The Major Herpes Simplex Virus Type-1 DNA-binding Protein Is a Zinc Metalloprotein*

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The primary amino acid sequence of the major herpes simplex virus type 1 (HSV-1)-infected cell polypeptide 8 (ICP8) deduced from the DNA sequence of the unique long open reading frame 29 (UL29 ORF) contains a potential metal-binding domain of the form Cys-X_{2,5}-Cys-X_{2-10}-A-X_{2-4}-A where A may be either histidine or cysteine and X is any amino acid. The putative metal-binding sequence in ICP8 encompasses residues 499–512 as follows: -C-N-L-C-T-F-D-T-R-H-A-C-V-H.

Atomic absorption analysis of several preparations of ICP8 indicates the presence of 1 mol of zinc/mol of protein. The zinc is resistant to removal by dialysis against concentrations of EDTA which deplete zinc from alcohol dehydrogenase. The bound zinc can be removed by reaction with the reversible sulfhydryl reagent p-hydroxymercurimethylsulfonate and the zinc-depleted protein transiently retains DNA binding activity. Digestion of both native and zinc-depleted ICP8 with V8 protease indicates that the bound zinc is required for the structural integrity of the protein.

The major herpes simplex virus type 1 (HSV-1)* DNA-binding protein commonly designated infected cell polypeptide 8 (ICP8) is one of seven HSV-1 gene products required for origin-dependent replication of HSV DNA (1, 2). A large body of work with conditional lethal mutants mapping in the vicinity of the ICP8 gene (6) indicates that the protein is composed of 19,186 amino acids and has a predicted molecular weight of 128,341. This value is in good agreement with previous estimates of 120,000–130,000 for the molecular weight of ICP8 by SDS-PAGE (7).

ICP8 has been isolated and preferentially to single-stranded DNA. This interaction is cooperative based on electron microscopic analysis of ICP8-DNA complexes prepared with sub-saturating amounts of protein (8–10) and by Scatchard analysis (11). ICP8 also binds to duplex DNA and polyribonucleic acid but with a lower affinity than that observed with single-stranded DNA, as determined by salt elution from DNA-cellulose matrices and/or by competitive filter binding assays (12–14). ICP8 has also been shown to have the ability to melt duplexes of complementary homopolymers and to protect bound single-stranded DNA from nucleosome digestion (15, 16). These properties are reminiscent of those displayed by the T4 gene 32 protein and the Escherichia coli SSB, both of which also bind cooperatively to single-stranded DNA and are required for DNA replication (17, 18). Further evidence that ICP8 is an analogue of these prokaryotic proteins comes from recent work by Hernandez and Lehman (19) who have shown that ICP8 is required for the complete processive synthesis of singly primed qX-174 DNA in the presence of the gene products of the HSV origin dependent replication system.

Following its synthesis in the cytoplasm, ICP8 moves to the nucleus where it initially interacts with the nuclear matrix at discrete prereplicative sites. With the onset of DNA replication, ICP8 moves into regions of the nucleus which have been dubbed "replication compartments." More recently it has been shown that ICP8 is required for the organization of DNA replication proteins at discrete sites within the cell nucleus (20). Thus ICP8 appears to be responsible for the organization of the proposed HSV DNA replication complex. Finally, studies with temperature sensitive mutants indicate that ICP8 is involved in the regulation of expression of late genes during HSV infection. Specifically this protein appears to down-regulate expression of such genes by an as yet unknown mechanism (21).

Examination of the predicted amino acid sequence of ICP8 shows that residues 499–512 conform to the consensus sequence (Cys-X_{2,5}-Cys-X_{2-15}-Cys/His-X_{2-1}-Cys/His) for a divalent metal coordination site or "zinc finger" described by Berg (22). This sequence is -C-N-L-C-T-F-D-T-R-H-A-C-V-H-and occurs near the midpoint of the primary structure of the molecule (Fig. 1). We report here on experiments which show that ICP8 is a zinc metalloprotein and that the zinc atom appears to be required for the overall structural stability of ICP8.

MATERIALS AND METHODS

Cells and Viruses—Vero and U-35 cell lines were propagated in monolayer culture as described by Orberg and Schafer (23) using Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum and 8% Serum Plus (Hazelton Laboratories). Stocks of HSV-1 strain mP were propagated and titered in Vero cell monolayers.

Purification of ICP8 from HSV-1-infected U-35 Cells—ICP8 was purified from U-35 cells infected with HSV-1 strain mP at a multiplicity of infection of 1. This cell line is stably transformed and contains multiple copies of the ICP8 gene integrated into the cellular genome. The U-35 cell line has been used by several laboratories to generate large quantities of homogenous protein (16, 24). ICP8 was purified from U-35 cell pellets using protocols similar to those developed for purification of this protein from infected Vero cells (25).

Briefly, 2 × 10^6 infected U-35 cells which had been stored as pellets frozen at ~70 °C were resuspended in 200 ml of lysis buffer (1.7 M KCl, 50 mM Tris hydrochloride (pH 7.5), 5 mM EDTA, 0.5 mM dithiotreitol, 0.2% Nonidet P-40 (Sigma), 20 μg/ml phenylmethylsulfonyl fluoride, and 2.5 μg/ml leupeptin (Sigma)). The cells were lysed by gentle agitation, and the resulting viscous solution was clarified by centrifugation for 6 h at 24,000 rpm in a Beckman SW28 rotor. Subsequent dialysis against buffer containing 0.15 M KCl and purification by DNA-agarose chromatography and heparin-Sepharose chromatography were carried out as described previously (20). The
final yields of homogeneous ICP8 (homogeneity defined as the migration of the protein as a single band by SDS-PAGE) from two separate preparations were 8 and 10 mg. The protein was dialyzed against buffer containing 0.15 M KCl, 10 mM Tris.Cl (pH 7.6), 1 mM EDTA, 0.1 mM dithiothreitol, and 50% glycerol and stored at -20 °C prior to use.

Protein concentrations were determined using the method of Lowry (26) following precipitation of protein with 5% trichloroacetic acid and redissolution in 1 N NaOH.

Reaction of ICP8 with PMPS—Reaction of ICP8 with the reversible sulfhydryl reagent PMPS followed the procedure of Giedroc et al. (27). PMPS was dissolved in 100 mM NaCl, 10 mM Tris-Cl (pH 7.6) (TN buffer). The PMPS was added in 40-fold molar excess to ICP8 dialyzed in the same buffer, and the resulting mixture was incubated at 4 °C for 60 min. Following incubation the reaction mixture was divided and dialyzed against a variety of buffers as described under "Results and Discussion." A control sample of ICP8 was diluted without PMPS treatment and dialyzed in a similar fashion.

Zinc Analysis of ICP8—All buffers used for zinc analysis of ICP8 were treated by passage through a 200-ml Chelex-100 column (Bio-Rad). Prior to use, the Chelex-100 gel was activated by successive treatment with 4 volumes of 1.0 M NaOH, and an additional 4 volumes of deionized water (28). The buffers were Chelex-treated at 10 × concentration and stored in sterile plasticware (Bellco) which was found to be free of containing zinc. Zinc analyses were performed essentially as described by Foote and Delves (29) using a Perkin-Elmer model 2100 atomic absorption spectrometer equipped with an HGA-700 graphite furnace. A standard curve, linear from 0.1 to 1.0 pmol of zinc, was obtained for each set of experiments. Individual samples were assayed for the presence of zinc using a variety of free buffers and used as controls. Samples were removed upon completion of dialysis, and the zinc content of the proteins was determined as described under "Materials and Methods." The results are presented in Table I. ICP8 from both preparations contained, on the average, 1 mol of zinc/mol of protein. The zinc was resistant to exhaustive dialysis against EDTA concentrations of up to 10 mM in the presence of 0.1 mM dithiothreitol. In contrast, the zinc atoms bound to horse liver alcohol dehydrogenase were partially removed upon dialysis against 1.0 mM EDTA in the presence of dithiothreitol.

Giedroc et al. (27, 31) had considerable success in addressing the role played by zinc in the activity of the T4 gene 32 protein using the reversible and strongly dissociating sulfhydryl reagent PMPS. Reaction with this reagent resulted in the loss of zinc from the T4 gene 32 protein and allowed reconstitution with zinc and other divalent metals upon removal of PMPS. Our treatment of ICP8 with PMPS followed the scheme outlined by these authors as described under "Materials and Methods." The results are shown in Table II and indicate that reaction with PMPS and subsequent reconstitution of free sulfhydryl groups with dithiothreitol resulted in the efficient removal of zinc from ICP8. This result is in contrast with those obtained with the T4 gene 32 protein where complete removal of zinc required dialysis against both

**RESULTS AND DISCUSSION**

ICP8 derived from two separate preparations was dialyzed overnight against three 1-liter changes of zinc-free TN buffer, 0.1 mM dithiothreitol. The final concentration of ICP8 ranged from 125 to 300 µg/ml. The samples were then divided in half, and dialysis was continued against either TN buffer or against TN buffer containing 1.0 mM EDTA or 10 mM EDTA (0.1 mM dithiothreitol added to all samples). Horse liver alcohol dehydrogenase which is known to contain 4 mol of zinc/1 mol of protein of molecular weight 80,000 (30) and the SSB, which does not contain zinc (27), were also dialyzed against zinc-free buffers and used as controls. Samples were removed upon completion of dialysis, and the zinc content of the proteins was determined as described under "Materials and Methods." The results are presented in Table I. ICP8 from both preparations contained, on the average, 1 mol of zinc/mol of protein. The zinc was resistant to exhaustive dialysis against EDTA concentrations of up to 10 mM in the presence of 0.1 mM dithiothreitol. In contrast, the zinc atoms bound to horse liver alcohol dehydrogenase were partially removed upon dialysis against 1.0 mM EDTA in the presence of dithiothreitol.

**TABLE I**

Summary of ICP8 zinc determinations

| Protein     | Conditions                          | Moles zinc/mol protein |
|-------------|-------------------------------------|------------------------|
| ICP8        | Dialyzed without EDTA               | 1.22 ± 0.04            |
| ICP8        | 50% dilution of above               | 1.08 ± 0.02            |
| ICP8        | Dialyzed against buffer with 1.0 mM EDTA | 0.90 ± 0.09           |
| ICP8        | Dialyzed 4 days with buffer containing 10 mM EDTA | 1.05 ± 0.10          |
| ICP8        | Separate preparation dialyzed against buffer containing 1.0 mM EDTA | 1.29 ± 0.08         |
| ADH         | Diluted into buffer                 | 4.44 ± 0.16            |
| ADH         | Dialyzed against buffer without EDTA | 3.44 ± 0.14            |
| ADH         | Dialyzed against buffer with 1.0 mM EDTA | 1.28 ± 0.08         |
| E. coli SSB | Dialyzed against buffer with 1.0 mM EDTA | 0.04 ± 0.00          |

* ADH, horse liver alcohol dehydrogenase.
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TABLE II

| Protein       | Conditions                       | Moles zinc/mol protein |
|---------------|----------------------------------|------------------------|
| ICP8 fraction 1 | Native protein dialyzed against dithiothreitol and EDTA | 1.08 ± 0.01 |
| ICP8 fraction 2 | PMPS modified followed by dialysis against dithiothreitol | 0.10 ± 0.00 |
| ICP8 fraction 3 | PMPS modified followed by dialysis against dithiothreitol and EDTA | 0.06 ± 0.03 |

Although zinc was bound, the molar ratios varied from 0.1 to 3.2 mol of zinc/mol of protein, and no DNA binding activity was observed. These results suggest that the zinc atom in ICP8 is most likely not required for DNA binding but may play a role in maintaining the native tertiary structure of the protein. Thus loss of zinc would result in a destabilization of the protein tertiary structure ultimately leading to an irreversible loss of activity.

In order to further examine this possibility, native and zinc-depleted ICP8 were treated with V8 protease as described under "Materials and Methods." Time courses of reaction were carried out and the products analyzed by SDS-PAGE. The results show that the zinc-depleted ICP8 was highly susceptible to cleavage by the protease, whereas the native protein was relatively untouched (Fig. 4). This outcome is consistent with the idea that zinc is involved in the stabilization of the overall tertiary structure of ICP8 and suggests that the gradual loss of DNA binding activity noted above was most likely due to a slow denaturation of the protein.

In summary we have shown that ICP8 is a zinc-metalloprotein and contains 1 mol of zinc/mol of protein. The zinc atom is tightly bound and is resistant to removal by extensive dialysis against 10 mM EDTA. The zinc can be removed by reaction with the reversible sulfhydryl reagent PMPS and appears to be required for structural stability of ICP8 but not directly involved in the DNA binding activity of this protein as assessed by the nitrocellulose filter binding assay.

The question then arises as to whether the zinc atom is actually located in the putative zinc finger encompassing residues 499–512. While no direct evidence is available, data gathered by several investigators suggest that this region is important for the overall function of ICP8 in the replication of the virus and is separate from the apparent DNA-binding domain of ICP8. Gao, Knipe, and co-workers (32, 33) have shown that mutation of the ICP8 gene resulting in simultaneous substitution of cysteine with glycine at positions 499 and 502 gives rise to a protein which is 90% insoluble in infected cell extracts. Only one-third of the remaining soluble protein was capable of binding to single-stranded DNA cellulose matrices. This mutant designated pml was incapable of supporting virus growth on Vero cells. These investigators have also engineered a deletion mutant of ICP8 (d101) which lacks 546 amino acids from the amino-terminal portion of ICP8 including the zinc finger region. The protein product encoded by the d101 strain is also predominantly insoluble (78%) in infected cell extracts, but the remaining soluble portion did show DNA binding. Although the size of the deletion is such that it is difficult to draw specific conclusions, the data are compatible with a model in which the zinc and

Fig. 2. Nitrocellulose filter binding assays using fraction 1 (+), fraction 2 (△), and fraction 3 (○) from the PMPS experiment described in the text and Table II. All reaction mixes contained 38 ng of heat-denatured ³H-labeled λ DNA.

Fig. 3. The ratio of DNA binding activity of native (fraction 1) and zinc-depleted (fraction 2) ICP8 over a 48-h period following modification and dialysis. Proteins were stored under identical conditions at 4°C. All reaction mixes contained 0.25 μg of protein and 38 ng of heat-denatured DNA.

Fig. 4. SDS-PAGE analysis of time courses of V8 protease digestion of (A) native and (B) zinc-depleted ICP8. Lanes 1, 3, and 4 in A and lanes 2–4 in B represent 0-, 10-, and 20-min digestions. Lanes 2A and 1B contain molecular weight standards (116,250, 92,500, 66,200, 45,000, and 30,000).

dithiothreitol and EDTA (27). The positive control (sample 1) was dialyzed under nitrogen and in the presence of dithiothreitol and EDTA, and the results indicate that these procedures do not dissociate the bound zinc atom.

The single-stranded DNA binding activity of the zinc-depleted ICP8 was assessed by the nitrocellulose filter binding assay. The DNA-binding activity of the zinc-depleted protein remained intact in experiments carried out immediately after treatment with dithiothreitol (Fig. 2) but was gradually lost (as compared with native ICP8) after several days storage at 4°C (Fig. 3). Attempts to reconstitute zinc-depleted ICP8 by dialysis against zinc-containing buffers were not successful.
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DNA-binding sites are separate. Finally, data from several laboratories based upon work with the above deletion mutants (33), proteolytic fragments (16), and specific portions of ICP8 expressed from recombinant plasmids (34) indicate that the DNA-binding domain of ICP8 most likely lies between residues 564 and 849. Thus the retention of DNA binding activity upon removal of zinc from ICP8 presented here is again consistent with the evidence that the putative zinc-binding domain is outside the DNA-binding domain. Site-specific mutagenesis of the zinc finger region of ICP8 such as that carried out by Gauss et al. (35) in the case of the gene 32 protein and zinc analysis of the resulting mutant proteins may be useful in the study of this problem and such work is currently underway. However, unambiguous location of the zinc atom in ICP8 will require x-ray crystallographic analysis of the protein.

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