The Exonuclease Activity of Herpes Simplex Virus 1 UL12 Is Required for Production of Viral DNA That Can Be Packaged To Produce Infectious Virus

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ABSTRACT The herpes simplex virus (HSV) type I alkaline nuclease, UL12, has 5'-to-3' exonuclease activity and shares homology with nucleases from other members of the *Herpesviridae* family. We previously reported that a UL12-null virus exhibits a severe defect in viral growth. To determine whether the growth defect was a result of loss of nuclease activity or another function of UL12, we introduced an exonuclease-inactivating mutation into the viral genome. The recombinant virus, UL12 D340E (the D340E mutant), behaved identically to the null virus (AN-1) in virus yield experiments, exhibiting a 4-log decrease in the production of infectious virus. Furthermore, both viruses were severely defective in cell-to-cell spread and produced fewer DNA-containing capsids and more empty capsids than wild-type virus. In addition, DNA packaged by the viral mutants was aberrant, as determined by infectivity assays and pulsed-field gel electrophoresis. We conclude that UL12 exonuclease activity is essential for the production of viral DNA that can be packaged to produce infectious virus. This conclusion was bolstered by experiments showing that a series of natural and synthetic α -hydroxytropolones recently reported to inhibit HSV replication also inhibit the nuclease activity of UL12. Taken together, our results demonstrate that the exonuclease activity of UL12 is essential for the production of infectious virus and may be considered a target for development of antiviral agents.

IMPORTANCE Herpes simplex virus is a major pathogen, and although nucleoside analogs such as acyclovir are highly effective in controlling HSV-1 or -2 infections in immunocompetent individuals, their use in immunocompromised patients is complicated by the development of resistance. Identification of additional proteins essential for viral replication is necessary to develop improved therapies. In this communication, we confirm that the exonuclease activity of UL12 is essential for viral replication through the analysis of a nuclease-deficient viral mutant. We demonstrate that the exonuclease activity of UL12 is essential for the production of viral progeny and thus provides an attractive, druggable enzymatic target.

KEYWORDS DNA recombination, DNA replication, HSV, UL12, drug discovery, exonucleases, viral DNA

Herpes simplex virus 1 (HSV-1) contains a 152-kbp linear DNA genome housed within an icosahedral capsid shell surrounded by a lipid envelope. Encapsulation of viral genomes into infectious virus requires production of longer-than-unit-length

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genomes, or concatemers, which are recognized by the viral packaging machinery (1–5). Although the essential viral replication proteins have been identified (reviewed in reference 6), the mechanism by which HSV replicates its DNA is poorly understood. It has long been recognized that recombination occurs frequently between coinfecting viral genomes (7–10). Electron micrographs have revealed that replicating DNA contains multiple X- and Y-shaped structures reminiscent of recombination intermediates (3, 11). Furthermore, replicating viral DNA does not enter a pulsed-field gel and remains in the well, suggesting that well DNA is highly branched (12–14). These observations cannot be explained by a simple rolling-circle mechanism of DNA replication, and we have suggested that these structures may arise as a result of recombination-dependent DNA replication (15–19).

The mechanism of HSV DNA replication appears to be distinct from that of other DNA viruses such as simian virus 40 (SV40), which replicates by a theta mechanism to produce two circular daughter molecules (20–22). Interestingly, when SV40 genomes were replicated by the HSV replication machinery and SV40 large T antigen in cell culture, concatemers composed of X-shaped DNA structures reminiscent of HSV DNA replication intermediates were observed (23). This result suggests that the HSV replication machinery is responsible for the complex structure of the replicating DNA. An additional distinction between HSV and other DNA viruses is that incoming HSV genomes contain nicks and gaps (17, 24–28). The genome structure has implications for the mechanism of replication and manipulation of host DNA damage response (DDR) pathways (19). DDR pathways such as classical nonhomologous end joining (C-NHEJ) and homologous recombination repair (HRR) have been shown to be antiviral (17, 29, 30). To establish a lytic infection, HSV must produce concatemers that can be packaged into a procapsid to produce infectious virus while at the same time avoid triggering host antiviral DDR pathways.

HSV encodes two proteins that together are capable of performing single-strand annealing. The 5′-3′ exonuclease (Exo) (UL12) and major single-strand DNA binding protein (ICP8) form a complex that is functionally similar to the λ phage Red α/β recombinase. UL12 is not only functionally similar but also shares sequence homology with the lambda phage exonuclease, Red α . ICP8 is functionally related to Red β , the λ phage single-strand DNA annealing protein. Reminiscent of λ phage Red α/β , UL12 and ICP8 are capable of mediating recombination events such as strand exchange *in vitro* (31, 32). Recent experiments from our laboratory demonstrate that HSV-1 activates the single-strand annealing pathway (SSA) and that this activity requires the exonuclease activity of UL12; moreover, HRR and C-NHEJ were inactivated (16). We have suggested that HSV utilizes SSA to generate concatemers during DNA replication (16, 18).

Viral mutants that cannot express UL12 exhibit severe defects in the production of infectious progeny virus (33–35), indicating that UL12 is important for viral growth. AN-1 is a UL12-null virus that is able to synthesize viral DNA; however, replicating DNA produced in AN-1-infected cells is fragile and prone to fragmentation (14, 36). Furthermore, AN-1 is severely defective in its ability to stably package this DNA, resulting in an increase of “A” or empty capsids and fewer DNA-containing “C” capsids (14, 34). Nuclear egress is also severely compromised. The observation that viral DNA made in the absence of UL12 is aberrant is supported by a report that encapsidated DNA isolated from mutant virions is not infectious (35). Thus, despite the ability to synthesize DNA, UL12-null mutants are defective in the production of viral DNA that can be packaged into infectious virus.

HSV-1 UL12 contains seven motifs that are highly conserved among nucleases of the *Herpesviridae* family (Fig. 1). Motif II contains a particularly well-conserved region, which is shared by other members of the Exo family, including lambda Exo (37–39) (Fig. 1). This motif is common to a larger superfamily of enzymes known as nucleotidyltransferases (NTases) that play important roles in several types of reactions involved in processing genetic material. The active site of UL12 contains a catalytic triad of conserved acidic residues shown to be important for coordinating Mg^{2+} common to other NTases (Fig. 1) (40, 41). Interestingly, NTase enzymes such as HIV integrase have

FIG 1 The seven conserved motifs among the herpesvirus alkaline nuclease homologs highlighting motif II. The highly conserved aspartic acid residue is indicated by a blue asterisk in the WebLogo plot (see <http://weblogo.berkeley.edu/logo.cgi>). Amino acid numbers correspond to the HSV-1 UL12 protein sequence. VZV, varicella-zoster virus.

been successfully targeted using small molecules that coordinate strongly to the catalytic Mg^{2+} in the active site and prevents substrate binding (42).

We previously reported the isolation of two mutants in highly conserved residues of motif II: one with a single mutation, D340E, and the other with a double mutation, G336A S338A (37). Both mutant proteins were expressed and purified from insect cells infected with recombinant baculovirus, and surprisingly, they exhibited differential biochemical activities. The D340E mutant protein lacked exonuclease activity but retained endonuclease activity, whereas the G338A S338A mutant protein lacked both exonuclease and endonuclease activities. Both of these UL12 mutant proteins expressed from amplicon plasmids were unable to complement the growth of the UL12-null virus (37). In this paper, we have focused on the D340E mutation because this aspartic acid is part of the catalytic triad and this mutation would be expected to disrupt Mg^{2+} coordination. The consequences of the D340E mutation in the context of viral infection has never been addressed. Here, we report genetic and pharmacological data that indicate that the exonuclease activity of UL12 is essential for virus production. These results highlight UL12 as a target for development of antiviral agents.

RESULTS

The exonuclease activity of UL12 is required for infectious virus production and cell-to-cell spread. Viral mutants that cannot express the UL12 protein exhibit severe defects in production of progeny virus (33–35), indicating its importance for viral growth. In this paper, the question of whether the exonuclease activity or another function of UL12 is responsible for this phenotype was examined in the context of viral infection. To determine the importance of UL12 exonuclease activity, we generated the recombinant D340E virus (37, 43, 44). This virus was propagated on permissive 6-5 cells, and homogeneity was confirmed via sequencing (data not shown). In addition, the wild-type KOS bacmid (WT-Bac) was purified from the recombinant *Escherichia coli* strain GS1783 (containing a KOS bacmid) to enable a direct comparison between the D340E mutant and a WT version derived from GS1783.

FIG 2 AN-1 and D340E mutant growth on Vero cells is impaired. (A) Growth curves of WT, WT-Bac, AN-1 or D340E mutant virus propagated on Vero cells at an MOI of 0.1 and titrated on permissive 6-5 cells. (B) Vero cells were infected with WT, AN-1, WT-Bac, or D340E mutant virus at an MOI of 5 and harvested at the times indicated above the gels (in hours). Cells were lysed in SDS loading buffer and resolved by SDS-PAGE. Protein kinetic classes are represented by ICP4 (immediate early), ICP8 and UL12 (early), and UL32 (late). Lanes M, mock-infected sample harvested at time zero.

The growth properties of the D340E mutant were compared to those of wild-type KOS (WT), WT-Bac, and the UL12-null mutant AN-1 in a virus yield assay in which nonpermissive Vero cells were infected at a multiplicity of infection (MOI) of 0.1 (Fig. 2A). WT and WT-Bac displayed similar growth properties. Consistent with previous reports, AN-1 growth was significantly reduced compared to that of the WT or WT-Bac, reaching a difference of 4 logs by 48 h postinfection (hpi). The D340E mutant growth recapitulated the behavior of AN-1, demonstrating that UL12 exonuclease activity is essential for promoting lytic infection. To better characterize the growth defect in the D340E mutant, we evaluated the effect on viral gene expression. Expression of all three kinetic classes of viral protein was analyzed in Vero cells infected at an MOI of 5 with WT or mutant viruses. The expression kinetics of AN-1 and the D340E mutant were similar to those of WT and WT-Bac (Fig. 2B), indicating that a decrease or delay in gene expression was not responsible for the growth defects of AN-1 and the D340E mutant.

The virus yield assay reflects virion production within the first 12 to 48 hpi; however, another important parameter of virus growth is the ability to spread. We previously observed that although AN-1 is capable of initiating infection in nonpermissive Vero cells, plaques that develop are very small and oddly shaped (33). To measure cell-to-cell spread, Vero cells were infected with KOS, AN-1, or the D340E mutant at a low MOI in the presence of human serum and monitored by immunofluorescence (IF). ICP4 antibody was used to visualize infected cells, and TO-PRO-3 stain was used to visualize nuclei of both infected and uninfected cells. Figure 3 shows that the size of WT plaques

FIG 3 AN-1 virus and D340E mutant virus initiate infection but do not spread on Vero cells. (A) Vero cells were infected at 100 PFU with WT virus or at 1,000 PFU with AN-1 or D340E mutant virus and overlaid with medium containing 1.25% human serum to prevent reinfection. Cells were fixed at the indicated times and stained for ICP4 to identify infected cells (red channel) and TO-PRO-3 nuclear DNA to visualize all cells (blue channel). Images were captured using a confocal microscope and then analyzed and merged using Adobe Photoshop CS3 and Illustrator CC.

expanded between 24 and 48 hpi. On the other hand, AN-1 and the D340E mutant were able to initiate the formation of plaques that did not increase in size during this time frame. At 72 hpi, all WT-infected cells had detached from the plate; however, the small mutant plaques did not expand (data not shown). These observations indicate that both AN-1 and the D340E mutant are severely defective in cell-to-cell spread, supporting the essential role of UL12 in viral replication.

We previously reported that AN-1 was able to synthesize DNA near wild-type levels (33). To test the ability of D340E mutant virus to replicate viral DNA, total DNA was isolated from Vero cells infected at an MOI of 5, and viral DNA synthesis was measured via dot blot and quantitative PCR (qPCR) analysis (Fig. 4). Although we confirmed that AN-1 produced near-WT levels of replicating DNA, the amount of viral DNA in the D340E mutant-infected cells was approximately 60% of that seen in WT- and AN-1-infected cells (Fig. 4A). This decrease was also observed in qPCR analysis quantifying the relative copy number of the UL9 gene from the replicating viral DNA preparations (Fig. 4B). One possible explanation for the decrease in viral DNA synthesis is that the Exo-dead (Exo[−]) protein may act as a transdominant inhibitor, perhaps by binding DNA in a nonproductive fashion.

Packaging of viral DNA into capsids is defective in cells infected with the Exo[−] D340E mutant. During HSV infection, three types of capsids are found in the nucleus: procapsids containing scaffold, C capsids containing viral DNA, and A capsids, which are missing both the scaffold and DNA (45). Procapsids contain scaffold but are unstable and angularize, forming B capsids when infected cells are lysed. Previously, AN-1 has been reported to produce fewer C capsids and more A capsids than WT, suggesting that either DNA packaging was initiated but not completed or that DNA was prematurely released from capsids made in AN-1-infected cells (34).

To determine whether the D340E mutant virus exhibited similar packaging defects, Vero and 6-5 cells were infected with mutant and WT virus, and lysates were subjected

FIG 4 DNA synthesis is compromised in D340E mutant-infected Vero cells compared to cells infected with WT or AN-1 virus. Vero were cells infected at an MOI of 5, and total replicated DNA was purified 18 hpi. (A) Threefold serial dilutions of total DNA were prepared in denaturing buffer and subjected to dot blot analysis. The Southern blot was probed with a biotin-labeled genomic DNA. (B) Quantification of the relative copy number of UL9 in the total DNA preparations measured by qPCR. The copy number was measured using a standard curve ranging from 10^2 to 10^6 copies of UL9.

to sucrose gradient centrifugation (Fig. 5A and B). In Vero and 6-5 cells infected with WT virus, the expected pattern of B and C capsids and very few A capsids was observed. In contrast, lysates from AN-1-infected Vero cells exhibited very few C capsids and an increased production of “abortive” A capsids. A similar pattern was observed in D340E mutant-infected Vero cells. C capsids produced in mutant-infected Vero cells were not only fewer but appeared more diffuse than those produced in WT-infected cells

FIG 5 AN-1 and the D340E mutant produce fewer C capsids and more A capsids in nonpermissive cells than WT HSV. Capsids were separated by centrifugation on a sucrose density gradient (20 to 50%) from WT-, AN-1-, or D340E mutant-infected cells. Infection on nonpermissive Vero cells (A) and permissive 6-5 cells (B) is shown.

FIG 6 AN-1- and D340E mutant-packaged DNA in Vero cells migrates aberrantly in PFGE. Infected Vero cells and 6-5 cells were embedded in agarose, treated with proteinase K, and subjected to PFGE, and the gel was stained with ethidium bromide. (A) Equal numbers of cells, $\sim 3 \times 10^5$, were embedded in plugs and either treated or not with *PacI* to digest host cellular DNA, as indicated by the minus and plus symbols. (B) The numbers of AN-1- or D340E mutant-infected Vero cells embedded in plugs were increased to 10-fold more than the wild type to adjust the amount of packaged DNA released into the gel.

(Fig. 5A). The defect in C capsid production was rescued when mutant viruses were grown in 6-5 cells (Fig. 5, compare panels A and B).

Packaged DNA produced in the absence of functional UL12 is aberrant. The diffuse nature of C capsids from AN-1 and D340E mutant virus suggested a difference in composition between mutant and WT capsids. We therefore compared packaged DNA from WT-, AN-1-, and D340E mutant-infected Vero and 6-5 cells by pulsed-field gel electrophoresis (PFGE) (Fig. 6). Equal numbers of infected cells were embedded in agarose gel plugs. To facilitate visualization of viral DNA, the plugs were treated with the restriction enzyme *PacI*, which specifically cleaves host but not viral DNA (Fig. 6A, even-numbered lanes). Ethidium bromide staining revealed that the DNA in *PacI*-treated plugs migrated as a smear, indicating extensive digestion of host DNA (Fig. 6A, compare even-numbered to odd-numbered lanes). WT-, AN-1-, and D340E mutant-infected cell plugs released a DNA fragment that migrated to the expected size of the packaged viral genome. This fragment was not observed when plugs were prepared from Vero cells infected with the UL32-null virus *hr64FS*, which is able to produce viral DNA similar to WT levels but is defective for packaging (Fig. 6A, lanes 15 and 16). In addition, no viral DNA was observed in mock-infected cells (Fig. 6A, lanes 1, 2, 17, and 18).

The amount of packaged DNA was less in the AN-1- and D340E mutant-infected Vero cells than in cells infected with WT virus (Fig. 6A, compare lanes 7, 8, 11, and 12 to lanes 3 and 4). This result is consistent with the decrease in C capsids observed in Fig. 5. Another difference between the mutant and WT is that packaged DNA from mutant-infected Vero cells appeared more diffuse and migrated faster than viral genomes seen in WT-infected cells, suggesting that DNA packaged during mutant infection is abnormal. To rule out the possibility that the difference between WT and mutant DNA reflected unequal amounts of packaged viral DNA, the numbers of AN-1- and D340E mutant-infected Vero cells embedded in plugs were normalized by approximating the number of cells required to load an equivalent amount of packaged DNA. Figure 6B confirms that the band representing packaged viral DNA from AN-1- and D340E mutant-infected Vero cells was more diffuse and migrated faster than DNA packaged by WT virus or by mutant virus grown in 6-5 cells. Thus, viral genomes packaged in the absence of functional UL12 are aberrant.

FIG 7 Packaged DNA is not infectious when produced in the absence of functional UL12. Vero or 6-5 cells were transfected with 200 ng of packaged viral DNA and 800 ng of salmon sperm carrier DNA. Medium was replaced with methylcellulose 24 h posttransfection and incubated at 37°C until plaques formed (3 to 6 days). Cells were fixed with formaldehyde and stained with crystal violet. (A) Packaged DNA used for transfection was isolated from infected Vero cells; (B) packaged DNA used for transfection was isolated from infected 6-5 cells.

Packaged DNA produced in the absence of functional UL12 is not infectious.

The diffuse nature of C capsids and packaged DNA from AN-1- and D340E mutant-infected Vero cells (Fig. 5 and 6) led us to question the infectivity of this DNA. Packaged DNA from mutant- and WT-infected Vero or 6-5 cells was isolated and used to transfect either Vero or 6-5 cells (Fig. 7). DNA isolated from AN-1- or D340E mutant-infected Vero cells was not infectious on either Vero or 6-5 cells (Fig. 7A). When mutant viruses were grown on 6-5 cells, the DNA isolated was not infectious on Vero cells but was infectious on 6-5 cells (Fig. 7B). These results demonstrate that the exonuclease activity of UL12 must be present during DNA replication and that in its absence, the product of viral DNA synthesis was aberrant and not efficiently packaged. Furthermore, the DNA that was packaged under these conditions was not infectious. The observation that infectivity could not be rescued even in cells in which functional UL12 was provided supports the suggestion that nuclease activity is required during DNA synthesis.

Inhibition of UL12 nuclease activity by natural and synthetic α -hydroxytropolones. The genetic experiments presented in this paper indicate that the exonuclease activity of UL12 is essential for the production of infectious virus. Based on these findings, we hypothesize that small-molecule inhibitors of the nuclease activity are antiviral. As mentioned in the introduction, the active site of UL12 contains a catalytic triad of conserved acidic residues found within the PD(D/E)XK superfamily of nucleases and includes D340 of motif II (Fig. 1) and E364 and K366 of motif III (40, 41). For nucleases such as UL12, coordination of Mg^{2+} is required to direct the cleavage of the phosphodiester bond at the 5' end of the double-strand DNA (dsDNA) substrate. HIV integrase inhibitors, such as raltegravir, are known to sequester the catalytic Mg^{2+} in the active site and prevent substrate binding (42).

Intriguingly, NTase inhibitors such as β -thujaplicinol, manicol, and synthetic hydroxytropolone analogs were effective in suppressing HSV infection in cell culture models (46, 47); however, the viral targets of these compounds have not been identified. Although four HSV-1 viral proteins, UL30, ICP8, UL15, and UL12, have been identified as possible members of the NTase superfamily (48–50), it is unclear whether

FIG 8 UL12 nuclease activity can be inhibited by α -hydroxytropolones. (A) Structure of hydroxytropolone derivatives used. Compound numbers are taken from reference 46. (B) UL12 was incubated in the presence of DMSO or a 15 μ M concentration of the indicated compound for 5 min at 37°C. The nuclease reaction was initiated by the addition of dsDNA, and aliquots were quenched at 0 and 10 min with EDTA. Inhibition of nuclease-mediated DNA digestion was determined using PicoGreen, which selectively binds dsDNA and fluoresces. Error bars represent the standard error of results from two independent experiments.

any of these proteins are the targets responsible for their anti-HSV activity. Interestingly, a recent report demonstrated that the HSV viral terminase, UL15, can be inhibited by α -hydroxytropolones (51).

In order to address whether UL12 could also be a target for these compounds, β -thujaplicinol, manicol, and a series of substituted α -hydroxytropolones prepared by Meck and coworkers (52, 53) were tested for their ability to inhibit UL12 nuclease activity (Fig. 8A). A nuclease assay was developed based on PicoGreen fluorescent DNA dye, which binds to double-strand DNA by associating with the minor groove (54). Baculovirus-expressed UL12 was incubated in the presence of dimethyl sulfoxide (DMSO) or a 15 μ M concentration of each compound for 5 min at 37°C to allow complexes to form. The nuclease reaction was initiated by the addition of a linear dsDNA fragment, and aliquots were removed and quenched at 0 and 10 min. Figure 8B demonstrates that several compounds showed significant inhibition of enzymatic activity, whereas input DNA was digested to near completion after 10 min in the untreated control. Compounds 118, 115, 146, and 114 were the most potent and inhibited UL12 activity by 80% at 15 μ M. It is notable that the most potent inhibitors contained relatively hydrophobic appendages attached to the tropolone core (Fig. 8A). Importantly, those hydroxytropolones with the greatest potency against UL12 also

displayed the strongest antiviral activity against HSV in cell culture (46). The correlation between inhibition of UL12 activity and HSV infection provides further evidence that UL12 is essential and can serve as a good drug target.

DISCUSSION

We previously reported that an HSV UL12-null mutant exhibited a severe growth defect in virus yield assays (33, 34); however, it was not possible to assess whether the growth defect reflected loss of nuclease activity or another UL12 function. We have now introduced the motif II Exo⁻ point mutation, D340E, of UL12 into the viral genome and determined that the phenotype of the D340E mutant resembles that of the null mutant, AN-1. In a viral yield assay, both the D340E mutant and AN-1 exhibited a 4-log decrease compared to the WT, and both viruses exhibited severe defects in cell-to-cell spread. In addition, the D340E mutant resembled AN-1 in the impaired production of DNA-containing C capsids, an increase in the number of abortive empty A capsids, and packaging of diffusely migrating noninfectious viral DNA. Taken together, our results indicate that loss of exonuclease activity is responsible for the growth defects of the null mutant. Furthermore, these data suggest that exonuclease activity is essential for the production of viral DNA suitable for packaging into an infectious virion, suggesting that HSV nuclease is a promising target for antiviral drug discovery.

Growth phenotype of UL12 mutants. The phenotype of UL12 mutants is complex. We previously reported that the UL12-null virus, AN-1, was compromised for growth on nonpermissive cells, with viral yields 2 to 3 logs lower than that of wild-type virus (33–35, 55). In this paper, we show that the growth defect of AN-1 in nonpermissive cells is even more serious than previously recognized. When mutant virus stocks were plated on Vero cells at a low MOI, plaques with a small number of infected cells (~5 to 10 cells) were detected (Fig. 3); however, even after 48 h, limited cell-to-cell spread occurred. The inability of mutant viruses to spread raises a question of how an infection could initiate in the first place to generate the small plaques seen in Fig. 4A. One explanation is that mutant viral stocks grown on 6-5 cells package viral genomes that had been replicated in the presence of functional nuclease. Thus, in the experiment shown in Fig. 3, the initially infected Vero cell would have been infected with viruses whose genomes were relatively intact. The inability of mutant virus to spread through the Vero cell culture may be explained by the aberrant nature of DNA produced in the absence of a functional nuclease. The observation that mutant viral genomes packaged from infected Vero cells were noninfectious (Fig. 7) also supports the notion that the nuclease activity of UL12 is essential for the production of infectious virus.

In this paper, the D340E mutation has been incorporated into the viral genome, allowing us for the first time to analyze this Exo⁻ mutant in the context of viral infection. The D340E mutant exhibited the same profound growth and cell-to-cell spread defects as AN-1. Based on these results, we conclude that exonuclease is essential for viral growth and cell-to-cell spread. These results are not consistent with a report by Fujii and coworkers, who generated a viral mutant containing the G336A S338A, motif II double mutation. In contrast to the results reported in this paper, they reported that the G336A S338A double mutation only slightly reduced viral replication in cell culture (56). The reason for the difference in the growth phenotype is not clear; however, as mentioned above, these mutant proteins exhibit distinct properties. The D340E mutant is defective for exonuclease and retains endonuclease activity, while the G336A S338A mutant is defective for both exonuclease and endonuclease activities. This biochemical difference may produce differential effects on viral replication and viral growth. On the other hand, as mentioned in the introduction, when the D340E and G336A S338A mutations were expressed on viral amplicons, they both failed to complement the null virus for viral growth. Moreover, Henderson et al. have also reported that Exo⁻ mutants failed to complement null virus for viral growth (57). Thus, the majority of evidence, at this time, points to the essentiality of the exonuclease activity of UL12 for virus growth.

DNA phenotype of UL12 mutants. In many ways, the phenotype of the D340E mutant resembles that of the null mutant, with at least one interesting difference. D340E mutant-infected Vero cells produced approximately 40 to 60% of viral DNA in comparison to the WT and AN-1 (Fig. 4). As described above, the D340E mutant is defective for exonuclease but not endonuclease activity. It is possible that the D340E protein binds DNA and, in the absence of Exo activity, this nonproductive binding could exert a transdominant effect on DNA synthesis. It is also possible that other functions associated with UL12 could be responsible for the defect in DNA synthesis. We have reported that UL12 interacts with several components of the host cell DNA damage response (DDR) pathways, including MRN, Ku70, and MSH2/6 (58, 59). Furthermore, Karttunen and coworkers have reported an interaction between UL12 and both FANCI and FANCD2 proteins of the Fanconi anemia genomic stability pathway (60). It will be of interest to determine whether the ability of the D340E protein to bind DNA or DDR proteins is responsible for inhibition of viral DNA synthesis.

The nature of the viral DNA that accumulates in cells infected with UL12 mutants is also of interest. We have shown previously that replicating DNA from AN-1-infected Vero cells is fragile and prone to fragmentation, suggesting an aberrant structure (36). In this paper, we report that in Vero cells infected with either AN-1 or the D340E mutant, encapsidation of DNA into C capsids is much less efficient than for WT or for UL12 mutants grown in 6-5 cells. Furthermore, packaged DNA from UL12-infected Vero cells migrates faster and more diffusely in a pulsed-field gel and is not infectious (Fig. 6 and 7). Taken together, these results suggest that in the absence of UL12 nuclease activity, replicating DNA is aberrant and only a small fraction can be stably packaged; furthermore, the DNA that is packaged is also aberrant. This is consistent with our observation that fewer C and more A capsids were isolated from AN-1- and D340E mutant-infected cells (Fig. 5). We suggest that during DNA replication, UL12 exonuclease activity is required to produce concatemers that can be recognized by the packaging machinery. The structure of replicating DNA produced in cells infected with null or Exo[−] mutants is under investigation.

Role of UL12 in DDR pathway choice. Herpesviruses have evolved a complex relationship with host DDR pathways (reviewed in reference 19). In response to DNA damage or replication stress, mammalian cells activate various DDR pathways to repair double-strand breaks (DSBs) (61–63). The two most prominent DSB repair pathways are classical nonhomologous end joining (C-NHEJ) and homologous recombination repair (HRR). If HRR is not available, the single-strand annealing (SSA) pathway is used (64–67). HRR and SSA are dependent on end resection to generate single-strand DNA ends that participate in strand invasion and strand annealing, respectively (64, 68–71). One important mechanism by which cells control DDR pathway choice following DNA damage involves competition between repair proteins for binding at the DSB. For instance, if the MRN complex along with exonucleases EXO and DNA2 binds at a DSB before components of the C-NHEJ pathway (Ku and DNA-PK), end resection occurs (72, 73). This end resection would be expected to promote homologous recombination, either HRR or SSA, and inhibit C-NHEJ. If Ku and DNA-PK bind to DSBs, the C-NHEJ pathway is favored.

HSV replicates in the nucleus, and introducing the viral genome into this compartment would be expected to induce a DDR response. It is now recognized that components of the DDR pathways are antiviral and that HSV must inhibit these antiviral components in order to initiate a successful lytic infection (19). For instance, HSV replication is more efficient in cells lacking DNA-PKcs (29, 30), and in Ku-deficient murine embryonic fibroblasts, viral yields are increased by almost 50-fold (74). To counteract antiviral activities of DNA-PK, the viral immediate early protein ICP0 can hinder C-NHEJ by two mechanisms, one that induces degradation of DNA-PK (29, 30) and another that inhibits the activity of DNA-PK by another mechanism (17). ICP0 can also inhibit the HRR pathway by inducing degradation of downstream HRR compo-

nents, RNF8 and RNF168 (75). We suggest that in order to carry out viral replication, HSV must influence pathway choice by inhibiting the pathways that are antiviral.

We have previously shown that HSV infection is capable of stimulating SSA in a chromosomally integrated reporter assay (16). The increase in SSA activity was abolished when cells were infected with the null virus AN-1. Furthermore, expression of UL12 alone increased SSA, while the Exo[−] protein was unable to do so (16). The ability of UL12 to influence pathway choice is intriguing because it suggests that HSV may have evolved an additional mechanism to inhibit C-NHEJ. Resection of DSBs by UL12 in HSV-infected cells would be expected to inhibit C-NHEJ similar to the way end resection by MRN/EXO/DNA2 can prevent C-NHEJ (72). Thus, in addition to the role of ICP0 in inhibition of C-NHEJ and HHR, it is possible that UL12 also plays a role in counteracting the antiviral C-NHEJ pathway.

We and others have proposed that HSV utilizes recombination-dependent DNA synthesis (15, 23, 32, 76–78), and as described above, SSA appears to be the pathway of choice in an HSV-infected cell (16, 18). The SSA pathway is generally believed to involve nucleases that resect DNA at a DSB and a single-strand DNA protein that promotes annealing. We suggest that HSV concatemers are formed by SSA reactions that involve the two-subunit viral recombinase, UL12 and ICP8. Replication through a nick or a gap in the viral genome can lead to the formation of double-strand DNA fragments that would be resected by UL12 to generate a free 3′ single-strand end that could be coated by ICP8 and then participate in a recombination reaction (as shown in Fig. 9). The ICP8-coated 3′ overhang could anneal at a gap on the lagging strand template to initiate formation of a DNA replication fork (Fig. 9A). Figure 9B depicts another possible scenario in which ICP8-coated DNA anneals at a single-strand DNA (ssDNA) gap in the HSV genome, generating a branched structure. Strand displacement synthesis, followed by DNA replication on the displaced strand, would result in the formation of a full replication fork. This fork can replicate out to the end of the DNA template, resulting in a linear DNA fragment that could participate in reactions leading to concatemer formation. We have depicted a simple case, but numerous annealing events can occur at additional gaps along each strand of the HSV genome such as that depicted in Fig. 9. These models could explain the generation of complex branched structures seen in replication intermediates during HSV infection (3, 13, 79). While additional experimentation is required to provide direct evidence for one or both of the scenarios depicted in Fig. 9, they are consistent with our current understanding of HSV DNA replication.

Results presented in this paper demonstrate that the exonuclease activity of UL12 is essential for the production of infectious virus. The conserved acidic triad resembles regions in the superfamily of NTases, suggesting that it may be possible to take advantage of the two coordinated metals in the active site for inhibition. The hydroxytropolones tested in Fig. 8 indeed show that UL12 is amenable to inhibition by small drug-like compounds. The molecules tested so far are relatively small and simple yet are generating moderate levels of inhibition, indicating that it should be possible to improve inhibition with additional chemical modification. Most importantly, conservation of this critical enzyme in all the herpesviruses makes it possible to produce a potent nuclease inhibitor that would have broad-spectrum antiviral activity against homologs in cytomegalovirus (CMV) (UL98), Epstein-Barr virus (EBV) (BGLF5), and Kaposi's sarcoma-associated herpesvirus (KSHV) (Sox).

MATERIALS AND METHODS

Cells and viruses. Vero cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified minimal essential medium (DMEM) (Gibco) containing 5% fetal bovine serum (FBS). The UL12-expressing Vero cell line, 6-5, which was previously described (34), was grown in DMEM supplemented with 5% FBS and G418 to maintain selection for the UL12 cassette (34). The KOS strain of HSV-1 was used as wild-type (WT) virus. A UL12-null virus, AN-1, was derived from KOS and lacks 917 bp of the UL12 gene due to replacement with a *lacZ* insertion under the expression of the ICP6 promoter (33). A UL32 mutant virus, *hr64F5*, containing a frameshift mutation that creates two tandem stop codons at amino acids 56 and 57 of UL32, was previously described (80).

FIG 9 Model for DNA replication in HSV-infected cells. UL12 resects DNA at the 5' end of a DSB. The 3' ssDNA overhang is coated by ICP8, which can anneal the fragment to its complementary sequence through either pathway. UL12 is represented as a major sector, and ICP8 is represented as a circle in which the ICP8-coated DNA is assimilated at the lagging end of a replication fork (A) or whereby ICP8-coated DNA anneals at a gap and leads to the formation of a replication fork (B). Figure adapted from Weller and Sawitzke (18).

Construction of recombinant UL12 D340E virus. *En passant* mutagenesis was used to introduce the D340E mutation into the KOS genome as described previously (43, 44). Recombinogenic *Escherichia coli* GS1783 (kindly provided by Greg Smith) harboring the KOS bacmid was subjected to a two-step recombination process. To promote recombination and introduce the D340E point mutation, a DNA fragment that contained a kanamycin-resistance cassette, I-SceI restriction site, and homologous nucleotide sequences to the UL12 gene was amplified from the pEPKanS2 vector (kindly provided by Greg Smith) using the following primers: 5'GGGCCAGGTAGCCGTGAATGTCCCGAGGACAGACGAGAATTCCAGGGACGCCCCGACCATAGGATGACGACGATAAGTAGGG3' and 5'TCATGGACGGTCACACGGGGATGGTCGGGCGCTCCCTGGAAATTCTCGTCTGTCTCGGGACAACCAATTACCAATTCTGATTAG3'.

The underlined 3'-nucleotide sequences are homologous to pEPKanS2. The remaining nucleotides are complementary sequences within the UL12 gene, with the mutagenic codon in bold font. The PCR product was amplified with Platinum *Taq* DNA polymerase high fidelity (Invitrogen catalog no. 11304) and purified with the Promega Wizard PCR cleanup kit (Promega catalog no. A9281). GS1783 was transformed with the PCR product to initiate the first round of recombination at the unique UL12 sequences, which resulted in the insertion of the entire fragment. Kan^r *E. coli* isolates were selected and used for the second round of recombination. Expression of *SceI* was induced to create a double-strand break at the I-SceI restriction site and promoted recombination at the duplicated homologous UL12 sequences. This resulted in removal of the Kan cassette and retention of the D340E mutation.

The recombinant UL12 D340E-bacmid was purified from the final Kan^s *E. coli* isolates and used to transfect permissive 6-5 cells to propagate virus production. Three isolated plaques were selected, and the purity of the D340E recombinant viral clones was confirmed by amplifying the UL12 fragment containing the point mutation and then sequencing this fragment (GeneWiz).

Viral growth curves and yields. Vero cells were seeded in 35-mm dishes and infected at a multiplicity of infection (MOI) of 0.1 PFU/cell. Cells and media were harvested together at 0, 6, 12, 24, and

48 h postinfection. After two freeze-thaw cycles, cellular debris was pelleted and the resulting supernatants were titrated on 6-5 cells. To count plaques, cells were fixed with 4% formaldehyde in $1\times$ PBS (approximately 2 to 4 days postinfection) and stained using 0.1% crystal violet.

Western analysis of viral proteins. Vero cells were seeded in 35-mm dishes and infected at an MOI of 3 PFU/cell. Cells were washed two times with PBS and scraped into $1\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% milk and probed with the following antibodies: rabbit polyclonal anti-UL32 (1:500; antibody to synthetic antigenic peptide was generated by Open Biosystems), rabbit polyclonal anti-UL12 antibody (1:10,000, a gift from Joel Bronstein and Peter Weber; Parke-Davis Pharmaceutical Research), mouse monoclonal anti-ICP8 antibody (1:2,000; catalog no. ab20194, Abcam), and mouse anti-actin antibody (1:10,000; catalog no. A5441, Sigma).

IF cell-to-cell spread. Viral stocks were harvested from supernatants of infected 6-5 cells, and titers were determined on 6-5 cells. To perform immunofluorescence (IF) experiments, 4×10^5 Vero cells in a 35-mm plate containing a glass coverslip were infected at 100 PFU/plate with WT virus and 1,000 PFU/plate with AN-1 and D340E mutant virus. After 1 h of absorption at 37°C, the cells were washed three times with PBS and overlaid with 1.5 ml of DMEM containing 5% FBS and 1.25% human serum. Cells were processed for immunofluorescence at 24, 48, and 72 hpi as previously described (58). Primary antibodies were used to detect the immediate early protein ICP4 (1:200; Santa Cruz catalog no. 56986) and the DNA marker TO-PRO-3 (1:1,000; Molecular Probes), diluted in 3% normal goat serum (NGS) for 1 h. Alexa Fluor anti-mouse antibody (1:500; Molecular Probes) diluted in 3% NGS was used as the secondary antibody. Images were captured using a Zeiss LSM 780 confocal microscope equipped with argon and HeNe lasers and a Zeiss 40 $\times$ objective lens (0.6 $\times$ zoom). Images were processed and arranged using Adobe Photoshop CS3 and Illustrator CC.

Quantification of viral DNA synthesis. Vero cells were seeded onto 60-mm plates and infected at an MOI of 5 PFU/cell for 18 h at 37°C. Infected cells were washed with PBS and pelleted. Cells were resuspended in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), pH 7.5, and lysed with the addition of $3\times$ lysis buffer (30 mM Tris-HCl [pH 7.5], 30 mM EDTA, 1.8% SDS) to a final 1-fold concentration. Proteinase K was added to a final concentration of 100 μ g/ml and incubated at 55°C for 4 h. DNA was isolated by phenol-chloroform/isoamyl alcohol extraction, followed by ethanol precipitation. Quantification of viral DNA was performed by serial dilution of DNA samples using a dot blot manifold in accordance with the manufacturer's protocol (PerkinElmer) using a GeneScreen Plus nylon membrane (catalog no. NCF10700). The membrane was probed with biotin-labeled KOS genomic DNA labeled with North2South biotin random prime labeling kit (Thermo Scientific catalog no. 17075).

Replicated DNA was used as the template for qPCR to amplify a 200-nucleotide fragment from the UL9 gene using the following primers: 5'-CGACCACCACATGCACGT-3' and 5'-CCTCGTTCTGGACGAGGTTATG-3'. A standard curve prepared from pCDNA3-UL9 was used to determine the relative copy number of UL9 from replicating DNA. Accumulation of DNA products was measured using iQ SYBR green Supermix (Bio-Rad catalog no. 170-8880) in a Bio-Rad CFX 96 thermal cycler.

Isolation of viral capsids. Vero and 6-5 cells were infected at an MOI of 3 PFU/cell for 24 h at 37°C. Cells were pelleted at $125\times g$ for 10 min at 4°C, washed with PBS, repelleted, and stored at -80°C . Frozen cell pellets were thawed at 37°C and suspended in 5 ml of 20 mM Tris-HCl, pH 7.5, and lysed by the addition of equal volumes of $2\times$ lysis buffer (1 M NaCl, 40 mM Tris-HCl [pH 7.5], 2% Triton X-100, 2 mM dithiothreitol [DTT], 2 mM EDTA) and $1\times$ protease inhibitor cocktail (Roche catalog no. 05056489001). This mixture was incubated on ice for 30 min and treated with 250 U of Benzonase (EMD Millipore catalog no. 71205-3) and MgCl_2 at a final concentration of 10 mM at room temperature for 15 min. Treated lysates were sonicated in a cup horn sonicator at 50% amplitude three times for 10 s and chilled on ice between pulses. Lysates were cleared by centrifugation at $10,000\times g$ for 10 min at 4°C. Capsids were isolated through a 30% sucrose cushion prepared in TNE buffer (20 mM Tris-HCl [pH 7.4], 500 mM NaCl, 1 mM EDTA) in a Beckman SW41 rotor at $100,000\times g$ for 1 h at 4°C. Supernatant was removed, and the capsid pellets were resuspended in TNE buffer containing 2 mM DTT and protease inhibitors. Capsids were separated by ultracentrifugation on a 20-to-50% sucrose density gradient and visualized with the halogen light of a piston gradient fractionator (BioComp).

Pulsed-field gel electrophoresis. Vero and 6-5 cells were seeded in 35-mm plates and infected the next day at an MOI of 3 PFU/cell with WT or mutant viruses. At 20 to 24 hpi, cells were pelleted and resuspended in 1% low-melting-temperature agarose (LMA) (Bio-Rad LMP) at 55°C in PBS buffer for casting into Bio-Rad plug molds. To increase the amount of packaged DNA from mutant-infected cell pellets, the volume of LMA was adjusted such that 10-fold more mutant-infected cells were added than wild-type-infected cells. Plugs were transferred to a 50-ml Falcon tube containing 5 ml of proteinase K lysis buffer (1% laurylsarcosine, 0.2% sodium deoxycholate, 0.1 M EDTA). Proteinase K was added to a final concentration of 1 mg/ml, and plugs were incubated for 20 h at 50°C. Plugs were washed four times for 30 min at room temperature in TE buffer and then embedded in a 0.9% molecular biology-grade agarose gel (SeaKem) prepared in $0.5\times$ TBE (45 mM Tris, 45 mM borate, 1 mM EDTA, pH 8.3) buffer. Electrophoresis was performed using a CHEF-DR III apparatus (Bio-Rad) with $0.5\times$ TBE running buffer. Samples were separated using 6 V/cm (approximately 200 V) for 18 h at 14°C. Switch times ramped from 1 to 60 s. Lambda Ladder PFG marker (Bio-Rad) was used for size standards. Gels were stained with ethidium bromide and imaged using a ChemiDoc MP imaging system (Bio-Rad).

Preparation of packaged DNA. Three 100-mm plates of Vero or 6-5 cells were infected with WT or mutant viruses at an MOI of 3 PFU/cell. Cells and growth medium were harvested at 18 to 20 hpi, and the cells were pelleted at $800\times g$ for 5 min at 4°C. The supernatant was collected, and virions were pelleted using a Beckman SW41 rotor at $100,000\times g$ for 1 h. The virion pellet was washed with TM buffer

(10 mM Tris-HCl [pH 7.4], 30 mM MgCl₂) and stored on ice. Cell pellets were subjected to two freeze-thaw cycles at -80°C and 37°C , resuspended in TM buffer, and incubated on ice for 20 min. Cells were sonicated in a cup horn sonicator at 40% amplitude for six 15-s pulses. Cell debris was pelleted in a microcentrifuge at maximum speed ($16,000 \times g$) for 5 min at room temperature, and the supernatant (containing capsids and intracellular virions) was collected and combined with the virion pellet. This mixture was treated with 250 U of Benzonase and 40 $\mu\text{g/ml}$ RNase at 37°C for 1 h. After incubation, Triton X-100 was added to each sample to a final concentration of 1% and incubated on ice for 20 min. Capsids were pelleted through a 30% sucrose cushion in TNE buffer in a Beckman SW55 rotor at $100,000 \times g$ for 1 h at 4°C . The supernatant was removed, and the capsid pellets were washed and resuspended with TNE buffer containing 100 mM NaCl. SDS and proteinase K were added to final concentrations of 1% and 100 $\mu\text{g/ml}$, respectively, and incubated at 50°C for 2 to 4 h. DNA was purified by phenol-chloroform-isoamyl alcohol (25:24:1) extraction, followed by ethanol precipitation. Precipitated DNA was washed with 70% ethanol, dried, and gently resuspended in TE buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA). The DNA concentration was determined using the Qubit 2.0 and dsDNA HS reagents (Thermo Fisher Scientific catalog no. Q32851). Purified packaged DNA was aliquoted and stored at -80°C .

Transfection of packaged DNA to measure infectivity. Vero or 6-5 cells were seeded in 35-mm plates at 85 to 90% confluence 24 h before transfection. Virion DNA isolated from WT-, AN-1-, or D340E mutant-infected Vero or 6-5 cells was used for Lipofectamine Plus-mediated transfection as recommended by the manufacturer (Invitrogen/Life Technologies). For each experiment, 200 ng of packaged DNA was mixed with 800 ng of denatured salmon sperm DNA, for a total of 1 μg per transfected plate. Mock samples were transfected with 1 μg of denatured salmon sperm DNA. Approximately 24 h posttransfection, the medium was removed and replaced with DMEM containing 2% methylcellulose. Cells were incubated at 37°C for an additional 3 to 6 days, depending on the appearance of plaques. When plaques were large enough to count, cells were fixed with 4% formaldehyde and stained with crystal violet.

UL12 protein purification. UL12 was purified as previously described, (37, 81) with minor modifications. Briefly, a suspension of Sf9 insect cells was grown in Sf-900 II medium (Gibco) supplemented with 5% FBS and infected with recombinant baculovirus. Infected cells were collected after 48 h of incubation at 27°C . Roche EDTA-free protease inhibitor cocktail (Roche catalog no. 05056489001) and 1 mM phenylmethylsulfonyl fluoride were used in place of the protease inhibitors described above.

PicoGreen nuclease assay. Nuclease activity was assessed using the Quant-iT PicoGreen dsDNA reagent dye (Molecular Probes by Life Technologies catalog no. P7581) to detect dsDNA as previously reported (82, 83). Nuclease reactions were performed in $1\times$ nuclease buffer (20 mM Tris-HCl [pH 8.2], 40 mM NaCl, 1 mM MgCl₂, 1 mM DTT) at 37°C . The DNA substrate was linearized pSAK vector (4 kbp) cut with PstI. Nuclease reaction mixtures contained 14 ng of purified UL12 protein, 1.7% DMSO, or 15 μM each α -hydroxytropone from a stock prepared in 100% DMSO. UL12 protein was incubated with DMSO or compound for 5 min at 37°C , and the reaction was initiated with the addition of 10 ng dsDNA per 30 μl of reaction mixture. Control reaction mixtures contained protein alone without DNA and dsDNA substrate without protein. At time zero and 10 min, 30 μl was removed from each reaction tube and quenched with 3 μl of 500 mM EDTA. The quenched reaction mixtures were stored on ice until all reactions were complete.

Each sample was processed for PicoGreen fluorescence in accordance with the manufacturer's protocol. Briefly, 67 μl of TE buffer was added to each quenched reaction mixture to bring the volume up to 100 μl . Then 100 μl of the PicoGreen reagent, diluted 1:200 in TE buffer, was added to each tube and vortexed briefly to mix. Fluorescence was measured with a SpectraMax 3 plate reader in a 96-well Fluotrac 200 black plate (Greiner Bio-One catalog no. 655076/077) with excitation and emission wavelengths of 485 nm and 520 nm (530-nm cutoff), respectively.

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