Physical interaction between the herpes simplex virus 1 origin-binding protein and single-stranded DNA-binding protein ICP8

(DNA replication/orient unwinding/protein-affinity chromatography/protein–protein interactions)

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ABSTRACT We had previously demonstrated that the herpes simplex virus 1 (HSV-1) single-stranded DNA-binding protein (ICP8) can specifically stimulate the helicase activity of the HSV-1 origin-binding protein (UL9). We show here that this functional stimulation is a manifestation of a tight interaction between UL9 protein and ICP8. By using protein-affinity chromatography, we have demonstrated the specific binding of purified UL9 protein to immobilized ICP8 and vice versa. Furthermore, ICP8 is specifically retained by a column on which the C-terminal 37-kDa DNA-binding domain of the UL9 protein was immobilized. The interaction between ICP8 and the DNA-binding domain of the UL9 protein was confirmed by cochromatography of the two proteins. These results suggest that the UL9 protein and ICP8 form a tight complex that functions in origin recognition and unwinding.

The herpes simplex virus 1 (HSV-1) chromosome is a linear duplex DNA of ~150 kbp, containing three origins of DNA replication (1). HSV-1 encodes seven gene products that are both necessary and sufficient for the replication of origin-containing plasmids in transient replication assays (for review, see ref. 2). These seven genes encode a highly processive heterodimeric DNA polymerase (UL30/UL42), a heterotrimetric helicase-primase (UL5/UL8/UL52), a single-stranded DNA-binding protein (SSB) (UL29), and an origin-binding protein (UL9) (2, 3).

Studies of the replication in vitro of plasmids containing the Escherichia coli origin (oriC) and simian virus 40 origin have shown that origin-specific initiation of DNA replication involves protein-mediated destabilization of the DNA duplex at the origin, thereby allowing access of the DNA synthetic machinist. The DNA polymerase (4). In the virus 40, the helicase protein, large tumor antigen, recognizes elements within the origin of replication and leads to the destabilization of the DNA duplex through its DNA helicase activity. This process also requires the action of the cellular SSB, RP-A, to maintain the DNA in a single-stranded conformation (2, 5, 6).

The ability of HSV-1 UL9 protein to recognize elements within the HSV-1 origin of replication (2, 3, 7) and to function as a DNA helicase (8, 9) suggests that it is involved in origin-specific DNA unwinding during HSV-1 DNA replication. Origin-unwinding and the maintenance of the single-stranded conformation should be aided by the helix-destabilizing properties of the HSV-1-encoded SSB ICP8 (10). Consequently, efficient destabilization of the DNA duplex at the origin might be expected to involve the concerted action of the UL9 protein and ICP8.

We had reported (9) that ICP8 can specifically stimulate the DNA helicase activity of UL9 protein. We show here that this functional stimulation is due to the tight interaction between ICP8 and UL9 protein.

MATERIALS AND METHODS

Materials. Cell culture media and reagents were obtained from GIBCO/BRL. Fetal calf serum was obtained from Gemini Biological Products (Calabasas, CA). SDS/PAGE and immunoblot analysis were done as described (11, 12). The anti-ICP8-specific rabbit serum 3-83 was from D. Knipe (Harvard University). The recombinant baculovirus AcUL29 (13) was from N. Stow (Medical Research Council Virology Unit, Glasgow). The E. coli over-expression plasmid pET-3a/HisΔOBP was from P. Elias (Gothenburg University). E. coli TOP10/pTrcHisCAT was obtained from Invitrogen. Protein standards for SDS/PAGE were obtained from Bio-Rad.

Buffers. The molar concentration of NaCl in each buffer is indicated separately according to use. Buffer A contained 20 mM Hepes-NaOH (pH 7.5), 10% (vol/vol) glycerol, 1 mM dithiothreitol, and 0.1 mM EDTA. Buffer B was the same as buffer A, except that the pH was adjusted to 7.6. Buffer C contained 10 mM sodium phosphate (pH 7.5), 10% glycerol, 1 mM dithiothreitol, and 0.1 M NaCl. Buffer D was identical to buffer C, except that the pH was adjusted to 7.0. Buffer E contained 20 mM Hepes-NaOH (pH 7.5), 10% glycerol, and 1 mM 2-mercaptoethanol. Buffer F was identical to buffer A, except that EDTA was omitted. Protease inhibitors (Sigma) were added at the following final concentrations: leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), and phenylmethylsulfonyl fluoride (0.1 mM).

Cell Culture and Preparation of Cell Extracts. Spodoptera frugiperda (SF9) cells and stocks of Autographa californica nuclear polyhedrosis virus (AcNPV) recombinant for the HSV-1 UL9 and UL29 genes were maintained and propagated as described (13–15). SF9 cells were grown in 20-225-cm2 tissue culture flasks to a cell confluency of ~80% and infected with recombinant AcNPV at a multiplicity of infection of ~15. Sixty hours after infection, the cells were harvested, washed in phosphate-buffered saline, and frozen at -80°C. Nuclear extracts were prepared as described (16).

Vero cells (African green monkey kidney fibroblasts) were propagated at 37°C in Dulbecco's modified Eagle's minimal essential medium/10% fetal calf serum/0.1 mM nonessential amino acids/2 mM glutamine. HSV-1-infected cell extracts were prepared by infecting 20 150-cm2 tissue culture flasks with HSV-1 strain Δ305 (17) at a multiplicity of infection of 10. Ten hours after infection, the cells were harvested.

Abbreviations: SSB, single-stranded DNA-binding protein; AcNPV, Autographa californica nuclear polyhedrosis virus; HSV-1, herpes simplex virus 1; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; ΔUL9, C-terminal DNA-binding domain of UL9 protein.

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washed in phosphate-buffered saline, and frozen at −80°C. All subsequent steps were done at 4°C. The cells, ≈1.3 g, were thawed and resuspended in buffer A (1.2 M NaCl) containing 1 mM EDTA. The cells were transferred to a Dounce homogenizer and lysed with 10 strokes of the tight-fitting pestle. The lysed cells were cleared by centrifugation at 55,000 rpm for 60 min in a Beckman 70.1 Ti rotor. Nucleic acid was precipitated from the supernatant by the drop-wise addition of 0.05 vol of 0.3 M spermidine-HCl/1 M NaCl/0.31 vol of 5% (wt/vol) streptomycin sulfate, both in buffer A. After a 30-min incubation, the nucleic acid precipitate was removed by centrifugation at 25,000 rpm for 30 min in a Beckman 70.1 Ti rotor. The supernatant, which was dialyzed against 0.1 M NaCl, was applied to a 5-ml HiTrap heparin column (Pharmacia) equilibrated with the same buffer. The column was washed, and proteins were eluted with a 60-ml linear gradient of NaCl (0.1–0.8 M) and 1-ml fractions were collected. The peak of UL9 protein, eluting at ≈0.7 M NaCl, was applied onto a 5-ml Econo-Pac HTP cartridge (Bio-Rad) equilibrated with buffer C, and proteins were eluted with a 50-ml linear gradient of sodium phosphate (0.01–0.3 M). Peak fractions, 1 ml each, eluting at ≈0.09 M sodium phosphate, were dialyzed against buffer B (0.1 M NaCl) and loaded onto a Mono S HR 5/5 column (Pharmacia) equilibrated with the same buffer. The column was washed, and proteins were eluted with a 15-ml linear gradient of NaCl (0.1–1.0 M). Fractions, 0.25 ml, containing near-homogeneous (>95% pure) UL9 protein eluting at ≈0.55 M NaCl, were divided into aliquots and stored at −80°C. The yield of purified UL9 protein was ≈2.5 mg from 12 225-cm² tissue culture flasks.

ICP8 was purified either from HSV-1-infected U253 cells, essentially as described by Hernandez and Lehman (18), or from nuclear extracts prepared from SF9 cells infected with AcUL29. Cleared nuclear extracts, dialyzed against buffer B (0.1 M NaCl), were loaded onto a 5-ml HiTrap heparin column (Pharmacia) equilibrated with the same buffer. The column was washed, and proteins were eluted with a 50-ml linear gradient of NaCl (0.1–0.8 M), and 1-ml fractions were collected. The peak of ICP8, eluting at ≈0.35 M NaCl, was applied onto a 5-ml Econo-Pac HTP cartridge equilibrated with buffer D, and proteins were eluted with a 50-ml linear gradient of sodium phosphate (0.01–0.2 M). Peak fractions, 1 ml each, eluting at ≈0.08 M sodium phosphate, were dialyzed against buffer A (0.1 M NaCl) and loaded onto a Mono Q HR 5/5 column (Pharmacia) equilibrated with the same buffer. The column was washed, and proteins were eluted with a 16.5-ml linear gradient of NaCl (0.1–0.75 M). Fractions, 0.25 ml, containing near-homogeneous (>95% pure) ICP8 eluting at ≈0.4 M NaCl were divided into aliquots and stored at −80°C. The yield of purified ICP8 was ≈20 mg from 20 225-cm² tissue culture flasks.

The histidine-tagged chloramphenicol acetyltransferase (CAT) protein and C-terminal DNA-binding domain of the UL9 protein (ΔUL9) were purified from E. coli ICP8/pTrCHisCAT and BL21(DE3)pLysS/pET-3a/Pichia, respectively. Extracts from 1 liter of isopropyl β-D-thiogalactoside-induced cells grown at 30°C were prepared as described (19). Cell extracts were diluted to 50 ml with buffer E (0.15 M NaCl) and cleared by centrifugation at 29,000 rpm for 45 min in a Beckman 45 Ti rotor. Cleared extracts were mixed batchwise with 4 ml of Ni-NTA resin (Qiagen, Chatsworth, CA) equilibrated in buffer E (0.15 M NaCl). After 60 min with gentle stirring, the mixture was poured into an Econo-Pac column and washed extensively with buffer E (0.15 M NaCl). Proteins were eluted with 10-ml steps of buffer E (0.15 M NaCl) containing 10, 20, 40, 60, and 200 mM imidazole. Fractions, 1 ml, containing near-homogeneous (>95% pure) histidine-tagged ΔUL9 and CAT proteins eluted in the 80 and 200 mM imidazole fractions and were stored at −80°C.

E. coli SSB was from E. H. Lee (this department), and its concentration was determined by the method of Bradford (20). Bovine serum albumin (BSA) was obtained from Pharmacia.

Protein concentrations were determined by using extinction coefficients of 82,720, 89,220, and 43,626 M⁻¹·cm⁻¹ at 280 nm for ICP8, UL9 protein, and BSA, respectively. Concentrations of the histidine-tagged ΔUL9 and CAT proteins were determined by assuming that one A₂₈₀ unit corresponds to a 1 mg/ml solution.

Protein-Affinity Chromatography. One milliliter of 1 mg/ml solutions of UL9 protein, ICP8, or BSA was dialyzed extensively against 0.2 M NaHCO₃/0.5 M NaCl, pH 8.3. The dialyzed proteins were then immobilized on 1 ml of N-hydroxysuccinimide (NHS)-activated HiTrap columns (Pharmacia), as recommended by the manufacturer.

HiTrap protein-affinity column chromatography was done with a Pharmacia fast protein liquid chromatography system. The columns were run at a flow rate of 0.05 ml/min, and 0.35-ml fractions were collected. Protein mixtures containing 100 μg of each protein species were dialyzed against buffer F (0.1 M NaCl) and loaded onto the HiTrap protein-affinity columns equilibrated with the same buffer. After sample application, the columns were washed with 2 ml of buffer F (0.1 M NaCl) and developed with 10-ml linear gradients of NaCl (0.1–1.0 M) followed by elution with 1 ml at 1.0 M NaCl and 4 ml at 0.1 M NaCl.

Purified histidine-tagged ΔUL9 and CAT proteins (1 mg) were dialyzed against buffer E (0.05 M NaCl) and adsorbed batchwise to 100 μl of Ni-NTA resin (Qiagen) equilibrated with the same buffer. The resin was pipetted into a packed, long 400-μl Eppendorf microcentrifuge tube, plugged with glass beads (212–300 μm; Sigma) and tipped with a 0.5 × 16 mm syringe needle. The columns were washed with buffer E (0.05 M NaCl/20 mM imidazole). A mixture of proteins in buffer E (0.05 M NaCl/20 mM imidazole), consisting of 50 μg each of ICP8, BSA, and E. coli SSB in ≈100 μl, was applied to the columns, and the flow-through fraction was collected. The columns were washed with 500 μl of buffer E containing increased concentrations of NaCl and imidazole as indicated in Fig. 3, and the eluant at each step was collected.

Gel-Filtration Chromatography. A Superose 12 HR 10/30 gel-filtration column (Pharmacia) was equilibrated with buffer F (0.2 M NaCl) at 0.2 ml/min. Protein samples, ≈1 nmol, were dialyzed into chromatography buffer and injected onto the column. The column was developed at 0.2 ml/min, and 0.2-ml fractions were collected.

RESULTS

Protein-Affinity Chromatography Demonstrates a Tight Physical Interaction Between ICP8 and UL9 Protein. Protein-affinity chromatography was used to detect a specific interaction between ICP8 and the UL9 protein. Purified ICP8, UL9 protein, and BSA were covalently attached to activated agarose beads. A mixture of purified proteins was then chromatographed on the three protein-affinity columns. The ability of an ICP8-agarose column to specifically bind UL9 protein is shown in Fig. 1A. SDS/PAGE analysis of the column fractions indicated that the control proteins, BSA and E. coli SSB, failed to adsorb to the column, whereas the UL9
Fig. 1. Protein-affinity chromatography of ICP8 and UL9 protein. Proteins were chromatographed on the indicated columns, as described. Column fractions were analyzed by SDS/PAGE followed by Coomassie blue staining. Diagonal lines represent linear gradients of NaCl. (A) ICP8-agarose. (B) UL9 protein-agarose. (C) BSA-agarose. Positions of ICP8, UL9 protein, BSA, and E. coli SSB are as indicated. M, Mr standards; L, load fraction; FT, flow-through fraction.
protein was specifically retained, eluting at \( \approx 0.6 \) M NaCl. This result indicates a tight physical interaction between ICP8 and the UL9 protein. Similarly, immobilized UL9 protein could specifically retain ICP8 (Fig. 1B). When a mixture of proteins consisting of ICP8, BSA, and \( E. \ coli \) SSB was applied to the UL9 protein column, the control proteins, BSA and \( E. \ coli \) SSB, were not retained, whereas ICP8 adsorbed to the column and was eluted as a broad peak at \( \approx 0.6 \) M NaCl. In contrast, there was no significant retention of ICP8, UL9 protein, or \( E. \ coli \) SSB by a column consisting of immobilized BSA (Fig. 1C).

To determine whether the interaction between ICP8 and the UL9 protein could be detected in a crude cell extract, an HSV-1-infected cell extract was chromatographed on a column containing immobilized UL9 protein. ICP8 in the column fractions was monitored by immunoblotting with an anti-ICP8-specific rabbit serum. Fig. 2 shows that ICP8 was retained by the immobilized UL9 protein and then eluted after increasedionic strength, with a peak appearing at \( \approx 0.5 \) M NaCl. These results show that the tight interaction seen between purified ICP8 and UL9 protein can also be detected when ICP8 is present in a crude cell extract.

ICP8 Binds to the 37-kDa C-Terminal DNA-Binding Domain of UL9 Protein. The C-terminal DNA-binding domain of UL9 protein was expressed in \( E. \ coli \) as a histidine-tagged 37-kDa protein. The purified \( \Delta U9 \) protein was immobilized on Ni-NTA resin because of its histidine tag. Fig. 3A shows that the immobilized \( \Delta U9 \) protein could specifically retain ICP8; most of the ICP8 eluted in the 1.0 M NaCl steps. Control proteins BSA and \( E. \ coli \) SSB, on the other hand, both appeared in the flow-through and wash fractions. No interaction between ICP8, BSA, or \( E. \ coli \) SSB was seen with resin alone (data not shown) or with immobilized, histidine-tagged CAT protein (Fig. 3B). These results show that the specific interaction between ICP8 and UL9 proteins is confined to the C-terminal DNA-binding domain of UL9 protein.

CoChromatography of ICP8 and the DNA-Binding Domain of UL9 Protein. To confirm the interaction between ICP8 and the \( \Delta U9 \) protein detected by protein-affinity chromatography, the behavior of the two proteins during gel filtration was examined. The UV-absorbance profiles of proteins eluting from a Superose 12 HR 10/30 column, as well as SDS/PAGE analysis of the peak protein fractions, are shown in Fig. 4. Fig. 4A and B show the elution profiles of ICP8 and the \( \Delta U9 \) protein, eluting after 11.6 and 14.3 ml of buffer, respectively. Fig. 4C depicts the elution profile of a mixture of ICP8 and \( \Delta U9 \) protein. The UV-absorbance profile indicates that the elution position of the \( \Delta U9 \) protein shifted to that of ICP8. Coeluion of \( \Delta U9 \) protein and ICP8 was confirmed by the coincidence of both proteins upon SDS/PAGE. The ability of the two proteins to cochromatograph verifies that they associate to form a tight complex.

**DISCUSSION**

We have investigated the physical interaction between the HSV-1 UL9 protein and ICP8 in an attempt to elucidate their mode of action in promoting origin-specific DNA unwinding.

We had reported (9) that ICP8 can specifically stimulate the DNA helicase activity of the UL9 protein. Here we have used protein-affinity chromatography to show directly a tight and specific physical interaction between these two proteins. Purified UL9 protein was specifically retained by immobilized ICP8 and was eluted only at high ionic strength (0.6 M NaCl). Similarly, ICP8 was specifically retained by immobilized UL9 protein. Furthermore, immobilized UL9 protein could retain ICP8 from an HSV-1-infected cell extract. These
We have localized the site of interaction between the UL9 protein and ICP8 to the C-terminal 37-kDa DNA-binding domain of UL9 protein (ΔUL9). ICP8 was specifically retained by immobilized ΔUL9 protein and was released only at high ionic strength (>0.5 M NaCl). The interaction between ICP8 and ΔUL9 proteins was confirmed by their coelution upon gel filtration.

The primary amino acid sequence of the UL9 protein reveals two leucine zipper motifs (21), one near the N terminus and the other closer to the C terminus, that may be involved in protein–protein interactions (22). In fact, Elias et al. (23) have shown the N-terminal portion of UL9 protein to be required for cooperative binding to its recognition sequences within ori. These cooperative protein–protein interactions between UL9 protein monomers may be mediated through the N-terminal leucine zipper. Our findings are consistent with the possibility that the heterologous UL9 protein–ICP8 interaction is mediated through the C-terminal leucine zipper. Further studies are needed to localize more precisely the site of interaction between ICP8 and the UL9 protein and to address the stoichiometry of the ICP8–UL9 protein complex.

Specific interactions between proteins involved in origin-specific initiation of DNA replication are not unprecedented. For example, in simian virus 40 DNA replication, specific protein–protein interactions have been shown to exist between the viral initiator protein, large tumor antigen, and the cellular SSB RP-A (24), as well as with DNA polymerase α-primase (25).

This paper is dedicated to the memory of Hatch Echols, who was among the first to point out the importance of protein–protein interactions at a replication origin. This work was supported by Grant AI 26538 from the National Institutes of Health.


**Fig. 4.** Coelution of ICP8 and ΔUL9 protein on Superose 12. Gel filtration was done as described. The UV-absorbance profiles are shown as well as Coomassie blue-stained SDS/polyacrylamide gels of peak protein fractions from each chromatogram. (A) ICP8 alone. (B) ΔUL9 protein alone. (C) ICP8 and ΔUL9 protein. The start of each chromatogram is indicated, as are the positions of ICP8 and ΔUL9 protein. M, M₀ standards.

Findings suggest that the UL9 protein and ICP8 associate to form a tight complex and that the stimulation of the UL9 protein helicase by ICP8 results from this association.

Previous work has shown that the UL9 protein is an origin-binding protein (2, 3, 7) as well as a DNA helicase (8, 9). These properties are well-suited to a role in unwinding the HSV-1 chromosome at an origin of DNA replication. ICP8, by virtue of its ability to destabilize duplex DNA (10) and to stimulate the DNA helicase activity of UL9 protein (9), should also be suited for a role in origin activation. Our present studies show that the UL9 protein and ICP8 exist as a complex that might function in activating the HSV-1 origin of DNA replication.