Identification of a Divalent Metal Cation Binding Site in Herpes Simplex Virus 1 (HSV-1) ICP8 Required for HSV Replication

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Herpes simplex virus 1 (HSV-1) ICP8 is a single-stranded DNA-binding protein that is necessary for viral DNA replication and exhibits recombinase activity in vitro. Alignment of the HSV-1 ICP8 amino acid sequence with ICP8 homologs from other herpesviruses revealed conserved aspartic acid (D) and glutamic acid (E) residues. Amino acid residue D1087 was conserved in every ICP8 homolog analyzed, indicating that it is likely critical for ICP8 function. We took a genetic approach to investigate the functions of the conserved ICP8 D and E residues in HSV-1 replication. The E1086A D1087A mutant form of ICP8 failed to support the replication of an ICP8 mutant virus in a complementation assay. E1086A D1087A mutant ICP8 bound DNA, albeit with reduced affinity, demonstrating that the protein is not globally misfolded. This mutant form of ICP8 was also recognized by a conformation-specific antibody, further indicating that its overall structure was intact. A recombinant virus expressing E1086A D1087A mutant ICP8 was defective in viral replication, viral DNA synthesis, and late gene expression in Vero cells. A class of enzymes called DDE recombinases utilize conserved D and E residues to coordinate divalent metal cations in their active sites. We investigated whether the conserved D and E residues in ICP8 were also required for binding metal cations and found that the E1086A D1087A mutant form of ICP8 exhibited altered divalent metal binding in an in vitro iron-induced cleavage assay. These results identify a novel divalent metal cation-binding site in ICP8 that is required for ICP8 functions during viral replication.

Herpes simplex virus 1 (HSV-1) is a double-stranded DNA virus that replicates its genome in the nuclei of infected cells. HSV-1 encodes seven gene products that are directly involved in the replication of viral DNA, all of which are essential for viral DNA replication (44). These proteins are the DNA polymerase (which consists of U5,30, the catalytic subunit, and U7,42, its processivity factor), an origin binding protein (U4,9), a helicase-prime- mase complex (which consists of the U5, U52, and U7,8 proteins), and the single-stranded DNA binding protein ICP8 (also known as U2,9), which is the focus of this report.

ICP8 is an early gene product that nonspecifically binds single-stranded DNA in a cooperative manner (22, 38). In addition to its essential role in viral DNA replication, ICP8 has also been shown both to repress transcription from the parental genome (13–15) and to stimulate late gene transcription (12). ICP8 is known to bind zinc (17), and several biochemical activities of ICP8 require magnesium cations (9, 24, 34), suggesting that the protein also binds magnesium. Zinc binding by ICP8 is thought to be involved in its DNA binding activity, because altering residues in the ICP8 zinc finger region results in an abolishment of DNA binding (11). The role of magnesium binding by ICP8 is much less well understood, and no mutations have been made that specifically disrupt ICP8 magnesium binding in order to directly assess its importance to ICP8 function.

ICP8 has also been reported to mediate several activities involved in DNA recombination in vitro, including destabilization of DNA duplexes (1), facilitation of the annealing of single-stranded DNA (9), mediation of strand exchange (2, 29, 34), and mediation of strand invasion (28). ICP8 interacts with the HSV-encoded alkaline nucleases U5,12, and U5,12 is proposed to play a role in the initiation and/or the resolution steps of the DNA recombination mechanism (34, 36). The interaction of ICP8 with U5,12 also stimulates the processivity of U5,12-mediated digestion of DNA (35). Other nucleases, such as Escherichia coli exonuclease III and lambda phage Red α exonuclease, can also stimulate the strand exchange activity of ICP8 in vitro (36). The HSV-1 helicase-prime-mase complex has also been reported to cooperate with ICP8 to promote strand exchange activity in vitro (29). Furthermore, ICP8 is required for long-chain DNA synthesis in an in vitro recombination-dependent replication assay (30, 31), although it is not clear that ICP8 recombinase activity is required in this assay. ICP8 is a major component of HSV-1 replication compartments, which are nuclear domains where viral DNA replication and late gene expression occur. ICP8 also interacts with several cellular proteins known to be involved in recombination, including DNA-PKcs (DNA-dependent protein kinase, catalytic subunit), Rad50, and Ku86, and recruits these proteins to viral replication compartments (40), where they may play important roles in mediating the recombination of the HSV-1 genome.

In this study, we report the identification of a conserved aspartic acid residue in ICP8 that is required for divalent metal cation binding and viral DNA replication. ICP8 is thought to bind Mg2+, and Mg2+ is required for ICP8 activity in some in vitro recombinase assays. Therefore, the conserved D residue in ICP8 may co-
ordinate Mg\(^{2+}\), and bound Mg\(^{2+}\) at this site may be required for ICP8 function during HSV-1 infection.

**MATERIALS AND METHODS**

**Cells and viruses.** Vero cells were obtained from the Type I Culture Collection (Manassas, VA). The ICP8-complementing cell lines V529 (5) and S2 (11) were generated as described previously. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 5% heat-inactivated newborn calf serum (NCS). The growth medium for the V529 and S2 cells was also supplemented with 500 μg/ml G418.

All experiments were performed with HSV-1 strain KOS as the wild-type (WT) virus (39) or with mutant virus 8lacZ (21), p1ml.a (11), or KOS.8DDEm (described below). Viruses were propagated and titrated on Vero or V529 cells by standard procedures.

**Plasmids.** This section describes the construction of plasmids used in the transient complementation assays. Plasmids used in other assays are described in the relevant sections. Plasmid pFastBac HTa-ICP8 was constructed by ligating the Avrl/EcoRI fragment containing ICP8 from pSV8.3 (12) into pFastBac HTa (Invitrogen) DNA digested with XbaI and EcoRI (all restriction enzymes were from New England Biolabs [NEB]). The pFastBac HTa-d105 plasmid was constructed in the same manner, except that the Avrl/EcoRI ICP8 fragment was cloned into pSVd105 (12). The pFastBac HTa-U1-29 and pFastBac HTa-U1-29.d105 plasmids were constructed by introducing an EcoRI site immediately upstream of the ICP8 initiation codon in pFastBac HTa-ICP8 and pFastBac HTa-d105, respectively, by site-directed mutagenesis, digestion of these plasmids with EcoRI, and self-ligation to remove the ICP8 5' untranslated region (5' UTR) and vector sequences between the EcoRI site in pFastBac HTa and the ICP8-initiating ATG (newly introduced EcoRI site). pCIΔ U1-29 was constructed by ligation of the ICP8-containing DNA fragment from pFastBac HTa-U1-29 that was digested with HindIII, followed by blunting of the sticky ends with the Klenow fragment of E. coli DNA polymerase I (NEB) and digestion with EcoRI, into pCIΔ (27) that had been digested with Smal and EcoRI. pCIΔ U1-29.d105 was constructed in the same manner, except that the HindIII/Klenow/EcoRI fragment containing the ICP8 gene originated from pFastBac HTa-d105. pCIΔ U1 was generated by digesting pCIΔ with BamHI, blunting the sticky ends with Klenow fragment, and self-ligating (thus destroying the BamHI restriction site). pCIΔ U1.d105 was constructed by ligating the ICP8-containing DNA fragment from pFastBac HTa-U1-29 that was digested with HindIII, followed by blunting of the sticky ends with Klenow fragment and digestion with EcoRI, into pCIΔ that had been digested with Smal and EcoRI. Several smaller regions of the ICP8 gene were subcloned into pBluescript II SK (+) (pBS; Stratagene) to be used as the template for introducing D or E codon mutations into the ICP8 gene by performing PCR-based site-directed mutagenesis. pBS-ICP8-B5 contains the BamHI/Sall fragment of the ICP8 gene cloned into the BamHI and Sall sites in pBS, pBS-ICP8-N contains the NotI fragment of the ICP8 gene cloned into the NotI site in pBS.

**Generation of recombinant baculoviruses.** To generate recombinant baculoviruses that express ICP8, we utilized the pFastBac Dual-based vector (Invitrogen) pFBd.GFP, which expresses green fluorescent protein (GFP) under the control of the p10 promoter. We obtained pFBd.GFP.S2 (unpublished plasmid), which expresses the reovirus S2 protein, as a gift from Kenneth Murray (Florida International University). The pFastBac HTa-U1-29 and pFastBac HTa-U1-29.E1086A/D1087A weredigested with EcoRI and HindIII, and the DNA fragment containing ICP8 was cloned into pFBd.GFP.S2 that had been digested with EcoRI and HindIII, which removed the S2 coding sequence. The resulting plasmids were named pFBd.GFP.U1-29 and pFBd.GFP.U1.E1086A/D1087A. ICP8 was epitope tagged at its N terminus with the 6× His epitope by ligating oligonucleotides encoding six histidine residues into the EcoRI site upstream from the ICP8-initiating ATG codon, generating pFBd.GFP.U1-29.His and pFBd.GFP.U1.E1086A/D1087A.His. The presence of the 6× His se-
quence in the correct reading frame was confirmed by DNA sequencing. Recombinant baculovirus bacmids were generated by transforming chemically competent *E. coli* DH10B cells (InvitroGen) with either pFBd.GFP.U2.29.His or pFBd.GFP.U2.29.E1086A/D1087A.His according to the manufacturer’s instructions.

S21 insect cells were transfected with recombinant bacmids that express either wild-type ICP8 or the DDE mutant by using the Cellfectin transfection reagent (InvitroGen) according to the manufacturer’s instructions. The supernatant from transfected cells was collected and was designated the P1 stock. This was used to infect new S21 cells and to generate the P2 stock. The P2 stock was used to infect S21 cells for the expression and purification of recombinant ICP8 proteins.

**Protein purification.** Baculovirus-infected S21 cells were harvested for protein purification by washing once with PBS, and then cells were collected by scraping from the flask into PBS. Cells were lysed by incubating with a buffer consisting of 20 mM Tris (pH 7.5), 300 mM NaCl, 20% glycerol, and 0.5% NP-40 for 30 min on ice. The cell-free supernatant containing the soluble recombinant protein was collected following centrifugation of the samples, applied to a column containing Talon affinity resin (Clontech) that was equilibrated with lysis buffer, and allowed to proceed through the column by gravity flow. Bound protein was washed with a buffer consisting of 20 mM Tris HCl (pH 7.5), 300 mM NaCl, 5% glycerol, and 0.1% NP-40 and was then eluted with a buffer consisting of 50 mM Tris HCl (pH 7.5), 300 mM NaCl, 15% glycerol, and 150 mM imidazole. Ten fractions of the eluate were collected. The fractions containing the purified protein were identified by resolving a portion by SDS-PAGE and staining the gel with Coomassie stain, and those fractions were combined. The pooled fractions were concentrated, and the buffer was exchanged to ICP8 storage buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 20% glycerol) using Vivaspin sample purification columns (Vivascience). Protein concentrations were measured using the Bio-Rad protein assay reagent (Bio-Rad). Protein concentration in the P2 stock was used to calculate the yield of recombinant ICP8 proteins.

**Viral yield assay.** Vero or V529 cells were infected with the indicated virus at an MOI of 10 in PBS-ABC containing 1% bovine serum and 0.1% glucose for 1 h in a shaking incubator at 37°C. Following the 1-h adsorption step, cells were washed twice with acid wash buffer (40 mM citric acid [pH 3], 10 mM KCl, 135 mM NaCl) and once with DMEM containing 1% FBS, and then DMEM containing 1% FBS was added. Provirus was harvested at the indicated times postinfection by scraping the infected-cell monolayer and collecting the cells and supernatant. Samples were frozen at −80°C following harvesting. Viral yield was determined by performing plaque assays on V529 cells.

**Viral DNA replication assay.** Vero cells were infected with the indicated virus as described above for the viral yield assay. Following infection for the indicated time, samples were harvested by washing the cell monolayers with PBS-ABC, and the cells were scraped in PBS-ABC and were then collected by centrifugation. Total DNA (including both cellular and viral DNA) was purified by using the Generation Capture Column kit (Qiagen, formerly Gentra) according to the manufacturer’s instructions. Viral DNA was quantified by performing real-time PCR using primers specific for the ICP27 promoter (3). Real-time PCR was performed using PowerSYBR green reagents (Applied Biosystems) and an Applied Biosystems 7300 sequence detection system according to the manufacturer’s instructions. The viral DNA levels were normalized to the levels of a GAPDH pseudogene in each sample (20).

**Iron-induced protein cleavage assay.** The iron-induced protein cleavage assay was performed essentially as described previously (19). Briefly, 20 pmol of either wild-type ICP8 or the DDE mutant form of ICP8 was either mock treated or incubated with ammonium iron(II) sulfate hexahydrate at a final concentration of 20 μM and ascorbic acid at a final concentration of 20 mM, either in the presence or in the absence of 20 pmol of the oligo(dt)25 oligonucleotide, in ICP8 storage buffer for 30 min. The samples were resolved on a 5% native polyacrylamide gel, and the gel was dried and exposed to a phosphor storage screen.

**Immunofluorescence.** Indirect immunofluorescence was performed as described previously (4). Briefly, Vero cells on glass coverslips were either mock infected or infected with the indicated virus at an MOI of 10 PFU/cell for 10 h prior to fixation with 3.65% formaldehyde. The antibodies used were 3-83, to detect total ICP8, and the conformation-specific antibody 395, which detects correctly folded ICP8. The fixed and stained cells were imaged using a Zeiss Axiosplan 2 microscope, a Photometrics CoolSNAP HQ2 charge-coupled device (CCD) camera, and Zeiss AxiosVision 4 image acquisition software.

**Construction of mutant viruses.** Plasmid p8-8GFP, which encodes ICP8 fused to GFP at its C terminus (41), was linearized by digestion with EcoRI and was cotransfected into S2 cells with 8lacZ infectious DNA to generate the recombinant virus KO8.8GFP. Plaques expressing GFP were identified by fluorescence microscopy, and these recombinant viruses were plaque purified 5 times prior to use in experiments. To generate KO8.8DDEm, KO8.8GFP infectious DNA was cotransfected into V529 cells together with EcoRI-linearized pBS.8flank.8DDEm, which contains the E1086A/D1087A mutation in ICP8. To generate pBS.8flank.8DDEm, we constructed pBS.8flank by ligating a blunted BglII/Ndel fragment from pSG18 (16), which contains the ICP8 locus and the surrounding region, into EcoRV-digested pBS.8BS.8flank.8DDEm was then generated by ligating a Bpu101I fragment containing the E1086A/D1087A mutation from pCLAU.29.E1086A/D1087A into Bpu101I-digested pBS-8flank. Plaques that did not express GFP were identified by fluorescence microscopy and were purified 3 times prior to use in experiments. The presence of the D1086A E1087A mutations in the ICP8 gene was confirmed by sequencing a PCR product from the appropriate region of ICP8. A recombinant virus that rescued the ICP8 DDE mutation, KO8.8DDEm-R, was also generated in a similar manner. In this case, infectious KO8.8DDEm DNA was cotransfected into V529 cells together with EcoRI-linearized pBS.8flank. Recombinant viruses were selected by their ability to form plaques on noncomplementing Vero cells and were plaque purified 4 times prior to use in experiments. The presence of wild-type sequence was confirmed by sequencing a PCR product from the appropriate region of ICP8.

**RESULTS**

**Presence of conserved aspartic acid and glutamic acid residues in HSV-1 ICP8.** To identify highly conserved regions (and therefore potential new functional domains) in HSV-1 ICP8 and its homologs in other herpesviruses, we performed an alignment of amino acid sequences from nine ICP8 homologs, with representatives from alpha-, beta-, and gammaherpesviruses. We discovered aspartic acid (D) and glutamic acid (E) residues that were conserved in many or all of the ICP8 homologs (some conserved residues are shown in Fig. 1; a complete list is given in Fig. S1 in the supplemental material), leading us to hypothesize that these conserved residues are important for ICP8 function.
Several of these conserved residues were located in or near the DNA binding groove in the ICP8 crystal structure (25), suggesting that they would be available to carry out enzymatic functions on bound DNA. Interestingly, members of a family of enzymes called DDE recombinases, including transposases, RAG-1, and retroviral integrases, also have conserved D and E residues that coordinate magnesium ions that are important for mediating the recombination reactions, leading us to speculate that ICP8 may share biochemical and pharmacological properties with these well-studied proteins. Numerous ICP8 residues were further investigated, including D545 (amino acid residue positions are based on the KOS ICP8 sequence [10]), D547, D625, E627, D645, E735, E860, D861, E1086, and D1087.

An ICP8 molecule with an altered conserved D residue has a decreased ability to support HSV-1 replication. To determine whether the conserved D and E residues in ICP8 were required for ICP8 function during HSV-1 infection, specific D and E codons were altered to encode alanine in a plasmid encoding HSV-1 ICP8. The ability of the encoded proteins to complement the replication of an ICP8 mutant virus was then determined in a transient complementation assay. All D/E mutant forms of ICP8 were expressed to similar levels in transfected HeLa cells (Fig. 2A). At 24 h after transfection, the cells were infected with the ICP8-null mutant virus 8lacZ at an MOI of 10. Samples were harvested at 24 h postinfection (hpi), and the viral yield was determined by performing plaque assays on V529 cells, which contain the ICP8 gene and can complement the replication of the 8lacZ mutant virus. The viral yield observed in cells transfected with the plasmid expressing wild-type ICP8 was designated 100% complementation, and complementation by the ICP8 mutants was compared to that value. The d105 mutant form of ICP8, which is not able to complement the replication of an ICP8 mutant virus (12), did not complement 8lacZ replication to levels above background. The E860A D861A mutant form of ICP8 complemented 8lacZ replication to approximately 55% of the level of wild-type ICP8 (Fig. 2B).
indicating that residues 860 and 861 are important, but not essential, for wild-type activity of ICP8. The D645A mutant form of ICP8 also complemented 8lacZ replication to approximately 50% of the level of wild-type ICP8 (Fig. 2B), indicating that residue 645 is also important, but not essential, for wild-type activity of ICP8. The E1086A D1087A mutant form of ICP8 did not complement the replication of 8lacZ to levels above the background observed when cells were transfected with the empty-vector plasmid (Fig. 2B). These results indicated that either residue 1086 or 1087, or both, is absolutely critical for ICP8 to function during HSV-1 replication in this assay.

To determine whether both residues 1086 and 1087 are required for ICP8 activity, we changed them each individually to alanine residues and assayed their abilities to complement 8lacZ replication. The E1086A mutant form of ICP8 complemented 8lacZ replication to an extent similar to that of wild-type ICP8 (Fig. 2B), indicating that this residue is not critical for ICP8 activity. However, the D1087A mutant form of ICP8 did not complement 8lacZ to levels above background (Fig. 2B), indicating that residue 1087 is absolutely essential for ICP8 to complement the replication of an ICP8 mutant virus. Furthermore, the inability of the E1086A D1087A double point mutant to complement 8lacZ replication can be attributed entirely to the D1087A mutation. Cysteine residues are known to be able to coordinate divalent metal cations in DDE recombinases; therefore, to investigate whether divalent metal cation binding played a role in the D1087A phenotype, we tested a mutant form of ICP8 in which residue D1087 was changed to cysteine. The D1087C mutant form of ICP8 was able to complement 8lacZ to much higher levels than the D1087A mutant (Fig. 2B), suggesting that D1087 coordinates divalent metal cations and that this activity is required for ICP8 function. Other amino acid residue mutants (the D545A, D547A, D625A, E627A, E629A, and E735A mutants) complemented 8lacZ replication to near-wild-type levels when expressed in HeLa cells (Fig. 2B), indicating that these residues are not absolutely required for ICP8 function in viral replication.

ICP8 molecules with altered conserved D/E residues are not globally misfolded. To rule out the possibility that the E1086A D1087A mutation in ICP8 reduced the activity of ICP8 by simply destroying the overall folding of the protein, we investigated the ability of purified recombinant wild-type and E1086A D1087A (DDE mutant) ICP8 to bind DNA in vitro by performing electrophoretic mobility shift assays. Both wild-type and DDE mutant forms of ICP8 were purified to near-homogeneity (Fig. 3A). Increasing concentrations of both proteins in the binding reactions resulted in a slower-migrating form of the labeled oligo(dT)25 DNA (Fig. 3B), indicating that the DNA was bound by both forms of purified ICP8 and that the DDE mutant form of ICP8 is not globally misfolded. However, higher concentrations of the DDE mutant form of ICP8 than of wild-type ICP8 were required to bind the oligonucleotide, arguing that the mutant form bound DNA with decreased affinity. To assess the folding of the mutant form of ICP8 by another method, we investigated whether mutant ICP8 expressed during infection with the 8DDEm mutant virus was recognized by the conformation-specific 39S antibody (42). Mock-infected cells were not stained with either the 39S antibody or the 3-83 α-ICP8 antibody, which detects total ICP8, demonstrating the specificity of these antibodies (Fig. 4, top row). Cells infected with wild-type HSV-1 were stained with both antibodies, and mature replication compartments were observed (Fig. 4, second row). Cells infected with the ICP8 mutant virus pm1.a, which expresses an altered form of ICP8 that is defective for DNA binding and is incorrectly folded (11), were not stained by antibody 39S but were stained by antibody 3-83 (Fig. 4, third row). 8DDEm-infected cells were stained by both 39S and 3-83 antibodies (Fig. 4, bottom row), further demonstrating that this mutant form of ICP8 maintained the correct conformation. In addition, we observed that the mutant form of ICP8 expressed during 8DDEm infection did not form mature replication compartments, indicating that the altered residues were required for this activity.

The conserved D/E residues in ICP8 are required for efficient HSV-1 replication and viral DNA replication. We constructed a

![Effect of ICP8 DDE mutation on DNA binding. (A) His-tagged wild-type and DDE mutant ICP8 proteins were expressed from recombinant baculoviruses and were purified from infected Sf21 cells. Purified proteins were resolved by SDS-PAGE, and the gel was stained with Coomassie blue stain. (B) The indicated concentration of each protein was incubated with radiolabeled oligo(dT)25 DNA, and protein-DNA complexes were resolved on a 5% native polyacrylamide gel.](http://jvi.asm.org/content/jvi/86/12/6829/F3.large.jpg)
mutant virus, 8DDEm, containing the E1086A D1087A mutations in the ICP8 gene, to investigate whether this mutant form of ICP8 affected HSV-1 replication when expressed from the viral genome. We observed that replication of this mutant virus was decreased 10- to 100-fold from that with wild-type HSV-1 strain KOS in noncomplementing Vero cells (Fig. 5A). However, 8DDEm replicated better than the ICP8 mutant virus pm1.a (Fig. 5A) (11) in Vero cells, suggesting that the replication functions of the altered ICP8 in the 8DDEm mutant virus were not completely destroyed. A virus in which the E1086A D1087A mutation was restored to the wild-type sequence, 8DDEm-R, replicated similarly to the wild-type virus, indicating that the decrease in replication observed for 8DDEm was likely due to the specific mutation. All viruses replicated to nearly wild-type levels in V529 cells, which express wild-type ICP8 and therefore complement ICP8 mutant viruses, suggesting that the replication defects of these viruses in Vero cells were due to the changes in ICP8.

We also determined the plating efficiencies for the wild-type virus, the 8DDEm mutant, and the 8DDEm-R rescued virus by plating serial dilutions of each virus on either noncomplementing Vero cells or the complementing cell line V529. The wild-type virus and the rescued virus 8DDEm-R had similar titers on both cell lines, but the 8DDEm mutant virus had an approximately 70-fold-lower titer on Vero cells than on V529 cells (Fig. 5B). These results are consistent with a replication defect of the 8DDEm mutant virus.

We next investigated the levels of viral DNA replication in Vero cells infected with either the wild-type virus, the 8DDEm mutant virus, the 8DDEm-R rescued virus, or the pm1.a ICP8 mutant virus, which is defective for DNA binding and viral DNA replication (11). For the wild-type virus, we observed that levels of viral DNA increased ~1,000-fold during the course of infection (Fig. 6). We observed only a very small increase in viral DNA levels between 4 and 12 h postinfection (~2-fold), and an approximately 10-fold increase in viral DNA levels by 24 h postinfection, with the 8DDEm mutant virus (Fig. 6), indicating that DNA synthesis during 8DDEm infection was severely decreased relative to that of the wild-type virus and strongly suggesting that the E1086 and D1087 residues in ICP8 are essential for efficient HSV-1 DNA replication. Viral DNA synthesis by the 8DDEm-R rescued virus was similar to that by the wild type (Fig. 6). Viral DNA levels did not increase at all during the course of infection with pm1.a (Fig. 6). Because viral DNA levels in cells infected with 8DDEm were greater than those in pm1.a-infected cells, it was clear that the mutant ICP8 expressed by 8DDEm supported low levels of DNA replication.

**Effect of altered ICP8 D/E residues on viral gene expression.**

Because the 8DDEm mutant virus exhibited defects in viral replication and DNA synthesis, we next investigated whether this mutation also had an effect on viral gene expression. We assayed the accumulation of the immediate-early ICP27 gene product, the early ICP8 gene product, and the late glycoprotein C (gC) gene

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**FIG 4** Effect of the ICP8 DDE mutation on recognition by the conformation-specific antibody 39S. Vero cells were either mock infected or infected with the indicated virus at an MOI of 10 PFU/cell. At 10 hpi, the cells were fixed and stained with either the α-ICP8 39S conformation-specific antibody (green; left) or the 3-83 antibody, which detects total ICP8 (red; center).
product by performing immunoblot assays with Vero cells that were infected with either the wild-type virus, the ICP8 mutant virus 8DDEm, or the rescued virus 8DDEm-R. We observed that the levels of ICP27 and ICP8 in 8DDEm-infected Vero cells were slightly lower than those in Vero cells infected with the wild-type virus but comparable to those in cells infected with the 8DDEm-R rescued virus (Fig. 7), arguing that the E1086/D1087 residues in ICP8 are not required for the expression of viral immediate-early or early genes but may be required for maximal levels of expression. We observed that the late gC protein accumulated to much lower levels in Vero cells infected with the 8DDEm virus than in cells infected with the wild-type or 8DDEm-R virus (Fig. 7). Although ICP27 expression appeared to be relatively late in this experiment, this was not observed in other experiments that confirmed the reduced expression of gC. In RNA hybridization assays, we observed patterns of viral transcript accumulation similar to the patterns of viral protein accumulation seen in immunoblot assays (results not shown). It is known that gC expression requires HSV-1 DNA replication (18), and the decreased levels of gC are consistent with the defect in viral DNA replication that we observed.

Effect of altered ICP8 D/E residues on divalent cation binding. Because D and E residues in DDE recombinases function to coordinate the binding of magnesium ions (reviewed in reference 7), we hypothesized that the defect in viral DNA replication with the 8DDEm mutant virus was due to an altered metal cation-binding site in ICP8. One approach that has been used to investigate metal-binding sites of DDE recombinases is to measure the ability of reactive iron ions to cleave proteins when bound (19). Because DDE recombinases coordinate magnesium ions with their catalytic residues, iron ions can replace magnesium and promote the cleavage of the bound protein by reactive hydroxyl radicals. This technique was used to successfully map the active-site residues in RAG1 (19), one of the most extensively characterized DDE recombinases. We observed that purified recombinant ICP8 underwent substantial iron-dependent cleavage in this assay (Fig. 8), demonstrating that the wild-type protein bound the iron ion...
and therefore suggesting that it is also competent for binding magnesium ions. The amount of iron-dependent cleavage of the E1086A D1087A mutant form of ICP8 was dramatically reduced relative to that of the wild-type protein, indicating that this mutant protein was defective for iron ion-induced cleavage and thus would also be defective for magnesium binding.

**DISCUSSION**

In this report, we have identified a divalent metal cation-binding site in HSV-1 ICP8 that is essential for efficient viral replication. Identification of this site was based on strong conservation with ICP8 homologs. Plasmids encoding ICP8 with E1086A D1087A and D1087A mutational alterations failed to complement the replication of an ICP8-null virus, arguing that the 1087 residue is essential for ICP8 function and viral replication. A mutant virus, 8DDEm, containing the E1086A D1087A mutations in the U1,29 gene, which encodes ICP8, exhibits a severe defect in viral replication and viral DNA synthesis. The mutant virus also shows reduced late gene expression. The 8DDEm mutant ICP8 shows evidence of reduced dative cation binding, consistent with the notion that this is a DDE recombinase enzymatic domain. ICP8 has been proposed to be a DDE recombinase (7, 8), a member of a class of enzymes that catalyze recombination using a catalytic triad of aspartic acid and glutamic acid residues that coordinate divalent metal cations, usually Mg$^{2+}$. The 8DDEm mutant virus could provide a new reagent for the functional analysis of ICP8 recombinase activity during infection.

**Nature of the functional defect in 8DDEm ICP8.** ICP8 is a multifunctional protein that binds to single-stranded DNA, facilitates the unwinding of DNA at viral origins of DNA replication, and interacts with the U1,9 origin-binding protein and the viral helicase-primase complex to promote viral DNA replication. Additionally, ICP8 exhibits recombinase activities in vitro (1, 2, 28–31, 34, 36). The 8DDEm mutant ICP8 could have defects in one or more of these activities. The 8DDEm ICP8 is capable of binding to DNA in a gel shift assay, demonstrating that it is not completely functionally destroyed. The 8DDEm ICP8 is also recognized by the conformation-specific 39S monoclonal antibody, evidence that its conformation and interaction with other HSV DNA replication proteins are normal (42). However, our initial results indicate that the affinity of DNA binding by 8DDEm ICP8 is lower than that of WT ICP8. Thus, the reduced DNA replication could be due to reduced DNA binding and promotion of viral DNA replication. Alternatively, the recombinase function could be defective, and this defect could be responsible for the defect in viral replication. Further studies of the DNA binding properties and recombinase activities of this and other ICP8 mutant proteins will be necessary in order to parse out the precise roles of DNA binding and recombinase activity in viral replication.

**Potential function of ICP8-mediated recombination in replication.** The recombinase activity of ICP8 could play any of several roles in HSV-1 biology. First, recombination could be involved in the circularization of the viral genome upon entry into the nucleus. However, ICP8 is expressed as a delayed early protein, and ICP8 has not been demonstrated to be present within the virion, so it is difficult to postulate a role for ICP8 this early in the viral replication process. Second, recombination could be involved in the initiation of viral DNA replication or in the transition from theta-form replication to rolling-circle replication (37, 43). This would be consistent with our observation of a limited amount of viral DNA replication by the 8DDEm mutant virus. Third, recombinase activity may also be required to resolve the branched DNA intermediates that have been observed during HSV-1 infection, perhaps to generate the linear genomes that are packaged into virions. In support of this, ICP8 is associated with progeny viral DNA (23). Fourth, the recombinase activity of ICP8 could play a role in the isomerization of the viral genome, which results in inversion of the genome at the repeated sequences. The role of genome isomerization is not known; however, viruses in which the internal repeated sequences are deleted and which are therefore noninverting retain their ability to replicate (32). Fifth, the ICP8 recombinase activity could lead to conformational changes in viral progeny DNA that allow efficient late gene transcription. cis-acting changes in viral genomes following viral DNA replication are known to activate late gene transcription (26). ICP8 is known to bind to viral progeny DNA (23), and the recombinase activity of ICP8 could act to resolve concatemers and increase viral late gene transcription. This would be consistent with the strong defect in gC expression observed with the 8DDEm mutant virus. Finally, general recombination could enhance the exchange of genetic information to allow novel genotypes that could be more evolutionarily fit; however, it is not apparent how general recombination could enhance replication in cultured cells other than through the roles described above.

**Identification of a potential new drug target.** The HIV integrase, a DDE recombinase, has been exploited as a very effective antiviral target, and multiple small molecules and compounds that bind the DDE active site and inhibit the activity of this viral enzyme have been designed. HSV-1 ICP8 may have a DDE active site similar to the HIV integrase active site, and one of the putative DDE residues in ICP8 (D1087) was required for efficient HSV-1 DNA replication, suggesting that this site in ICP8 may represent a novel antiviral target. We are currently working to test this hypothesis and to develop molecules that bind to ICP8 and inhibit its activity. If these drugs do target a domain including the D1087 residue, the conservation of this residue in human alphaherpesviruses (including HSV-1, HSV-2, and varicella-zoster virus [VZV]), human betaherpesviruses (including human cytomegalovirus [HCMV] and human herpesvirus 7 [HHV7]), and human gammaherpesviruses (including Epstein-Barr virus [EBV] and Kaposi’s sarcoma-associated herpesvirus [KSHV]) (Fig. 1) indicates that this class of drugs might be applicable to multiple human herpesviruses.

It has been known for several years that ICP8 has recombinase activity in vitro, and this study provides evidence that ICP8 shares
some similarities with enzymes in the DDE family of recombinases. Additionally, while it is well established that recombination of the HSV-1 genome occurs at a high frequency, the viral and cellular factors involved in mediating this recombination, as well as the role of recombination in viral replication, remain unclear. This report is the first description of an amino acid residue in ICP8 that may be involved in its recombinase activity, and this study provides reagents with which to investigate this residue in the mechanism of ICP8-mediated recombination during HSV-1 replication.

We have identified amino acid residues in ICP8 that are important for divergent metal cation binding, and we provide evidence that metal ion binding by ICP8 is required for efficient viral replication. More work is clearly required to define the remaining residues involved in magnesium binding and to determine whether these residues constitute a bona fide DDE recombinase active site in ICP8, as well as to define the other proteins that may be involved in ICP8-dependent recombination. Furthermore, the precise mechanism of recombination is still unclear, as is the specific role of recombination during viral DNA replication. The results in this report provide experimental evidence consistent with the hypothesis that ICP8 is a DDE recombinase.

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