The Crystal Structure of the Herpes Simplex Virus 1 ssDNA-binding Protein Suggests the Structural Basis for Flexible, Cooperative Single-stranded DNA Binding*

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All organisms including animal viruses use specific proteins to bind single-stranded DNA rapidly in a non-sequence-specific, flexible, and cooperative manner during the DNA replication process. The crystal structure of a 60-residue C-terminal deletion construct of ICP8, the major single-stranded DNA-binding protein from herpes simplex virus-1, was determined at 3.0 Å resolution. The structure reveals a novel fold, consisting of a large N-terminal domain (residues 9–1038) and a small C-terminal domain (residues 1049–1129). On the basis of the structure and the nearest neighbor interactions in the crystal, we have presented a model describing the site of single-stranded DNA binding and explaining the basis for cooperative binding. This model agrees with the beaded morphology observed in electron micrographs.

Viruses of the *Herpesviridae* family infect almost all vertebrates, including man, causing a variety of diseases. Of the seven viruses identified as human infectious agents, herpes simplex virus-1 (HSV-1) is the prototype of the herpesvirus subfamily and of the family as a whole. The HSV-1 single-stranded DNA (ssDNA)-binding protein (SSB), ICP8, is a nuclear protein that, along with the six other HSV replication proteins (the viral polymerase (UL30) and its accessory factor (UL42), the trimeric helicase-primase complex (UL5-UL8-UL52), and the origin-binding protein (OBP), coded by the gene (UL42), is required for viral DNA replication (1) during lytic infection. Replication has been thought to proceed by a rolling circle mechanism (2), although the observation of highly branched replication intermediates could be explained by other mechanisms that would link recombination and replication. ICP8 is a 128-kDa multifunctional zinc metalloprotein (3) encoded by the *ul29* gene. It preferentially binds ssDNA over double-stranded DNA in a non-sequence-specific and cooperative manner (4). ICP8 has been reported to interact either directly or indirectly with several other viral proteins. There is evidence that it binds to the C terminus of the OBP and stimulates its helicase activity (5, 6), that it promotes the helicase activity of the viral helicase-primase complex (UL5-UL8-UL52) (7), and that it modulates the processivity of the viral polymerase (UL30) (8). Before viral DNA replication commences, these proteins are thought to be co-localized with ICP8 at small punctate foci called pre-replicative sites. With the onset of viral genome amplification, these proteins become redistributed into a larger globular replication compartment (9) whose location is defined by the preexisting host cell nuclear architecture, most probably at the periphery of the nuclear matrix-associated ND10 domains where the viral transactivator ICP0 and the viral input genome are believed to migrate in the early stages of infection (10). ICP8 is also involved in several other events of the DNA metabolism. It can promote DNA strand transfer (11), catalyze strand invasion in an ATP-independent manner (12), and re-nature complementary DNA strands (13), which indicates that ICP8 plays an important role in HSV genome recombination. The replication of HSV-1 DNA is also associated with a high degree of homologous recombination. Recently it was shown that ICP8 works together with alkaline nuclease (UL12), which is a 5’–3’-exonuclease, to effect strand exchange (14). In addition to its role in DNA synthesis, ICP8 has been shown to regulate viral gene expression by repressing transcription from the parental genome (15) and stimulating late gene expression from progeny genomes (16).

Genetic and biochemical analyses have failed to identify functionally independent domains within ICP8. Even the extent of the minimal DNA binding region has remained unclear. It has been placed in the C-terminal half of the protein (18). It has been placed in the C-terminal half of the protein (17) or the nearest neighbor interactions in the crystal, we have presented a model describing the site of single-stranded DNA binding and explaining the basis for cooperative binding. This model agrees with the beaded morphology observed in electron micrographs.

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The atomic coordinates and structure factors (code 1URJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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* The abbreviations used are: HSV, herpes simplex virus; ssDNA, single-stranded DNA; SSB, ssDNA-binding protein; OBP, origin-binding protein; SeSm, selenomethionine; MAD, multiwavelength anomalous diffraction; MMA, methyl mercury acetate; OB, oligonucleotide/oligosaccharide binding; HsmtSSB, human mitochondrial SSB.
has provided insight into the likely mechanism of cooperative ssDNA binding and tempted us to speculate about the possible interaction with the origin-binding protein.

**EXPERIMENTAL PROCEDURES**

The preparation and crystallization of the ICP8 protein missing the last 60 amino acids of the C terminus and with the mutations C254S and C455S (ICP8ΔCcc) have been described previously (23).

**Protein Expression and Purification of Selenomethionine-ICP8ΔCcc—**Selenomethionine (SeMet)-enriched ICP8ΔCcc was expressed in High 5 insect cells grown as monolayer culture. Confluent cells were infected with a dithiothreitol-free buffer, then against a 5 mM MMA-containing buffer (methyl mercury acetate (MMA), the protein was first dialyzed against native protein (20), with the only exception that all buffers were flushed before harvesting. The purification protocol was the same used for the methionine-free medium was renewed after 4 h with SeMet-containing (50 mg/l) IPL-41 medium. Cells were incubated for a further 26 h before harvesting. The purification protocol was the same used for the native protein (20), with the only exception that all buffers were flushed with N<sub>2</sub> and supplemented with 10 mM reducing agent (dithiothreitol or β-mercaptoethanol) to overcome a more pronounced tendency of the self-oxidizing protein to oxidize and aggregate.

**Crystallization—**Selenomethionine-ICP8ΔCcc crystals were grown at 22 °C in hanging drops by equilibration of 5 mg/ml protein in 10 mM Tris-HCl (pH 8.0), 300 mM NaBr, 20% glycerol, 10 mM dithiothreitol against 12–14% polyethylene glycol 3000 and 100 mM sodium-potassium phosphate, pH 6.3. This crystallization condition is similar to that for the native crystal growth. Within about a week, fragile, plate-like crystals (~0.3 × 0.2 × 0.1 mm) grew by subsequent addition of methyl mercury acetate (MMA), the protein was first dialyzed against a dithiothreitol-free buffer, then against a 5 mM MMA-containing buffer at pH 7.5, and was subsequently used to set crystallization drops. Plate-like crystals appeared in a week in conditions similar to the ones used for native crystal growth. Crystals formed in space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, with two molecules in the asymmetric unit.

**Crystallization and Structure Determination—**Both Se-Met- and MMA-containing ICP8ΔCcc crystals were cryoprotected by brief soaks in 20% glycerol buffered at pH 6.3 before cryocooling in liquid nitrogen. Multiwavelength anomalous diffraction data from crystals of SeMet-ICP8ΔCcc were collected at 100 K using synchrotron radiation at the 17-ID IMCA-CAT beamline of the Advanced Photon Source (Argonne) at three/two different wavelengths around the selenium absorption edge. A full diffraction data set was collected for the MMA derivative at 100 K, using the BW7B beam line of the European Molecular Biology Laboratory Hamburg Outstation. The diffraction data were processed using the HKL program package (24). Data collection statistics are shown in Table 1.

The structure was solved by the MAD method (25). Initially from the first crystal (CRYST-1), F<sub>o</sub> - F<sub>c</sub> values were obtained using XPREP (Bruker-AXS Inc.) to 4.0 Å, enabling the selenium substructure to be solved (50 of 56 seleniums) using the program SHEXL (26). Phases were then obtained to 4.0 Å from the two wavelength MAD data. The phases were extended to 3.2 Å by using density modification procedures and 2-fold non-crystallographic symmetry averaging (27). 55% of the model was built using a semiautomatic procedure with the programs MAID (28), RESOLVE (29), and O (30). Later, phases were extended to 3.0 Å using data from another crystal (CRYST-3, see Table 1) by applying multiple crystal averaging (31). The resultant phases allowed the Se substructure of CRYST-2 to be determined using an anomalous difference Fourier at 4.0 Å. Then single isomorphous replacement with anomalous scattering was used to calculate phases, and phase combination was performed to 4.0 Å with the phases generated from multiple crystal averaging. Finally, phases were extended to 3.0 Å using density modification and 2-fold non-crystallographic symmetry averaging. At this stage, the quality of the map improved significantly. Model building was continued in a similar manner to that described above, and 70% of the model could be built. Refinement of the structure was performed using simulated annealing, followed by positional and restrained B-factor refinement as implemented in CNS (32). As the model became more complete, a new mask was calculated and used in the multiple crystal averaging and phase combination. Density modification and 2-fold non-crystallographic symmetry averaging were repeated, followed by the semiautomatic procedure for model building. The model produced in this way was nearly complete except for some missing loops, and there was interpretable density for 90% of the residues. In the final stage, refinement was continued using non-crystallographic symmetry restraints and a bulk solvent correction in the program CNS (32). The refinement was monitored using the free R-factor calculated with 10% of observed reflections. The refinement statistics for CRYST-3 (which, although a mercury derivative, were the best 3.0 Å data) are shown in Table 1. Of 1136 residues, 107 residues in chain A and 105 in chain B are not visible in the electron density and are probably disordered. The major disordered loops are located at the interface of the neck and head. The rest of the disordered loops are situated at different parts of the shoulder region and are shown as dotted lines in Fig.

**Overall Structure—**The structure of ICP8ΔCcc (9–1129) (Fig. 1) is composed of a large N-terminal domain (9–1038) and a smaller α-helical C-terminal domain (1049–1129). The first 8 residues and the last 7 residues of the construct are not visible in the electron density and are presumed to be disordered. The N-terminal domain can be described as consisting of head, neck, and shoulder regions. The head consists of the eight helices...
The crystal structure of the ssDNA-binding protein of HSV-1

**TABLE I**

Summary of data collection and refinement statistics for ICP8

|          | CRYST-1 | CRYST-2 | CRYST-3 |
|----------|---------|---------|---------|
| Wavelength (Å) | 0.979454 | 0.97968 | 0.97944 |
| Unit cell (Å)  | a = 103.12 | a = 102.01 | a = 101.97 |
|             | b = 147.60 | b = 146.13 | b = 146.09 |
|             | c = 170.11 | c = 168.39 | c = 168.39 |
| Resolution (Å) | 4.0 | 4.0 | 4.0 |
| Complete (%) | 98/(99) | 99/(99) | 100/(100) |
| Rmerge (%) | 6.2/(9.4) | 5.7/(12.2) | 7.4/(13.1) |
| Rfree (%) | 6.2/(9.4) | 5.7/(12.2) | 7.4/(13.1) |
| Rmerge (%) | 6.2/(9.4) | 5.7/(12.2) | 7.4/(13.1) |
| Rfree (%) | 6.2/(9.4) | 5.7/(12.2) | 7.4/(13.1) |
| Resolution (Å) | 4.0 | 4.0 | 4.0 |
| Complete (%) | 98/(99) | 99/(99) | 100/(100) |
| Rmerge (%) | 6.2/(9.4) | 5.7/(12.2) | 7.4/(13.1) |
| Rfree (%) | 6.2/(9.4) | 5.7/(12.2) | 7.4/(13.1) |
| Rmerge (%) | 6.2/(9.4) | 5.7/(12.2) | 7.4/(13.1) |
| Rfree (%) | 6.2/(9.4) | 5.7/(12.2) | 7.4/(13.1) |

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**Fig. 1.** A, structure of ICP8. Overall view of the ICP8 structure. Dotted lines represent disordered regions with blue and red balls signifying the N- and C-terminal ends of the disordered regions. (Sequence information using the same color code is given Fig. 4). The shoulder region is colored blue; the zinc binding region is green; the part of the polypeptide chain linking the neck and shoulders as a single folding unit is orange. The neck is colored yellow (front) and gray (back). The head is red, and the C-terminal helical domain is purple. B, the structure rotated 60° along x-axis relative to Fig. 1A. The blue to red color gradient follows from the N to the C terminus. In this orientation, the C-terminal domain is behind the neck.

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α14, α15, α16, α21, α22, α23, α24, and α25 (Fig. 1B). The front side of the neck region consists of a five-stranded β-sheet (β16, β17, β23, β26, and β27) and two helices (α17 and α27), whereas the back side is a three-stranded β-sheet (β24, β25, and β28) (Fig. 1). The shoulder part of the N-terminal domain contains an α-helical and β-sheet region. The head, neck, and shoulders are interconnected in such a way that their individual structural folds are not formed by contiguous polypeptide chains. From the N terminus, the polypeptide chain forms a first helical region in the head and then one of the two β-sheet regions.
belonging to the neck. The strands $\beta 16$ and $\beta 17$ in the neck lead to strands $\beta 18-\beta 22$ in the shoulders before returning to strands $\beta 23-\beta 26$ in the neck. The strands $\beta 18-\beta 22$ are involved in interaction with residues in other strands from the N terminus (see Fig. 1A). This explains why limited proteolysis experiments have never yielded either soluble or functionally active fragments (20) and why so many mutant proteins have proven to be insoluble (see, for example, Ref. 18).

The C-terminal domain (1049–1129) is entirely helical ($a 28$, $a 29$, $a 30$, $a 31$, and $a 32$) and is connected to the N-terminal domain — 17 Å away by a disordered linker (residues 1038–1049) (Figs. 1 and 2).

**DNA Binding Region**—No structurally related protein can be retrieved from the DALI (40) or SSM (www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmserver) servers using the whole ICP8 molecule or the individual subdomains as search models. Although no structural homology is detectable for any of the ICP8 regions (Fig. 1), the front side of the neck region shows some structural resemblance to the oligonucleotide/oligosaccharide binding (OB) fold (41), which is responsible for ssDNA binding in all SSBs so far described with the exception of the adenoviral SSB (41). The topology is different (39), but the principle is the same, namely a crossed $\beta$-sheet with disordered connecting loops containing conserved basic and aromatic residues. The direction of each $\beta$-strand of the neck region that resembles the OB-fold is similar to that of HsmtSSB (38) (Fig. 3A). The proposed DNA binding region on the front side of the neck (Fig. 3A) contains elements of the sequence between amino acids 530 and 1028, similar to the boundaries suggested by Gao and Kippe (18). Limited proteolytic analysis studies had suggested that the putative boundaries of the minimal DNA binding region are between residues 300 and 949 (19). More recent evidence, based on ICP8 photo-affinity labeling with oligonucleotides, indicated a slightly different region, namely between residues 386 and 902 (43). There are a number of aromatic and positively charged residues from the front side of the neck that are exposed to the surface or lie in the disordered loops that are relatively well conserved across the *Herpesviridae*. These are Tyr<sub>543</sub>, Asn<sub>551</sub>, Arg<sub>722</sub>, Lys<sub>727</sub>, Arg<sub>726</sub>, Tyr<sub>988</sub>, Phe<sub>998</sub>, and Asn<sub>1002</sub> (Fig. 4), which we believe are involved in ssDNA binding either by base stacking or electrostatic interactions.

**The Role of Zinc Binding Region**—ICP8 is a zinc metalloprotein containing one zinc atom/molecule (3) that, as predicted, is coordinated by three cysteines (Cys<sup>499</sup>, Cys<sup>502</sup>, and Cys<sup>516</sup>) and a histidine (His<sup>512</sup>). Four of these residues, the cysteines are totally conserved among the *Herpesviridae* SSBs, but the histidine is only conserved in the *Simplexvirus* genus (Fig. 4). Thr<sup>513</sup> is, however, fully conserved and stabilizes the zinc loop further by hydrogen bonding to the main chain oxygen of residue 507 (Fig. 5A). It has been shown that mutation of Cys<sup>499</sup> and Cys<sup>502</sup> produces a non-functional protein that fails to complement a temperature-sensitive UL29 mutant at the non-permissive temperature (44) and that zinc-depleted ICP8 molecules transiently retain DNA binding activity (3), suggesting the zinc binding confers structural integrity to the protein. This is confirmed by the crystal structure. The loop containing the zinc finger interacts with two regions of the protein. The first is
FIG. 4. Structure-based sequence alignment of the ICP8 of Herpesviridae from three subfamilies. Representatives from three genus of the Alphaherpes virus subfamily (Simplexvirus, Varicellaviruse, Marek’s disease-like viruses), three genus from the Betaherpes virus subfamily (Roseolovirus, Cytomegalovirus, Marek’s disease-like viruses), and two genus from the gammaherpes virus subfamily (Rhadinovirus, Lymphocryptovirus) are used in the sequence alignment. The Swiss-Prot codes of ICP8 orthologues from these sources are P04296, Q89549, Q89690, O56282, P17147, P30672, O36860 and P03227, respectively. Horizontal cylinders above the sequences indicate α-helices (labeled α1–α32). Horizontal arrows indicate β-strands (labeled β1–β28). The secondary structure elements are colored red for the head, blue and orange for the shoulder, yellow and gray for the neck region of the N-terminal domain (a similar color code is used in Fig. 1A), purple for the C-terminal helical domain, and light green for the zinc binding loop, including two helices (α12 and α13) that are involved in interaction with part of the N and C termini. Three cysteines and a histidine involved in binding to zinc are shown by a bar above the corresponding residues. The dotted lines indicate regions that are disordered in
toward the N terminus and includes the region between β7 and α2. It is primarily a hydrophobic interface formed by residues Leu112 and Leu115 packing against Leu501, Val511, and the hydrophobic residues on one face of helix H11, but there is also a hydrogen bond between Asn111N and the carbonyl oxygen of Leu501. The second is toward the C terminus and contains the region between H24 and H26 (Fig. 1, sequence numbering as in Fig. 4). The interaction involves the positioning of His508, which hydrogen bonds with the carbonyl oxygen of Ser934 and is stabilized by a hydrogen bond between Trp933N and Glu470Oe1. The zinc binding region is remote to the proposed ssDNA binding region and therefore unlikely to be important for DNA binding.

The Existence of a Protein Chain through the Crystal Suggests How Flexible Cooperative ssDNA Binding Is Achieved—In the crystal, the C-terminal domain of each monomer fits loosely into a concave surface of the back side of the neck region of the N-terminal domain that belongs to a non-crystallographic symmetry-related molecule. A continuous chain with a beaded appearance is then formed by a molecule related to the first by translation of one unit cell along a. Although relatively small (−919 Å²), both independent interaction surfaces are similar. However, the spatial arrangement between the two domains differs (Fig. 2), indicating the flexible nature of the protein chain that, in this case, is probably determined by the crystal packing. A continuous chain of molecules through the crystal lattice can also be observed in the case of T4 gene 32 (45) and the adenovirus ssDNA binding proteins (42) as well as in the organization of Escherichia Coli SSB tetramers (37). The arrangement of ICP8 molecules is similar to the beaded morphology observed in negatively stained electron micrographs (5) of ICP8 decorated ssDNA (Fig. 3C). We previously established that the 60-residue C-terminal region of the ICP8 is a principal determinant for cooperative DNA binding (23), at least on shorter oligonucleotides. Although the crystallized construct does not contain this region, we have postulated that the remaining weaker interactions generate the same chain in the crystal that would form on DNA in solution. Ser254 and Ser455 (Cys254 and Cys455 in the native protein) are located in the loop region between helices α6 and α7 and the C-cap of the α10

dashed line indicates that the region was absent in the construct. The triangle above 3 residues in the C-terminal region (last 60 residues) indicates the region encompassing the FNF motif. The star sign above two cysteines shows mutation to serine in the structure presented here.
helix, respectively. These residues are neither solvent exposed nor close to the region in the neck that we have proposed to be involved in ssDNA binding. It is therefore unlikely that these two cysteines are involved in cooperativity as has been previously suggested (21).

We believe that, because of the nature of the ICP8 domain connection, ssDNA is covered in a flexible manner while keeping the ssDNA in an extended form (Fig. 3, B and C) that prevents formation of secondary structures. On the basis of the ICP8-ssDNA model (Fig. 3 and supplemental material), it seems that −14 oligonucleotides would be covered per ICP8 molecule, and this is in good agreement with biochemical data (23).

Interactions of the C Terminus—There is evidence (20) that the deletion of the C terminus seriously reduces cooperative binding for ICP8, suggesting that there is an additional protein-protein interaction involving this region. An F(N/D)F motif (amino acids 1142–1144) in the C terminus is identifiable in the Alphaherpes viruses and possibly in the Roseolovirus genus of the Betaherpesvirus subfamily (Fig. 4). We believe that at least one of the phenylalanines is involved in a hydrophobic interaction with a hydrophobic region formed by Phe827, Phe843, Trp844, Leu857, and Ile865 (Fig. 6) of the head. This region of ICP8 is also very well conserved among Alphaherpes viruses (Fig. 4). The C-terminal residue (Glu1129) of the model is in the vicinity of the head of the N-terminal domain of another molecule. Modeling a continuation of the C terminus would allow it to pass around the head and dock part of the F(N/D)F motif into the hydrophobic region mentioned above (Fig. 6). A similar interaction is important for the formation of the protein chain in the adenovirus SSB (42). The last C-terminal 28 residues that contain the nuclear localization signal must presumably remain free to facilitate nuclear import.

The deletion of 27 residues at the C terminus of the HSV1 origin-binding protein has been shown to reduce its specific affinity for ICP8 (46), suggesting that there may be an interaction between this region of OBP and ICP8 (46). However, there is no biochemical evidence that would help locate the corresponding interaction region of ICP8. Because a number of hydrophobic residues in the 27-residue C-terminal region of the protein are conserved among those Herpesviridae for which an OBP is present and among these is a V(N/D)F sequence, we have tentatively suggested that the V(N/D)F motif of OBP interacts with the same hydrophobic patch described above. Thus we speculated that the C terminus of ICP8 and OBP could compete for the same site, depending upon the nature of the protein-protein and, importantly, the protein-DNA interaction. This is consistent with a model of initiation of DNA replication in which the ICP8-OBP interaction is required to complete origin unwinding but is “replaced” by ICP8-ICP8 intermolecular interactions upon replication onset when the OBP is released from DNA and processive replication ensues.

Potential Protein-Protein Interaction Sites—We have identified the function of head, neck, and the C-terminal helical region of ICP8; however, a large part of the shoulder region is not, according to our model, involved in cooperative ssDNA binding. It is unlikely that such a large part of the N-terminal domain would have no functional role, because the necessity of packaging the viral genome should tend to enhance the evolution of multifunctional proteins and reduce the likelihood of producing non-functional coding regions. Regulation of late gene expression could involve the ssDNA binding region, but
presumably nuclear positioning involves the exposed part of the protein chain. Recent work (47) has identified, by immunocoprecipitation, a number of cellular proteins that co-localize with ICP8. Some of these co-localizations are not dependent on mediation by DNA and are involved in (cellular) DNA replication, repair, and recombination. Structurally there are two regions that are likely to be involved in some of these interactions. The first is a deep pocket (Fig. 5B) with conserved aromatic residues (Tyr 	20, Phe 	61, Tyr 	90) at the base and capped by the hydrogen bonding interaction between Glu 	58 and Arg 	183. The second is a cleft (Fig. 5C) containing the fully conserved Cys 	116 and Arg 	120, both on α2, at the side.

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