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Conformational Change in the Herpes Simplex Single-Strand Binding Protein Induced by DNA

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Protease digestion of the herpes simplex virus type 1 major single-strand DNA binding protein ICP8 showed that the cleavage patterns observed in the presence and absence of single-stranded DNA oligonucleotides are substantially different with protection of cleavage sites between amino acids 293 and 806 observed in the presence of oligonucleotide. Experiments using ICP8 modified with fluorescein-5-maleimide (FM) showed that the fluorescence signal exhibited increased susceptibility to antibody quenching and a significant decrease in polarization of the FM fluorescence was observed in the presence compared to the absence of oligonucleotide. Taken together, these results indicate that ICP8 undergoes a conformational change upon binding to single-stranded DNA.

Key Words: **single-strand binding protein; herpes simplex virus; DNA–protein interaction; protein conformation; DNA replication; ICP8.**

The herpes simplex virus type 1 (HSV-1) UL29 protein, commonly designated ICP8, is one of seven HSV-1 proteins required for origin-dependent replication of viral DNA (1–3). ICP8 binds preferentially and cooperatively to single-stranded DNA (4–15). This binding is largely sequence independent, although some preference for deoxynucleotide homopolymers has been observed (8, 9, 14–17). The size of the binding site has been estimated to be between 10 and 40 nucleotides using a variety of techniques with the majority of the studies suggesting a functional occluded site size on the order of 14–25 nucleotides (4, 10, 13–15, 18–21). ICP8 destabilizes complementary homopolymer duplexes and can displace short oligonucleotides annealed to long single strands of DNA (4, 5, 10, 17, 18, 22). This latter property is due, in part, to the cooperative nature of ICP8 binding (4, 10, 18). ICP8 stimulates the activity of the HSV-1 DNA polymerase (15, 23, 24) and is required for origin specific unwinding by the viral origin binding protein, UL9 (21, 22). ICP8 also optimizes the helicase and primase activities of the heterotrimeric HSV-1 helicase-primase (composed of the UL5, 8, and 52 gene products) most likely through an interaction with the UL8 subunit of that complex (25–27).

Evidence for changes in protein conformation upon binding to single-stranded DNA has been gathered for other single-stranded DNA binding proteins including the T4 gene 32 protein, the *Escherichia coli* SSB, the adenovirus DNA-binding protein and human replication protein A (28–33). Such conformational changes have been shown to be important for cooperative binding along a DNA strand or for interaction with other proteins. Furthermore, White and Boehmer (34) and Mapelli *et al.* (11) have recently shown that the presence of single-stranded oligonucleotides results in protected protein fragments in trypsin digestions. Based on these findings and the current understanding of the functions of ICP8, we hypothesized that the interaction of ICP8 with a single-stranded DNA molecule representing a single binding site would result in a conformational change of the protein. In this communication we present evidence that such a conformational change does occur.

MATERIALS AND METHODS

Chemicals and reagents. Fluorescein-5-maleimide (FM) and anti-FM antibody were purchased from Molecular Probes, Inc. (Eugene, OR). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) or by the Biomedical Instrumentation Center, Uniformed Services University of the Health Sciences (Bethesda, MD). Endoproteinase Lys C (sequencing grade) was purchased from Roche (Mannheim, Germany).

Purification and labeling of ICP8. ICP8 was purified from either *S. frugiperda* 21 or High Five insect cells infected with recombinant baculovirus expressing the HSV-1 KOS strain UL29 gene and a portion of the purified protein was labeled with FM to the level of one

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mole of FM per mole of protein as previously described (18). The concentration of ICP8 and ICP8-FM was quantified by UV absorbance at 205 nm by the method of Scopes (35).

Protease protection assays. Reaction mixes for time course protease protection experiments contained 30 μg ICP8 in the absence or presence of 3.0 μg oligo dT_{25}. This ratio represents an approximately twofold molar excess of oligonucleotide to protein. Sample volumes were adjusted to 100 μl with buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 0.5 mM DTT. Lys C (0.04 units) was added to these samples and digestion was performed at 37°C. Aliquots of 20 μl were removed immediately after addition of the protease (time 0) and after 15, 30, and 60 min of incubation. Digestion was stopped by the addition of a 10-μl aliquot of 2× SDS–PAGE sample buffer and products were resolved by SDS–PAGE. Protection experiments comparing the efficiency of protection by oligo dA_{17}, oligo dC_{17}, oligo dC_{25}, and oligo dT_{25} were performed as described above at equivalent protein to DNA ratios and time points were taken at 0 and 30 min.

Spectrofluorometric analysis of modified ICP8. Spectrofluorometric analyses were performed using an SLM Aminco SPF-500C Spectrofluorometer. The excitation and emission wavelengths utilized for fluorescein-5-maleimide were 490 and 520 nm, respectively. External quenching studies were done with 5μg of ICP8-FM in the presence and absence of 500 ng of oligonucleotide in a reaction volume of 2.0 ml of buffer (150 mM KCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 0.5 mM DTT). Increasing amounts of anti-FM antibody (Molecular Probes, Eugene, OR) were added to the sample and the fluorescence signal monitored by emission wavelength scans from 500 to 600 nm. Initial fluorescence in the absence of antibody was set at 100%.

Fluorescence polarization experiments were performed using instrument parameters and excitation and emission wavelengths as described above. The polarization accessory (SLM Aminco) consisted of a single cell holder and two motor-driven polarizers mounted on a baseplate that fitted into the sample compartment of the spectrofluorometer. Two micrograms of ICP8-FM was mixed with 200 ng of oligo dT_{25} and incubated on ice for 5 min in 2.0 ml of TEK buffer (10 mM Tris, pH 7.6, 1 mM EDTA, and 150 mM KCl). Measurements were also taken from a reagent blank consisting of TEK buffer only. This value was automatically subtracted from each experimental sample reading.

RESULTS

Protease Cleavage of ICP8 in the Presence and Absence of Oligonucleotides

Two separate experimental approaches were taken to determine if ICP8 undergoes a conformational change upon interaction with oligodeoxyribonucleotides containing a single binding site. In the first series of experiments ICP8 was digested with endoproteinase Lys C in the presence and absence of single-stranded oligonucleotides. This enzyme cleaves on the carboxy-terminal side of lysine residues. There are 31 predicted Lys C cleavage sites in the amino acid sequence of KOS strain ICP8 (36) and it is unlikely that all of these sites are equally accessible to the protease. We reasoned that if ICP8 undergoes a conformational change upon binding to single-stranded DNA, an alteration of the Lys-C cleavage pattern would occur. Previous work from our laboratory showed, in EMSA experiments, that oligo dT_{25} represents a model site for the binding of one ICP8 molecule with a dissociation constant of 2.1×10^{-7} M (18). This oligonucleotide was used as the binding substrate in the majority of the experiments performed in this study. Use of an oligonucleotide containing a single binding site would mimic the situation which occurs upon the binding of the first ICP8 molecule along a stretch of single-stranded DNA. Furthermore, ICP8 interaction with a segment of single-stranded DNA on the order of a single binding site has been shown to be important in unwinding of the HSV DNA replication origin Ori_S in studies with model substrates (20, 21).

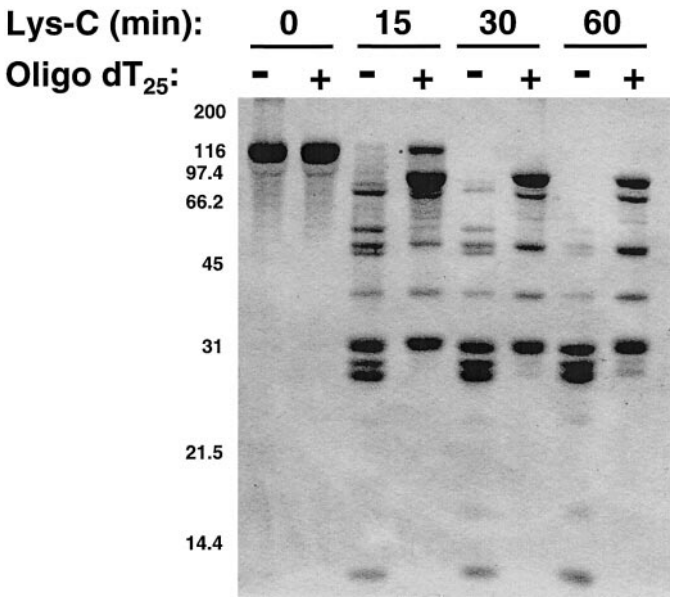

FIG. 1. Partial proteolysis of ICP8 with endoproteinase Lys-C in the presence and absence of a single-stranded DNA oligomer. SDS–PAGE comparison of ICP8 digestions in the absence and presence of the single-stranded DNA oligonucleotide dT_{25}. ICP8 was incubated without and with oligo dT_{25} at a 10:1 (w/w) ratio of protein to DNA for 30 min on ice. Lys-C was added after binding reached equilibrium and samples were incubated at 37°C. Aliquots were removed at 0, 15, 30, and 60 min and digestion was stopped by the addition of SDS sample buffer and incubation at 100°C for 5 min. Samples were run on a 10% acrylamide gel at 150 V for 1 h and bands were visualized by staining with Coomassie brilliant blue.

Results of time course protease digests performed at an approximately 2-fold molar excess of oligonucleotide to protein as described under Materials and Methods are presented in Fig. 1. The peptide profiles generated in the absence of oligo dT_{25} compared with those generated in the presence of oligonucleotide show significant differences at the 15-, 30-, and 60-min time points. Cleavage in the absence of the oligonucleotide for 30 or 60 min results in a pattern of resistant fragments with the most abundant species migrating at or below the 31-kDa marker. In contrast, several prominent high molecular weight fragments are present in the samples containing oligo dT_{25} indicating that cleavage sites are being protected. A small amount of cleavage is observed at time 0 in both samples indicating the presence of a highly susceptible site or sites which is/are

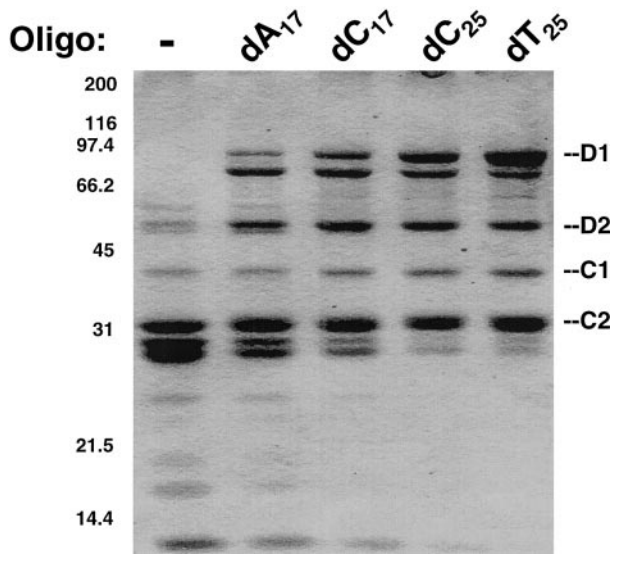

FIG. 2. Length and sequence dependence of protease protection. ICP8 was incubated with oligo dA_{17}, dC_{17}, dC_{25}, or dT_{25} at a 10:1 (w/w) ratio of protein to DNA for 30 min on ice. A control reaction was prepared without DNA. After incubation, samples were digested with Lys-C for 30 min at 37°C and digestion was stopped by the addition of SDS sample buffer at 100°C for 5 min. Samples were run on a 10% acrylamide gel at 150 V for 1 h and bands were visualized by staining with Coomassie brilliant blue.

accessible in the presence or absence of the oligonucleotide. Identical digestion patterns were obtained with 6-fold and 10-fold molar excess of oligonucleotide (data not shown).

Next, cleavage experiments were performed in the presence of oligo dA_{17}, oligo dC_{17}, oligo dC_{25}, as well as oligo dT_{25} at equivalent protein to DNA ratios in order to compare the effect of length and base composition. The Lys C cleavage patterns resulting from 30-min digestions in the presence of the oligonucleotides are presented in Fig. 2. All of the patterns observed in the presence of the oligonucleotides show substantial protection and are quite similar indicating that both lengths of oligonucleotide are reasonable model substrates for a single ICP8 binding site under these experimental conditions. The cleavage patterns observed with oligo dC_{25} and oligo dT_{25} were identical. The pattern observed with oligo dC_{17} was very similar showing only a slight increase in lower molecular weight fragments. The lowest level of protection under these conditions was observed with oligo dA_{17} although the pattern of protected fragments is clearly visible. The differences in protection observed with these oligonucleotides indicate that the strength of ICP8 binding shows a slight length dependence. More striking is the apparent preference of a oligopyrimidine over an oligopurine as was observed with oligo dC_{17} versus oligo dA_{17} suggesting that binding of ICP8 could nucleate at localized sequences rich in pyrimidines within HSV DNA.

In an effort to map the sites protected in the presence of the oligonucleotides, two ICP8 fragments common to digests performed in either the absence or presence of single-stranded DNA (bands C1 and C2, Fig. 2) and two of the high molecular weight bands detected in abundance only in the presence of DNA (bands D1 and D2, Fig. 2) were excised from gels, subjected to additional trypsin digestion and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Borealis Biosciences, Toronto, Canada). This analysis showed that the two fragments of the protein that were relatively resistant to digestion by Lys C in the absence of single-stranded DNA encompassed amino acids 807–1172 (C1) and amino acids 13–293 (C2). A trypsin resistant N-terminal ICP8 fragment (amino acids 1–253) was identified by Mapelli *et al.* (11). Thus, results from two independent laboratories indicate that this portion of the protein forms a separate, protease resistant domain. The C1 fragment contains the single-stranded DNA binding motif identified by Wang and Hall (17). In contrast, the protected fragments found in the presence of the oligonucleotide contained segments of the protein between amino acids 304–1174 (D1) and amino acids 304–727 (C2). Thus interaction with single-stranded DNA oligonucleotides of the size of a single ICP8 binding site resulted in protection of a 500 amino acid region in the center of the protein primary structure which contains eight Lys-C cleavage sites (Fig. 3). Clearly, a fraction of these sites could be masked by direct DNA contacts. However, the extent of the region and number of sites protected, strongly suggested that ICP8 undergoes a conformational change upon binding single-stranded DNA resulting in decreased accessibility of the protease to some of these sites. Based on these results, a second experimental approach was used to confirm a conformational change in ICP8 upon interaction with single-stranded DNA.

Analysis of Fluorescence Changes in ICP8-FM in the Presence of Oligonucleotides

These experiments involved the use of a fluorescent probe as a reporter for alterations in ICP8 structure. The use of fluorescent probes for the detection of conformational changes in proteins upon their interaction with ligands or other polypeptides has been employed in numerous systems as diverse as *E. coli* DNA polymerase, tubulin, fibronectin, and muscle proteins (37–41). We have previously shown that, under specific reaction conditions, one mole of the fluorescent sulfhydryl reagent, fluorescein-5-maleimide (FM), can be incorporated per mole of ICP8 (18). Greater than 85% of the FM moiety was attached to cysteine 254 in the protein sequence. This amino acid position falls outside the boundaries of fragments of ICP8 (amino acids 368–902 and amino acids 300–849) which have exhibited

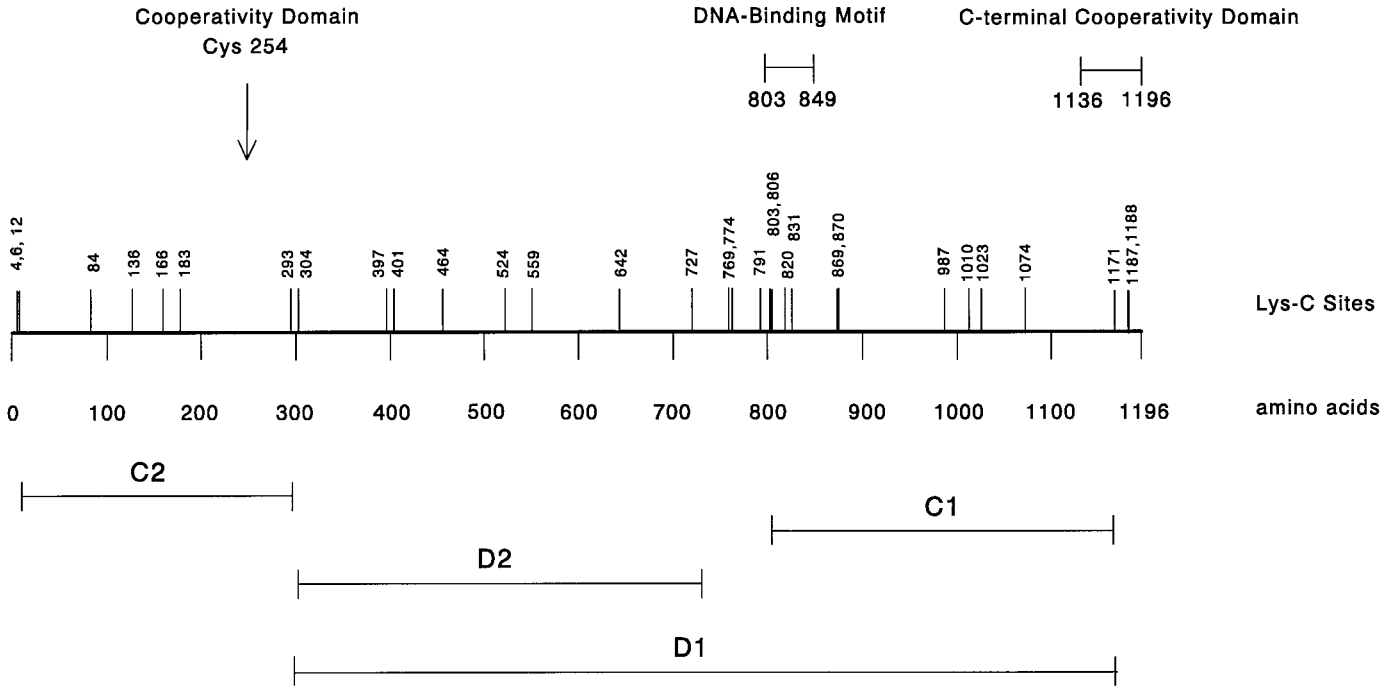

FIG. 3. Mapping of the protected region. The positions of all predicted Lys-C cleavage sites and the positions of the Lys-C peptide products (C1, C2, D1, and D2) analyzed by MALDI-TOF MS are shown relative to the linear amino acid sequence of ICP8. The positions of the cysteine 254 (18) and carboxy-terminal (11) cooperativity domains of ICP8 and the single-stranded DNA binding motif identified by Wang and Hall (17) are also indicated.

binding to single-stranded DNA (17, 34). In addition, EMSA experiments using oligo dT_{25} showed that the intrinsic DNA binding constant (i.e., binding to a single site) is unaltered by FM modification (18). Cys 254 is predicted to lie within an alpha helical segment not fully exposed to the solvent (11, 42). Thus changes in the accessibility of the FM probe and/or fluorescence signal upon interaction of the FM-modified ICP8 (ICP8-FM) with oligo dT_{25} would be indicative of a conformational change in the protein.

ICP8 was labeled with FM to the level of 1 mol of FM per mole of protein as previously described (18). External quenching experiments were performed to gain insight into the accessibility of the FM moiety on ICP8-FM and to determine if the accessibility is altered upon interaction of ICP8 with single-stranded DNA. A rabbit polyclonal anti-FM antibody (Molecular Probes Inc.) was used as the external quencher in experiments performed in the presence and absence of oligo dT_{25}. As shown in Fig. 4, the fluorescence of ICP8-FM in the absence of the oligonucleotide was quenched upon addition of antibody indicating that the FM group is accessible. In the presence of oligo dT_{25} the quenching curve was shifted to the left (i.e., less antibody was required for equivalent quenching) indicating that the FM group was now more readily accessible to the antibody.

Next, fluorescence polarization measurements were performed, as described under Materials and Methods, to determine if the relative motion of the region of ICP8 containing the FM group changed upon interaction with dT_{25}. Data from triplicate determinations resulted in an average polarization value of 0.146 ± 0.004 in the absence of oligo dT_{25} whereas the polarization value was 0.090 ± 0.002 in the presence of dT_{25}, a 38% decrease. This decrease in polarization is indicative of an increase in rotation or flexibility within the region of ICP8 to which the FM is attached upon interaction of ICP8 with the oligonucleotide (43). By comparison, decreases in polarization of 3–7% have been interpreted to show increases in rotation and flexibility in regions of tropomyosin (37) and fibronectin (40) which were labeled with fluorescent probes.

DISCUSSION

We have presented several lines of evidence indicating the occurrence of changes in the conformation of ICP8 upon its interaction with a single-stranded DNA oligonucleotide. The proteolytic cleavage analysis showed that upon interaction with oligo dT_{25}, the availability of Lys C cleavage sites within the ICP8 molecule was significantly altered. Proteolytic cleavage analysis assays such as these have been used to probe changes in the conformation and/or structure of several other single-stranded DNA binding proteins resulting from their interaction with single-stranded DNA (30, 32, 33, 44). In the case of ICP8, the fluorescence polarization

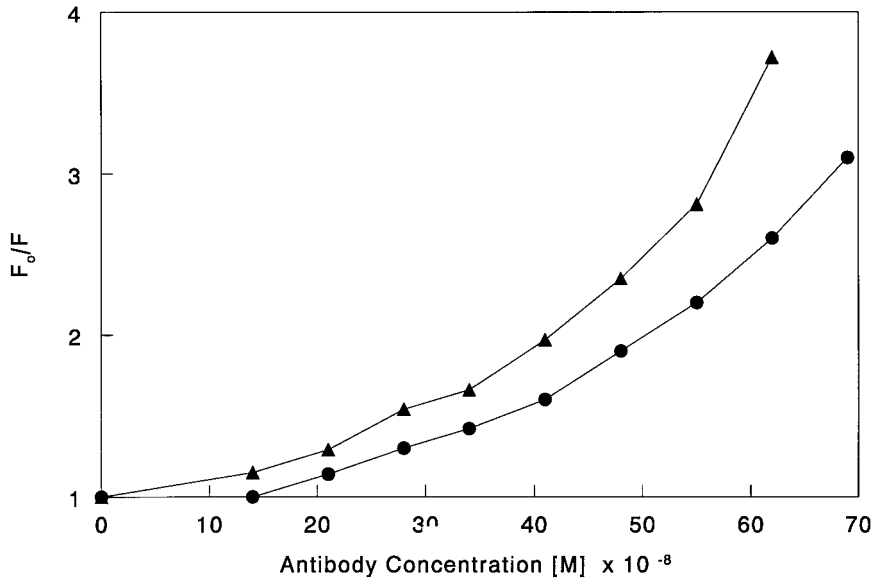

FIG. 4. Quenching of ICP8-FM fluorescence by anti-FM antibody. The results are plotted as the ratio of the initial fluorescence intensity (F_o) over the fluorescence intensity (F) obtained upon addition of antibody versus the final concentration of the added antibody in the absence (●) and presence (▲) of oligo dT_{25}. Five micrograms of ICP8-FM was used in these experiments resulting in a final concentration of 2×10^{-8} M. ICP8 was modified at cysteine 254 with FM to the level of 1 mol of FM per mole of protein. This modification does not affect the K_D of the ICP8 oligo dT_{25} interaction (18).Increasing amounts of anti-FM antibody were added to the samples and the fluorescence signal was monitored by emission wavelength scans from 500 to 600 nm. The initial fluorescence in the absence of antibody was set at 100%.

analyses of FM-modified ICP8 also yielded compelling evidence of a change in the structure of the protein upon binding to single-stranded DNA. A decrease in polarization (indicating an increase in rotation or flexibility within the region of ICP8 at or near the FM modification) is observed upon interaction of ICP8-FM with oligo $dT_{25.}$ Thus binding of ICP8 to a DNA molecule with a single binding site causes a change in the conformation of ICP8 at, or more likely near, the modified cysteine residue. Results from the antibody quenching experiments also support this conclusion. The FM fluorescence was more readily quenched (the FM adduct was more accessible to the antibody) when the protein was bound to oligo dT_{25} indicating binding to a single site increases the exposure of this portion of the protein to its environment. Direct interaction of this region with DNA would be expected to decrease accessibility and movement, in contrast to the experimental results we have obtained.

The concept that a conformational change occurs upon ICP8 binding to single-stranded-DNA has precedence in work with other single-strand DNA-binding proteins. An extensive X-ray crystallographic and mutational analysis of the adenovirus single-strand binding protein by Tucker and colleagues (29, 45) lead to a model where both a conformational change and a the presence of a flexible loop within the adenovirus SSB are required for binding. In the case of the T4 gene 32 protein, a region known to be involved in cooperative binding is proposed to be required to create a conformation of bound protein which can interact with other bound monomers (46). The major eukaryotic single-strand DNA binding protein, RPA, becomes more accessible to kinase activities upon binding to single-stranded DNA and the subsequent phosphorylation events influence its function (28).

The data presented in this study show that conformational changes also occur within ICP8 upon its interaction with single stranded DNA, resulting in protection of a 400- to 500-amino-acid stretch within the center of the predicted primary structure. They have also shown an increase in accessibility and movement at or near a region previously shown to influence ICP8 binding in either a cooperative (18) or anti-cooperative manner (11). These changes in conformation are likely to be important in the numerous functions of this essential herpesvirus protein and its interactions with itself and other HSV replication proteins. Moreover, the results presented here specifically suggest that ICP8 may be required to be bound to single-stranded DNA prior to the establishment of a physical or functional interaction with other viral proteins involved in DNA replication, with the possible exception of the HSV origin-binding protein where a physical interaction occurs in the absence of DNA (16). Therefore, elucidation of the function of the conformationally active region identified in this study, and the identification of other regions of the protein which alter in conformation upon its interaction with single-stranded DNA will be essential to our understanding of the numerous functions of ICP8 during herpes simplex virus DNA replication.

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