 0.1 mM EDTA, and 1 mM dithiothreitol was incubated with 1 nM ICP8 in 10 μl containing 25 mM EPPS-NaOH, pH 8.3, 2.5 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, and 100 μg/ml bovine serum albumin for 10 min on ice. The reactions were mixed with 3 μl of 50% glycerol, 40 mM Tris acetate, pH 7.2, 1 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol, and resolved by electrophoresis through nondenaturing 4% polyacrylamide-TBE gels at 100 V and 4 °C. Following electrophoresis, the gels were dried and autoradiographed or quantitated by storage phosphoranalysis with a Molecular Dynamics Storm 840. Binding affinities were determined by curve-fitting using the nonlinear cooperative ligand-binding equation of the GraFit version 4.09 program from Erithacus Software.

**Gel-mobility Shift Assay—**ICP8 was incubated with 1 nM (molecules) 5'-32P-labeled PB-9 in 10 μl containing 25 mM EPPS-NaOH, pH 8.3, 2.5 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, and 100 μg/ml bovine serum albumin for 10 min on ice. The reactions were mixed with 3 μl of 50% glycerol, 40 mM Tris acetate, pH 7.2, 1 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol, and resolved by electrophoresis through nondenaturing 4% polyacrylamide-TBE gels at 100 V and 4 °C. Following electrophoresis, the gels were dried and autoradiographed or quantitated by storage phosphoranalysis with a Molecular Dynamics Storm 840. Binding affinities were determined by curve-fitting using the nonlinear cooperative ligand-binding equation of the GraFit version 4.09 program from Erithacus Software.

**Helicase Assay—**Helicase assays were performed essentially as described (16). Unless otherwise stated, reactions were performed at 37 °C and contained 25 mM EPPS-NaOH, pH 8.3, 5.5 mM MgCl₂, 3 mM ATP, 3 mM dithiothreitol, 10% glycerol, 100 μg/ml bovine serum albumin, and DNA substrate and proteins as indicated. The reactions were terminated by the addition of 0.3 volumes of 90 mM EDTA, 6% SDS, 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol, and 0.6 mg/ml proteinase K, followed by a 15-min incubation at 37 °C. The reaction mixtures were resolved by electrophoresis through nondenaturing polyacrylamide-TBE gels. Following electrophoresis, the gels were dried and autoradiographed or quantitated by storage phosphoranalysis with a Molecular Dynamics Storm 840.

**ATPase Assay—**Rates of ATP hydrolysis were determined using an enzyme-linked assay as described (8). Reactions were performed at 37 °C and contained 20 mM EPPS-NaOH, pH 8.3, 2.5 mM MgCl₂, 2 mM ATP/MgCl₂, 200 μM NADH, 1.5 mM phosphoenolpyruvate, 40 units/ml L-lactic dehydrogenase, 40 units/ml pyruvate kinase, 10 μM (nucleotide) DNA cofactor, 3 mM dithiothreitol, 100 mM NaCl, 100 mM UL9 or UL9DM27, and 500 nM ICP8 as indicated. Rates of ATP hydrolysis were calculated by converting the absorbance change at 340 nm to moles of ATP hydrolyzed using an extinction coefficient of 6,220 M⁻¹ cm⁻¹ for NADH. Kinetic constants were determined using the nonlinear regression Michaelis-Menten kinetics curve-fitting program of Leaetherbarrow (24).

**RESULTS**

**Modification of the ssDNA-binding Site of ICP8—**Since specific residues required for the ssDNA binding activity of ICP8 have not been identified, it is not possible to construct mutants that specifically lack ssDNA binding activity. Consequently, ICP8 was covalently modified by cross-linking with (dT)₁₅. This procedure was repeated three times to yield a preparation that is not adsorbed while noncross-linked ICP8 is retained by the column. The reaction products were analyzed and quantitated by storage phosphoranalysis with a Molecular Dynamics Storm 840.

**Requirement for the ssDNA Binding Activity of ICP8 in the Stimulation of UL9—**To examine whether the stimulatory effect of ICP8 depends on its ability to interact with ssDNA, cross-linked ICP8 was compared with untreated and UV-treated ICP8 in their ability to stimulate the helicase activity of UL9. Fig. 3 shows that all three preparations of ICP8 could stimulate UL9 to the same final extent (∼9-fold stimulation) and that similar levels of stimulation were observed at given protein concentrations. Given that the preparation of cross-linked ICP8 possesses 4.5-fold lower ssDNA binding activity, a significant reduction in stimulatory activity should have been observed if the ssDNA binding activity of ICP8 is indeed required for stimulation. Fig. 3 also depicts two theoretical curves that show the predicted stimulatory activity of ICP8 whose activity is reduced by 1.5-fold (based on the physical extent of cross-linking) or 4.5-fold (based on the reduction in ssDNA binding activity).

To further examine the requirement for the ssDNA binding activity of ICP8, the stimulatory effect of ICP8 on UL9 was examined in the presence of a short (11-mer) oligodeoxynucleotide. In principle, given that the ssDNA size of ICP8 is 10 ± 1 nucleotides (17), the 11-mer should occupy the ssDNA-binding site of ICP8 but is too short to effectively interact with UL9. More importantly, excess concentrations of 11-mer (up to 50-fold more nucleotide than DNA substrate and 110-fold higher than the concentration of UL9) had no effect on the helicase activity of UL9, indicating that it is indeed too short to compete with DNA substrate binding to UL9. More importantly, excess concentrations of 11-mer (up to 50-fold more nucleotide than DNA substrate) 50-fold higher than the concentration of ICP8, and −10-fold higher than the Kᵣ for ssDNA for ICP8 (Ref. 17) did not reduce the stimulatory effect of ICP8. To demonstrate that the 11-mer can indeed interact with ICP8 under these conditions and therefore compete with DNA substrate binding, a variety of gel-mobility shift assays were performed. Fig. 5A shows that the concentrations of 11-mer that failed to disrupt the stimulatory effect of ICP8 block subsequent ssDNA binding. This is evident from the inability of cross-linked ICP8 to bind heparin-agarose or ssDNA-cellulose (data not shown). Fig. 1 shows the composition of cross-linked ICP8 compared with UV-treated and untreated ICP8. Cross-linked ICP8 is distinguished by its slightly slower electrophoretic mobility (25). Densitometric analysis revealed that 33% of ICP8 in the preparation was cross-linked to (dT)₁₅. The ssDNA binding activity of cross-linked ICP8 was measured directly using a gel-mobility shift assay. Fig. 2 shows ssDNA-binding isotherms for untreated ICP8, UV-treated ICP8, and cross-linked ICP8. The amount of protein required for half-maximal ssDNA-binding was 0.1 μg for ICP8 and UV-treated ICP8 and 0.45 μg for cross-linked ICP8. Consequently, cross-linked ICP8 exhibits 4.5-fold lower ssDNA binding activity. It is interesting to note that cross-linked ICP8 showed lower ssDNA binding activity than would be expected from its degree of cross-linking. This finding may be explained by the fact that some unsubstituted ICP8 is also deficient in ssDNA binding activity and that the preparation of cross-linked ICP8 is enriched for this form of ICP8 by repeated passage through heparin-agarose.

**Binding Specificity for ssDNA of ICP8—**To demonstrate that the ssDNA binding activity of ICP8 is indeed required for the ssDNA binding activity of ICP8, the stimulatory effect of ICP8 on UL9 was examined in the presence of a short (11-mer) oligodeoxynucleotide. In principle, given that the ssDNA size of ICP8 is 10 ± 1 nucleotides (17), the 11-mer should occupy the ssDNA-binding site of ICP8 but is too short to effectively interact with UL9. More importantly, excess concentrations of 11-mer (up to 50-fold more nucleotide than DNA substrate) 50-fold higher than the concentration of ICP8, and −10-fold higher than the Kᵣ for ssDNA for ICP8 (Ref. 17) did not reduce the stimulatory effect of ICP8. To demonstrate that the 11-mer can indeed interact with ICP8 under these conditions and therefore compete with DNA substrate binding, a variety of gel-mobility shift assays were performed. Fig. 5A shows that the concentrations of 11-mer that failed to disrupt the stimulatory effect of ICP8...
could in fact compete with \(^{32}\)P-labeled 22-mer in the formation of a 22-mer-ICP8 complex. Quantitation of the data shows that competition was 99% effective at the highest concentration of 22-mer (Fig. 5B). Similarly, the 11-mer could compete with M13 ssDNA in the formation of a M13 ssDNA-ICP8 complex and could itself be bound by ICP8 (data not shown). Collectively, these data show that stimulation of DNA unwinding by ICP8 does not depend on its ability to interact with the DNA template.

Helicase Activity of UL9DM27—UL9DM27 lacks the C-terminal 27 amino acids of UL9, which comprise the ICP8-binding site (19). Consistent with the failure of UL9DM27 to associate with ICP8, its helicase activity is not affected by ICP8 (19). Interestingly, UL9DM27 exhibits elevated ssDNA-stimulated ATPase and helicase activities compared with wild-type UL9 (19). To establish whether the properties of UL9DM27 mimic

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**Fig. 2.** ssDNA binding activity of cross-linked ICP8. ssDNA binding activity was determined by a gel-mobility shift assay as described under “Experimental Procedures.” A, untreated ICP8; B, UV-treated ICP8; C, cross-linked ICP8.

**Fig. 3.** Stimulation of UL9 helicase activity by cross-linked ICP8. Helicase activity was determined as described under “Experimental Procedures,” with 100 nM dithiothreitol. UL9 (150 nM) was preincubated with 1 nM (molecules) M13:100-mer substrate for 20 min at 37 °C. The reactions were initiated by the addition of ATP and untreated ICP8 (empty circle), UV-treated ICP8 (filled circle), or cross-linked ICP8 (filled square) and allowed to proceed for 15 min. The expected stimulatory activity of ICP8 that exhibits 1.5-fold (short dashes) or 4.5-fold (long dashes) reduced ssDNA binding activity is indicated. The data represent the average of three independent experiments.

**Fig. 4.** Stimulation of UL9 helicase activity by ICP8 in the presence of ssDNA competitor. Helicase reactions were performed essentially as described under “Experimental Procedures,” with 20 mg EPPS-NaOH, pH 8.3, 1 mM dithiothreitol, 5% glycerol, and 50 µg/ml bovine serum albumin. UL9 (150 nM) was preincubated with 0.5 nM (molecules) M13:100-mer substrate for 20 min at 37 °C. The reactions were initiated by the addition of ATP in the absence or presence of 326.5 nM ICP8 and 11-mer competitor as indicated, and allowed to proceed for 15 min for UL9 (columns 1–4) or 5 min for UL9 and ICP8 (columns 5–8). Column 1, UL9; column 2, UL9 and 326.5 nM (molecules) 11-mer; column 3, UL9 and 3.265 µM (molecules) 11-mer; column 4, UL9 and 16.325 µM (molecules) 11-mer; column 5, UL9, ICP8 and 326.5 nM (molecules) 11-mer; column 6, UL9, ICP8 and 3.265 µM (molecules) 11-mer; column 7, UL9, ICP8 and 16.325 µM (molecules) 11-mer; column 8, UL9 and ICP8.

**Fig. 5.** Interaction of 11-mer with ICP8. ssDNA binding activity was determined by a gel-mobility shift assay as described under “Experimental Procedures.” A, autoradiograph of reactions with 326.5 nM ICP8. Lane 1, no ICP8; lane 2, ICP8; lane 3, ICP8 and 326.5 nM (molecules) 11-mer; lane 4, ICP8 and 3.265 µM (molecules) 11-mer; lane 5, ICP8 and 16.325 µM (molecules) 11-mer. The positions of free 22-mer and of 22-mer-ICP8 complex are as indicated. B, quantitation of the data shown in A. Column 1, ICP8; column 2, ICP8 and 326.5 nM (molecules) 11-mer; column 3, ICP8 and 3.265 µM (molecules) 11-mer; column 4, ICP8 and 16.325 µM (molecules) 11-mer.
those of ICP8-stimulated UL9, we examined its processivity using two different substrates. The first substrate, consisting of strands of increasing length hybridized to M13 ssDNA, was preincubated with UL9 or UL9DM27, and DNA unwinding was initiated by the addition of ATP. At the same time, to capture helicase that dissociated during the course of the reaction, a competitor, and 207 nM E. coli SSB was added to trap unwound strands. Fig. 6 shows a time course of DNA unwinding under single-turnover conditions (enzyme > [DNA]) shows that, although the final extent of DNA unwinding catalyzed by UL9 and UL9DM27 approached the same level, the rate promoted by UL9DM27 was considerably faster (Fig. 6B). Moreover, addition of a 100-fold molar excess of a competitor 60-mer hairpin, which mimics the structure of the DNA substrate, 1.5 min after the reaction was initiated, inhibited further DNA unwinding by UL9. In contrast, there was significant DNA unwinding after the addition of the competitor with UL9DM27 (Fig. 6C). The faster rate of DNA unwinding by UL9DM27 and its relative resistance to challenge with competitor indicate that it possesses increased processivity as observed with ICP8-stimulated UL9 (8).

**ATPase Activities of UL9 and UL9DM27**—We have shown previously that ICP8 has no effect on the ATPase activity of UL9 in the absence of ssDNA cofactor (8). Similarly, the ssDNA-independent ATPase activity of UL9DM27 is not elevated. In fact, the specific activity of ATP hydrolysis in the absence of ssDNA cofactor (0.02 units/pmol, where 1 unit is that amount of enzyme that catalyzes the hydrolysis of 1 pmol of ATP/s) is the same for both UL9 and UL9DM27. This finding further supports the notion that UL9DM27 mimics the properties of ICP8-stimulated UL9 and indicates that the increased activity associated with deletion of the C-terminal 27 amino acids is not due to enhanced ATP hydrolysis.

Rates of ATP hydrolysis for UL9 and UL9DM27 were determined in the presence of (dT)$_{20}$, (dT)$_{60}$, and a 60-mer hairpin, in the absence or presence of ICP8 (Fig. 7). Consistent with previous results for UL9 (8), (dT)$_{60}$ supported a higher rate of ATP hydrolysis than (dT)$_{20}$, indicating a correlation between translocation and ATP hydrolysis. The 60-mer hairpin possesses a 20-nucleotide 3’ ssDNA extension to serve as a loading site for UL9. In the absence of DNA unwinding, it should support ATP hydrolysis to the same extent as (dT)$_{20}$. It is possible that, in the steady state, the rate of ATP hydrolysis due to efficient utilization of the 60-mer hairpin should be the sum of ATP hydrolysis required for DNA unwinding and that due to translocation. Moreover, addition of a 100-fold molar excess of a competitor 60-mer hairpin, which mimics the structure of the DNA substrate, 1.5 min after the reaction was initiated, inhibited further DNA unwinding by UL9. In contrast, there was significant DNA unwinding after the addition of the competitor with UL9DM27 (Fig. 6C). The faster rate of DNA unwinding by UL9DM27 and its relative resistance to challenge with competitor indicate that it possesses increased processivity as observed with ICP8-stimulated UL9 (8).

**Fig. 7. ATPase activity of UL9 and UL9DM27: effects of DNA length, structure, and ICP8.** ATP hydrolysis was determined as described under “Experimental Procedures.” Reactions contained 10 μM (nucleotide) (dT)$_{20}$ (columns 1, 4, 7, and 10), (dT)$_{60}$ (columns 2, 5, 8, and 11), and 60-mer hairpin (columns 3, 6, 9, and 12); UL9 (columns 1–3 and 7–9); UL9DM27 (columns 4–6 and 10–12); ICP8 (columns 7–12).
activity is probably due to inefficient unwinding of the hairpin by UL9. The ATPase activity of UL9DM27 exhibited a similar dependence on ssDNA length, although its overall activity was greater than that of UL9. In contrast to UL9, UL9DM27 exhibited a higher rate of ATP hydrolysis with the 60-mer hairpin than with (dT)60, indicating that it is more efficient at utilizing this substrate. The addition of ICP8 stimulated the activity of UL9 with (dT)20 and (dT)60. Interestingly, ICP8 resulted in a higher rate of ATP hydrolysis with the 60-mer hairpin than with (dT)60, indicating that it increases the efficiency with which UL9 unwinds this substrate. The addition of ICP8 to reactions with UL9DM27 resulted in weak inhibition of ATP hydrolysis, presumably because ICP8 can bind to the DNA and prevent subsequent association of UL9DM27. As a conservative estimate, in the steady state, the difference in ATP hydrolysis between (dT)60 and the 60-mer hairpin reflects the amount of ATP hydrolysis required for unwinding the hairpin. Interestingly, this difference (1.3-fold) is identical for UL9DM27 and ICP8-stimulated UL9, indicating equivalent efficiency.

The above studies on the helicase and ATPase activities of UL9DM27 indicate that it resembles ICP8-stimulated UL9. To examine whether the stimulatory effect of ICP8 is due to changes in substrate (ATP and ssDNA) affinity, the steady-state kinetic parameters $k_{cat}$ and $K_m$ were determined for UL9, ICP8-stimulated UL9, and UL9DM27 (Table 1). The values for $k_{cat}$ and $K_m$ for ATP and ssDNA cofactor for UL9 are consistent with those reported previously (22, 26). As expected, ICP8-stimulated UL9 exhibits an increased $k_{cat}$ (4-fold). However, there is no significant change in the $K_m$ for ATP. Owing to the fact that the ssDNA-stimulated ATPase activity of UL9 is inhibited at high ssDNA:DNA ratios, and the necessity to titrate (dT)60 while keeping ICP8 concentrations constant, it was not possible to determine $K_m$ for ssDNA cofactor for ICP8-stimulated UL9. Analysis of UL9DM27 shows that its $k_{cat}$ is increased $\sim$20-fold over UL9. Interestingly, there are no significant changes in $K_m$ for ATP or ssDNA cofactor for UL9DM27. DNA-cross-linking was used to ascertain whether ICP8 had a significant effect on the affinity for ssDNA. No significant increase in cross-linking was observed with UL9 in the presence of ICP8 or with UL9DM27 (data not shown).

**Effect of ICP8 on UL9 Mutants with Defects in DNA Unwinding**—UL9C111A and UL9C301A bear cysteine to alanine substitutions in the vicinity of the “Walker” type A ATP-binding motif, and helicase motif IV, respectively. UL9C111A shows a defect in coupling ssDNA binding to high affinity ATP binding and subsequent hydrolysis (22), while UL9C301A shows a defect in enzyme-DNA complex formation. If ICP8 exerts its stimulatory effect at either of these steps, these mutants should exhibit reduced stimulation in the presence of ICP8. Fig. 8 shows that ICP8 stimulated UL9C111A and UL9C301A to the same level as wild-type UL9.

**DISCUSSION**

The aim of this study was to further examine the mechanism by which ICP8 stimulates the helicase activity of UL9. It was postulated previously that ICP8, due to its function as an SSB and its association with UL9, increases the processivity of UL9 by tethering it to the DNA (8).

Two approaches were used to test the above prediction. First, ICP8 was modified such that its ssDNA-binding site was occupied with an oligodeoxyribonucleotide, preventing subsequent interactions with ssDNA. This was achieved by UV cross-linking (dT)10 to ICP8 using a method that is specific for the ssDNA-binding site of ICP8 (25). Cross-linked ICP8 that was severely impaired in its ssDNA binding activity was able to stimulate the helicase activity of UL9 to the same level as untreated or mock-treated ICP8. This finding clearly shows that the stimulatory effect of ICP8 does not depend on its ability to interact with the DNA template. In addition, the data also suggest that cross-linked ICP8 is still competent to interact with UL9 since their association is required for stimulation of DNA unwinding (19).

In the second approach, the ssDNA-binding activity of ICP8 was modulated by selectively blocking its ssDNA-binding site with competitor DNA. This was achieved by incubating ICP8 with excess concentrations of 11-mer oligodeoxyribonucleotide. Consistent with the results using cross-linked ICP8, excess 11-mer had no effect on the ability of ICP8 to stimulate the helicase activity of UL9. Taken together, these two approaches demonstrate that stimulation of UL9 does not depend on the ability of ICP8 to interact with the DNA template. It is conceivable that, to stimulate DNA unwinding, ICP8 must nevertheless interact with ssDNA in trans (i.e. independent of the DNA template). However, this would entail the unlikely scenario that ssDNA activates ICP8, which in turn activates the helicase activity of UL9. It is more feasible that the ssDNA-binding activity of ICP8 is completely dispensable for its stimulatory effect, and that ICP8 acts as an activator of UL9.

Previous studies have shown that maximal stimulation of helicase activity occurs at a stoichiometry of 1:1 UL9:ICP8, consistent with the existence of an equimolar complex (8). However, at this point it is not known whether the active species consists of a dimer of UL9 and two molecules of ICP8, or whether it involves an oligomeric species (16). In any case, the results presented in this study show that the active species does not require the ssDNA-binding activity of ICP8. However, it is likely that, following DNA unwinding by the UL9-ICP8 complex, stabilization of unwound DNA strands requires the ssDNA-binding activity of ICP8. It is also possible that the overall stimulatory effect of ICP8, especially that seen during the unwinding of long DNA strands (16, 27), is a composite of the action of a UL9-ICP8 complex (in which the ssDNA-binding activity of ICP8 is not required) and excess ICP8 (to stabilize unwound DNA).

**TABLE I**

**Kinetic parameters of the ssDNA-stimulated ATPase activities of UL9, ICP8-stimulated UL9, and UL9DM27**

|         | UL9                  | ICP8-stimulated UL9 | UL9DM27 |
|---------|----------------------|----------------------|---------|
| $k_{cat}$ (s$^{-1}$) | $0.34 \pm 0.02$       | $1.21 \pm 0.12$       | $6.28 \pm 0.37$     |
| $K_{cat,ATP}$ (M$^{-1}$s$^{-1}$) | $536 \pm 94$         | $646 \pm 126$         | $271 \pm 187$       |
| $K_{cat,ssDNA}$ (M$^{-1}$) | $1.05 \pm 0.29$      | ND                    | $1.16 \pm 0.33$     |

**FIG. 8. Effect of ICP8 on DNA unwinding by UL9, UL9C111A, and UL9C301A.** Helicase activity was determined essentially as described under “Experimental Procedures,” with 1 mM dithiothreitol and 5% glycerol. One hundred and fifty nM UL9 (columns 1 and 2), UL9C111A (columns 3 and 4), or UL9C301A (columns 5 and 6) were preincubated with 1 nM (molecules) M13:100-mer substrate for 20 min at 37 °C. The reactions were initiated by the addition of ATP in the absence (columns 1, 3, and 5) or presence (columns 2, 4, and 6) of 82 nM ICP8 and allowed to proceed for 15 min.
A complex of UL9 and ICP8 has been implicated in the unwinding of the viral origins of replication (20, 21). Here ICP8 may serve as a component of the active helicase and to stabilize unwound regions of DNA. It is possible that the former function of ICP8 is independent of its ssDNA-binding activity, as is the case for the unwinding of nonspecific DNA, whereas the latter function would require its ssDNA-binding activity. Consequently, it will be interesting to examine whether ICP8 deficient in ssDNA-binding activity can substitute for unmodified ICP8 during origin unwinding.

To further examine the mechanism by which ICP8 exerts its stimulatory effect, we characterized the properties of a UL9 mutant (UL9DM27) that lacks the C-terminal ICP8-binding site and therefore does not physically interact with ICP8 or respond to its stimulatory activity (19). Despite its inability to functionally or physically associate with ICP8, it retains the properties of ICP8-stimulated UL9 (increased ssDNA-stimulated ATPase and helicase activities, increased processivity, and increased efficiency of DNA unwinding). These observations suggest that the extreme C terminus of UL9 is an inhibitory region that normally suppresses the enzymatic activities of UL9. Furthermore, the role of ICP8 is to neutralize this inhibitory region thereby activating UL9. Consequently, we conclude that ICP8 functions as a positive regulator that mediates its effect through the C terminus of UL9.

To explain how ICP8 exerts its stimulatory effect, we entertained several possibilities that were tested against our results. First, it is possible that stimulation is brought about by improving the ratio of ATP hydrolyzed per base pair unwound (i.e., increasing the efficiency of DNA unwinding). Exact determination of the amount of ATP required per base pair unwound is not practical due to limitations in current technology. However, it is possible to calculate a ratio that relates rates of ATP hydrolysis and DNA unwinding. We find that neither ICP8-stimulated UL9 or UL9DM27 exhibit significant differences in this ratio when compared with UL9. Consequently, it appears unlikely that ICP8 acts in this capacity.

The second possibility involves a change in the step size of translocation/DNA unwinding. It is possible that, without changing the actual rate of movement along DNA, an increase in step size would lead to an increase in the overall rate of translocation/DNA unwinding. Given that the ssDNA-stimulated ATPase activity is a reflection of the translocation of UL9 along ssDNA, an increase in step size would actually decrease the effective length of DNA and result in a concomitant decrease in the rate of ATP hydrolysis for a DNA cofactor of a particular length. Moreover, since the stimulatory effect of ICP8 is substantial (~10-fold), a corresponding increase in step size would have to be equally extensive and may not be within the limits of a conformational change in UL9.

A third possibility is that ICP8 increases enzyme catalysis. This is unlikely since neither ICP8-stimulated UL9 or UL9DM27 display elevated ATPase activity in the absence of ssDNA cofactor.

The fourth possibility involves an increase in substrate affinity. Accordingly, if substrate (ATP or ssDNA) is limiting, an increase in affinity should enhance the rate of translocation/DNA unwinding. This mechanism has been proposed for Schizosaccharomyces pombe DNA helicase I, where RP-A increases the affinity of the enzyme for ATP (6). Similarly, E. coli ribosomal protein L3 has been shown to promote cooperative binding of Bacillus steaothermophilus FepA DNA helicase to its DNA substrate (7). It is unlikely that ICP8 exerts its effect on UL9 in this manner since neither ICP8-stimulated UL9 or UL9DM27 exhibit increased affinity for ATP or ssDNA.

Finally, the fifth possible mechanism by which ICP8 exerts its effect is by increasing the efficiency with which substrate-binding (ATP and ssDNA) and ATP hydrolysis are coupled to translocation/DNA unwinding. In this case, ATP binding, ssDNA binding, and ssDNA-independent ATP hydrolysis remain unaffected by ICP8, but more efficient coupling results in increased activity, which is reflected by increases in the rates of ssDNA-stimulated ATP hydrolysis and DNA unwinding. This step may entail a conformational change that allows UL9 to move along ssDNA more easily. In light of the evidence against the earlier possibilities, we favor this option. Precise determination of the step(s) affected by ICP8 will require the use of rapid kinetic techniques. Nevertheless, our present data with UL9C111A and UL9C301A, which possess defects in coupling ssDNA binding to ATP binding and subsequent hydrolysis, and ssDNA binding, respectively, suggest that ICP8 exerts its effect downstream of these steps.

In conclusion, our data indicate that stimulation of UL9 is independent of the ability of ICP8 to interact with the DNA template. Instead, ICP8 activates UL9 by binding to its extreme C terminus. Consequently, ICP8 acts as a positive regulator by neutralizing a region of UL9 that otherwise suppresses the enzyme. Rather than increasing substrate affinity, catalysis, efficiency of translocation/DNA unwinding, or step size, ICP8 appears to increase the efficiency with which substrate binding and ATP hydrolysis are coupled to translocation/DNA unwinding.

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