The Product of the Herpes Simplex Virus 2 UL16 Gene Is Critical for the Egress of Capsids from the Nuclei of Infected Cells

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ABSTRACT The herpes simplex virus (HSV) *UL16* gene is conserved throughout the *Herpesviridae* and encodes a poorly understood tegument protein. The HSV-1 *UL16* protein forms complexes with several viral proteins, including *UL11*, *gE*, *VP22*, and *UL21*. We previously demonstrated that HSV-2 *UL21* was essential for virus propagation due to the failure of DNA-containing capsids (C capsids) to exit the nucleus. We hypothesized that if a *UL16/UL21* complex was required for nuclear egress, HSV-2 lacking *UL16* would have a phenotype similar to that of HSV-2 lacking *UL21*. Deletion of HSV-2 *UL16* ($\Delta 16$) resulted in a 950-fold reduction in virus propagation in mouse L cell fibroblasts and a 200-fold reduction in virus propagation in Vero cells that was fully reversed upon the repair of $\Delta 16$ ($\Delta 16R$) and partially reversed by infecting *UL16*-expressing cells with $\Delta 16$. The kinetics of viral gene expression in cells infected with $\Delta 16$ were indistinguishable from those of cells infected with $\Delta 16R$ or the parental virus. Additionally, similar numbers of capsids were isolated from the nuclei of cells infected with $\Delta 16$ and the parental virus. However, transmission electron microscopy, fluorescence *in situ* hybridization experiments, and fluorescent capsid localization assays all indicated a reduction in the ability of $\Delta 16$ C capsids to exit the nucleus of infected cells. Taken together, these data indicate that, like *UL21*, *UL16* is critical for HSV-2 propagation and suggest that the *UL16* and *UL21* proteins may function together to facilitate the nuclear egress of capsids.

IMPORTANCE HSV-2 is a highly prevalent sexually transmitted human pathogen that is the main cause of genital herpes infections and is fueling the epidemic transmission of HIV in sub-Saharan Africa. Despite important differences in the pathological features of HSV-1 and HSV-2 infections, HSV-2 is understudied compared to HSV-1. Here we demonstrate that a deletion of the HSV-2 *UL16* gene results in a substantial inhibition of virus replication due to a reduction in the ability of DNA-containing capsids to exit the nucleus of infected cells. The phenotype of this *UL16* mutant resembles that of an HSV-2 *UL21* mutant described previously by our laboratory. Because *UL16* and *UL21* interact, these findings suggest that a complex containing both proteins may function together in nuclear egress.

KEYWORDS herpes simplex virus, tegument, *UL16*, *UL21*, nuclear egress

Herpes simplex virus (HSV) virions are complex assemblages containing almost 100 different viral and cellular proteins (1). Infectious virus particles are comprised of an icosahedral nucleocapsid, containing a linear double-stranded DNA genome, surrounded by a lipid envelope embedded with glycoproteins. Between the nucleocapsid and the envelope lies a complex proteinaceous compartment called the tegument. The initial stages of herpesvirus assembly take place in the nucleus where newly replicated virus genomes are packaged into preformed capsids. DNA-containing capsids (C capsids) and their associated tegument proteins gain access to the cytoplasm by undergoing a process called nuclear egress (2). Once in

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the cytoplasm, the tegument continues to assemble through the recruitment of tegument proteins to capsid components, interactions between the various tegument proteins, and interactions between tegument proteins and the cytoplasmic tails of membrane glycoproteins destined for the envelope of mature virions. The virion acquires its final envelope through the budding of capsid-tegument complexes into membranes derived from a post-Golgi compartment in a process referred to as secondary envelopment (3). Vesicles containing enveloped virus then traffic to, and fuse with, the plasma membrane of the cell, releasing mature virus into the extracellular environment (4).

This study concerns the functions of HSV-2 UL16, a tegument protein conserved throughout the *Herpesviridae* (5). Deletion of HSV-1 and pseudorabies virus (PRV) *UL16* orthologs led to a 10-fold reduction in virus propagation and defects in secondary envelopment (6–8). In contrast, deletion of the human cytomegalovirus (HCMV) *UL16* ortholog, *UL94*, and the murine gammaherpesvirus 68 (MHV-68) ortholog, *ORF33*, prevented virus propagation altogether and, consistent with a role for the encoded proteins in secondary envelopment, resulted in the accumulation of C capsids in the cytoplasm of infected cells (9, 10). Notably, the MHV-68 *ORF33* mutant also displayed defects in the egress of capsids from the nucleus to the cytoplasm, suggesting that *ORF33* has both nuclear and cytoplasmic functions (9, 11).

Work performed on HSV-1 UL16 revealed a number of interesting features of the protein. First, UL16 has the capacity to interact directly with several virion structural components, including the tegument proteins VP22 and UL21 (7, 12, 13), the membrane-associated tegument protein UL11 (14–16), and the envelope protein gE (17). The interactions of VP22, UL11, and gE with UL16 map to the N-terminal half of UL16, whereas the C-terminal portion of UL16 regulates the ability of UL16 to bind to these partners. There is strong evidence that UL16 can simultaneously interact with UL21, UL11, and gE, suggesting that there are multiple nonoverlapping binding sites for these proteins on the UL16 surface (12). Thus, UL16 might be expected to be a key structural component of the HSV-1 virion insofar as its interactions predict a capacity to link the virion envelope to the tegument and underlying capsid. Second, UL16 has the remarkable capacity to respond to signal transduction across the virion envelope (18, 19). Upon interaction of virion envelope proteins with cellular attachment receptors, UL16 within the virion loosens its association with the capsid. While UL16 localizes to the nucleus of infected cells at times when capsids are being assembled, viral genomes are being packaged, and nuclear egress is occurring (20), UL16 was not associated with A, B, and C capsids isolated from the nuclei of infected cells (18), although there is a possibility that UL16 has the capacity to interact with procapsids. In contrast, capsids isolated from the cytoplasm of infected cells are associated with UL16 (18, 21). The identity of capsid or capsid-associated proteins through which UL16 is recruited to capsids is not known.

A single report on HSV-2 UL16 suggested that it loosely interacts with C capsids but does not interact with A or B capsids that lack viral genomic DNA (22). In contrast to what was observed for HSV-1 UL16 (20), HSV-2 UL16 was not detected in extracellular virions, suggesting that UL16-capsid interactions are transient (22). The authors of that study also demonstrated that purified UL16 had DNA binding activity, possibly mediated by its putative zinc finger domain (23), and suggested that UL16 might function in the packaging and/or cleavage of viral genomic DNA.

Previous studies from this laboratory demonstrated that the tegument protein UL21 is required for the efficient nuclear egress of HSV-2 capsids, and its deletion from the HSV-2 genome prevented virus propagation (24). These findings were in stark contrast to what was observed with HSV-1 and PRV *UL21* deletion mutants, where comparatively modest defects in virus propagation were noted (8, 25–29). These results suggested that UL21 performs a unique role in HSV-2 infection. As both HSV-1 and PRV UL21 proteins interact with UL16 (8, 13), we hypothesized that if a UL21/UL16 complex was required for HSV-2 nuclear egress, HSV-2 lacking *UL16* would have a phenotype similar

FIG 1 Deletion of HSV-2 UL16 prevents the spread of infection between cells. (A) Vero cells were cotransfected with the HSV-2 WT BAC or Δ16 BAC along with pCI-neo (empty vector) or the UL16 expression plasmid pCI-16. Spread of infection was visualized at 70 h posttransfection by GFP expression encoded by BAC sequences. Representative images are shown. The arrowhead indicates a single Δ16 BAC-transfected cell from which infection failed to spread. (B, top) Extracts prepared from L and L16 cells infected with the WT, Δ16, and Δ16R viruses at 24 hpi were analyzed by Western blotting using chicken polyclonal antibodies produced against HSV-2 UL16. (Bottom) A blot identical to that shown at the top was probed for β-actin and served as a loading control. Migration positions of molecular mass standards (in kilodaltons) are indicated on the left, and arrowheads on the right indicate the positions of UL16 and β-actin, respectively. (C) Plaques formed by the WT, Δ16, and Δ16R strains on monolayers of L and L16 cells. Identical dilutions of each strain were used to infect L and L16 cell monolayers. Cells were fixed and stained with 0.5% methylene blue in 70% methanol at 3 days postinfection.

to that of HSV-2 lacking *UL21*. In this report, we demonstrate that this is indeed the case.

RESULTS

HSV-2 UL16 is critical for virus propagation. To determine the significance of UL16 during HSV-2 replication, we constructed an HSV-2 *UL16*-null bacterial artificial chromosome (BAC) (Δ16 BAC). Three days after cotransfection of the parental wild-type (WT) BAC into Vero cells along with the empty vector pCI-neo, plaque formation was evident and was visualized by enhanced green fluorescent protein (EGFP) fluorescence derived by BAC sequences (Fig. 1A). In contrast, cotransfection of the Δ16 BAC with the empty vector yielded no plaques; however, single fluorescent cells were seen (Fig. 1A, arrowhead). To test if UL16 provided in *trans* could enable the spread of Δ16 BAC-derived virus between cells, Vero cells were cotransfected with the Δ16 BAC along with the UL16 expression plasmid pCI-16. This resulted in the formation of small foci of

fluorescent cells, which suggested that the UL16 protein present in a transfected cell could enable $\Delta 16$ BAC-derived virus produced in that cell to spread to adjacent cells. This unexpected finding indicated that UL16 is important for HSV-2 spread between cells.

To further explore the possibility that UL16 is critical for virus spread, we constructed a UL16-expressing L cell line, L16. L16 cells were used to reconstitute and propagate virus from the $\Delta 16$ BAC. Additionally, the *UL16* deletion in the $\Delta 16$ BAC was repaired and used to reconstitute the $\Delta 16$ virus ($\Delta 16R$ virus). Extracts were prepared from L and L16 cells infected with the WT, $\Delta 16$, and $\Delta 16R$ viruses and analyzed by Western blotting using polyclonal antibodies produced against HSV-2 UL16 (Fig. 1B). Analysis of mock-infected L and L16 cell extracts identified a band of approximately 41 kDa in L16 cells that was absent in the L cell lysate, confirming that L16 cells produce UL16. Additionally, analysis of $\Delta 16$ - and $\Delta 16R$ -infected L cell extracts confirmed that $\Delta 16$ failed to produce UL16 and that UL16 production was restored in $\Delta 16R$.

To determine if UL16 was important for plaque formation, the WT, $\Delta 16$, and $\Delta 16R$ viruses were used to infect monolayers of L and L16 cells, and plaques were allowed to form in semisolid medium. Three days after infection, monolayers were fixed and stained (Fig. 1C). All three strains formed plaques on L16 cells, whereas only the WT and $\Delta 16R$ strains formed plaques on L cells. These data indicate that L16 cells were able to complement the ability of $\Delta 16$ to form plaques and that UL16 was critical for the cell-to-cell spread of the virus.

To measure the extent of the $\Delta 16$ virus replication defect, a multistep replication analysis was performed. L and L16 cells were infected with HSV-2 $\Delta 16$ and $\Delta 16R$ at a multiplicity of infection (MOI) of 0.01 for 1 h to enable the attachment and penetration of the virus. Residual virus from the inoculum was inactivated by incubating the cells briefly in a low-pH citrate buffer. Cells and medium were harvested together at 0, 24, 32, 48, and 72 h postinfection (hpi), and virus was titrated on monolayers of L16 cells. The results showed that at 72 hpi, $\Delta 16$ grown on L cells had a 946-fold (± 37 -fold) growth defect compared to $\Delta 16R$ grown on L cells (Fig. 2A). Additionally, the growth of $\Delta 16$ on L16 cells promoted $\Delta 16$ replication by 41-fold (± 7 -fold) compared to its replication in L cells. As expected, the replication of $\Delta 16R$ was not further enhanced in L16 cells.

To ensure that the failure of $\Delta 16$ to propagate on L cells was not cell type specific, Vero cells were tested for their ability to support $\Delta 16$ replication (Fig. 2B). Vero cells were infected with HSV-2 $\Delta 16$ and $\Delta 16R$ at an MOI of 0.01. Cells and medium were harvested together at 0, 24, 48, and 72 hpi, and virus was titrated on monolayers of L16 cells. Whereas WT- and $\Delta 16R$ -infected Vero cells produced similar amounts of infectious virus, the $\Delta 16$ strain displayed a 230-fold (± 44 -fold) growth defect compared to $\Delta 16R$. These findings indicate that $\Delta 16$ displays replication defects in multiple cell lines.

Kinetics of HSV-2 $\Delta 16$ gene expression. To explore the replication defect of $\Delta 16$ more closely, we examined the expression kinetics of virus proteins belonging to different kinetic classes in noncomplementing Vero cells by confocal microscopy and Western blotting. Vero cells were infected with the $\Delta 16$, $\Delta 16R$, and WT viruses at a low MOI (MOI = 0.01) and fixed at 4, 6, and 8 hpi. The expression of the immediate early (IE) protein ICP0, the early (E) protein ICP8, and the late (L) protein gD was detected by using rat polyclonal antisera against ICP0 and mouse monoclonal antibodies against ICP8 and gD. ICP0 was detected in nuclear puncta at 4 hpi in $\Delta 16$ -, $\Delta 16R$ -, and WT-infected cells (Fig. 3). The localization of ICP8 to replication compartments was observed at 6 hpi in $\Delta 16$ -, $\Delta 16R$ -, and WT-infected cells, and the L gene product gD was localized to the Golgi compartment by 8 hpi. To quantify these results, infected cells in 10 randomly selected fields were evaluated under each condition. At 6 hpi, the percentages of infected cells that were double positive for ICP0 and ICP8 were 37%, 40%, and 56% for the $\Delta 16$, WT, and $\Delta 16R$ viruses, respectively. At 8 hpi, 84%, 85%, and 88% of infected cells were double positive for ICP0 and ICP8 for the $\Delta 16$, WT, and $\Delta 16R$ viruses, respectively. From 6 to 8 hpi, the proportions of infected cells positive for ICP0

FIG 2 Replication kinetics of $\Delta 16$. (A) L and L16 cells were infected with $\Delta 16$ and $\Delta 16R$ at an MOI of 0.01. At the indicated time points, cells and medium were harvested together and titrated on monolayers of L16 cells. (B) Vero cells were infected with $\Delta 16$, $\Delta 16R$, or the WT at an MOI of 0.01. At the indicated time points, cells and medium were harvested together and titrated on monolayers of L16 cells. Each data point represents the average of data from two biological replicates, each of which had three technical replicates. Error bars are standard errors of the means.

and gD increased from 36% to 73% for $\Delta 16$, from 52% to 69% for the WT, and from 55% to 69% for $\Delta 16R$, respectively. These experiments did not reveal any substantial deficiencies in the kinetics of $\Delta 16$ gene expression.

To complement the microscopy experiments described above, Western blot analysis of protein expression was performed by using antisera against the IE protein ICP27, the E protein Us3, and the L proteins UL21 and UL16 (Fig. 4). Vero cells were infected with WT HSV-2, $\Delta 16$, or $\Delta 16R$, and cell lysates were prepared for SDS-PAGE analysis at 4 and 8 hpi. Unlike the microscopy experiments shown in Fig. 3, which utilized low multiplicities of infection, these experiments were performed at an MOI of 3 to ensure that the proteins of interest could be readily detected. While modest reductions in the levels of ICP27, Us3, and UL21 were observed at 4 hpi in $\Delta 16$ -infected cell lysates compared to WT- and $\Delta 16R$ -infected cell lysates, this reduction was not apparent at 8 hpi. As expected, UL16 expression was not detected in cells infected with $\Delta 16$. These data, combined with the microscopy data described above (Fig. 3), suggest that defects in virus protein production cannot explain the substantial defect in virus propagation observed for $\Delta 16$.

Capsids assemble efficiently in $\Delta 16$ -infected cells. The data so far demonstrated that the $\Delta 16$ strain, while exhibiting severe replication defects, produced viral gene products of each kinetic class. These findings raised the possibility that the $\Delta 16$ strain was defective in some aspect of virus assembly. To begin our analysis of $\Delta 16$ assembly,

FIG 3 Kinetics of HSV-2 gene expression are unaffected by deletion of UL16. Shown are confocal micrographs of Vero cells infected with the WT, $\Delta 16$, and $\Delta 16R$ strains for 4, 6, and 8 h. Cells were stained for the IE protein ICP0, the E protein ICP8, and the L protein gD. Nuclei were visualized by staining cells with Hoechst 33342 (blue).

we prepared and analyzed capsids from nuclei of infected Vero cells. A, B, and C capsids were separated by ultracentrifugation on 20 to 50% continuous sucrose gradients (Fig. 5A). The intensities of the light-diffracting bands representing A, B, and C capsids were very similar between the WT and $\Delta 16$ preparations, suggesting that similar quantities of each type of capsid were produced by each strain. The composition of A, B, and C capsids prepared from WT- and $\Delta 16$ -infected cells was analyzed by SDS-PAGE and silver staining (Fig. 5B). Although the band intensities were slightly lower for capsids isolated from $\Delta 16$ -infected nuclei, no obvious differences in protein composition between the capsid preparations derived from the WT and $\Delta 16$ strains were observed.

UL16 is critical for nuclear egress of capsids. Given that capsid assembly appeared normal in $\Delta 16$ -infected cells, it was of interest to determine if the loss of UL16 impacted virion maturation. Inspired by the observations that the deletion of UL21 from HSV-2 resulted in a nuclear egress defect and that UL16 and UL21 have been

FIG 4 HSV-2 protein expression in $\Delta 16$ -infected cells. Vero cells were infected with the WT, $\Delta 16$, and $\Delta 16R$ strains for 4 or 8 h, and cell lysates were prepared and analyzed for ICP27 (IE), Us3 (E), UL21 (L), and UL16 contents by Western blotting. The HSV-2 UL21 protein is labile. The slower-migrating band represents full-length UL21, and the faster-migrating band is a degradation product. Migration positions of molecular mass standards (in kilodaltons) are indicated on the left.

FIG 5 Similar amounts of A, B, and C capsids are produced in WT HSV-2- and $\Delta 16$ -infected cells. (A) At 18 hpi, nuclear capsids were extracted from Vero cells infected with the WT and $\Delta 16$ strains, and A, B, and C capsids were separated by ultracentrifugation through 20% to 50% linear sucrose gradients as described in Materials and Methods. Images of ultracentrifuge tubes after capsid separation reveal A, B, and C capsid bands of similar intensities on both WT and $\Delta 16$ gradients. (B) After fractionation of the gradients, fractions containing A, B, and C capsids were run on 10% SDS-PAGE gels that were subsequently silver stained to visualize the protein. No obvious differences in capsid protein composition between capsids isolated from WT-infected cells and those isolated from $\Delta 16$ -infected cells were observed. Migration positions of the capsid shell proteins VP5 and VP23 are indicated, as is the migration position of the scaffold protein VP22a, not found in A or C capsids. Images representative of results from three independent experiments are shown in panels A and B.

reported to interact (8, 13, 24), we hypothesized that UL16 may also function in nuclear egress. This possibility was first examined by transmission electron microscopy (TEM). Vero cells were infected with $\Delta 16$ and $\Delta 16R$ at an MOI of 3.0. At 16 hpi, the cells were fixed and processed for TEM as described in Materials and Methods. Whereas cytoplasmic capsids (Fig. 6A, arrowhead) and enveloped virions (Fig. 6A, arrow) were seen in the cytoplasm of $\Delta 16R$ -infected Vero cells, these structures were not seen in the cytoplasm of $\Delta 16$ -infected cells (Fig. 6C). Consistent with the findings shown in Fig. 5A, nuclear capsids were readily detected in nuclei of both $\Delta 16R$ - and $\Delta 16$ -infected cells (Fig. 6B to D, arrowheads). These data suggested that like UL21, UL16 might also function in the nuclear egress of capsids.

To corroborate the electron microscopy data, fluorescence *in situ* hybridization (FISH) was performed to localize viral DNA in $\Delta 16$ -infected cells. Vero cells were infected with the WT, $\Delta 16$, and $\Delta 16R$ strains. At 25 hpi, cells were fixed and processed for FISH. Viral DNA in cells was hybridized to a biotinylated probe directed against the HSV-2 unique short region and visualized by using a streptavidin-Alexa Fluor 568 conjugate. Nuclei were identified by using Hoechst 33342 (Fig. 7A). In cells infected with the WT and $\Delta 16R$, an abundant fluorescence signal was detected in both the nucleus and cytoplasmic puncta that represent DNA-filled capsids/virions. In contrast, while a significant signal was found in the nuclei of $\Delta 16$ -infected cells, cytoplasmic puncta were observed much less frequently. To quantify the results, WT ($n = 18$), $\Delta 16$ ($n = 19$), and $\Delta 16R$ ($n = 18$)-infected cells were imaged in three dimensions, and the total numbers of viral DNA puncta in the cytoplasm were determined for each entire cell (Fig. 7B). The differences between the numbers of cytoplasmic puncta in WT-, $\Delta 16R$ -, and $\Delta 16$ -infected cells were highly significant ($P < 0.0001$). These findings suggest that C capsids are not exported efficiently from the nuclei of $\Delta 16$ -infected cells.

As an additional approach to measure capsid localization in infected cells, we constructed viruses with mCherry fused to the N terminus of the capsid protein VP26 (mCh-VP26) in the WT and $\Delta 16$ viruses and quantified the cytoplasmic localization of fluorescent capsids in infected cells. Vero cells were infected with the $\Delta 16$ /mCh-VP26 or WT/mCh-VP26 strain and were fixed and stained with Hoechst 33342 at 24 hpi to identify nuclei. In these experiments, cytoplasmic capsids appear as red puncta (Fig. 8A, arrowheads). Whereas cells infected with the WT/mCh-VP26 strain had many cytoplas-

FIG 6 $\Delta 16$ is defective in nuclear egress. Vero cells were infected with $\Delta 16R$ (A and B) or $\Delta 16$ (C and D) at an MOI of 3. At 16 hpi, cells were fixed and processed for TEM as described in Materials and Methods. Cytoplasmic capsids (arrowheads) and enveloped virions (arrow) can be seen in the cytoplasm (Cyt) of $\Delta 16R$ -infected Vero cells (A); these structures were not seen in the cytoplasm of $\Delta 16$ -infected cells (C). Nuclear capsids were readily detected in the nuclei (Nu) of both $\Delta 16R$ - and $\Delta 16$ -infected cells (arrowheads in panels B to D).

mic capsids, capsids were rarely seen in confocal sections of cells infected with $\Delta 16/mCh-VP26$. To quantify the data from these experiments, infected cells were imaged in three dimensions, and the numbers of viral capsids in the cytoplasm were counted for each entire cell (Fig. 8B). Most WT/ $mCh-VP26$ -infected cells ($n = 17$) had roughly 100 cytoplasmic capsids, while most $\Delta 16/mCh-VP26$ -infected cells ($n = 17$) had <10 . The differences between the numbers of capsids in WT/ $mCh-VP26$ - and $\Delta 16/mCh-VP26$ -infected cells were highly significant ($P < 0.0001$). Collectively, these findings indicate that UL16 is required for the efficient nuclear egress of HSV-2 capsids.

FIG 7 Less viral DNA localizes to the cytoplasm of $\Delta 16$ -infected cells. (A) Representative images of WT-, $\Delta 16$ -, and $\Delta 16R$ -infected cells subjected to FISH analysis using a probe corresponding to the unique short region of the HSV-2 genome. Vero cells were infected with the WT, $\Delta 16$, and $\Delta 16R$ strains. At 25 hpi, cells were fixed and processed for FISH as described in Materials and Methods. The red signal identifies viral DNA. Nuclei were visualized by staining cells with Hoechst 33342 (blue signal). Note the abundant red puncta in the cytoplasm of WT- and $\Delta 16R$ -infected cells that represent viral genomic DNA associated with cytoplasmic C capsids. DIC, differential interference contrast. (B) Quantification of cytoplasmic DNA puncta. WT ($n = 18$), $\Delta 16$ ($n = 19$), and $\Delta 16R$ ($n = 18$)-infected cells were imaged in three dimensions by acquiring a series of z images taken from the bottom to the top of each cell by confocal microscopy using a step size of $0.4 \mu\text{m}$, and the total numbers of viral DNA puncta in the cytoplasm were determined for each entire cell (***, $P < 0.0001$; ns, not significant). Horizontal bars represent the mean numbers of viral DNA puncta in each specimen.

DISCUSSION

The herpes simplex virus *UL16* gene is conserved throughout the *Herpesviridae* and encodes a poorly understood tegument protein. Here we report that UL16 from HSV-2 plays an important role in virus replication insofar as the $\Delta 16$ mutant had an ~ 200 - to 950-fold replication defect depending on the cell type infected (Fig. 2). We noted that the kinetics of virus gene expression were not perturbed by the deletion of UL16 when cells were infected at a low MOI (Fig. 3); however, the abundance of select IE, E, and L gene products was somewhat diminished at 4 hpi in cells infected at a high multiplicity and had normalized by 8 hpi (Fig. 4). It may be that UL16 either directly or indirectly promotes protein synthesis at early times postinfection; however, such a modest deficiency cannot account for the dramatic replication defect observed for $\Delta 16$. Most importantly, our data indicate that HSV-2 UL16 plays a critical role in the efficient egress of C capsids from the nuclei of infected cells. HSV-2 UL16 may also function at later stages of virion maturation in the cytoplasm; however, such a role could not be

FIG 8 Quantification of cytoplasmic capsids in $\Delta 16$ - and WT HSV-2-infected cells. (A) Representative images of WT/mCh-VP26- and $\Delta 16$ /mCh-VP26-infected cells. Vero cells were infected with the $\Delta 16$ /mCh-VP26 or WT/mCh-VP26 strain, and at 24 hpi, they were fixed and stained with Hoechst 33342 to identify nuclei (blue). Cytoplasmic capsids appear as red puncta (arrowheads). (B) Quantification of cytoplasmic capsids in infected cells. Infected cells were imaged in three dimensions, and the numbers of viral capsids in the cytoplasm were determined for each entire cell. Most WT/mCh-VP26-infected cells ($n = 17$) had roughly 100 cytoplasmic capsids, while most $\Delta 16$ /mCh-VP26-infected cells ($n = 17$) had <10 (***, $P < 0.0001$). Horizontal bars represent the mean numbers of capsids in each specimen.

assessed in our electron microscopy experiments (Fig. 6C and D), likely due to the severity of the $\Delta 16$ nuclear egress defect. Our quantitative analysis of cytoplasmic capsid localization (Fig. 8) indicated a 10- to 20-fold deficiency in the number of cytoplasmic capsids present in $\Delta 16$ -infected Vero cells, whereas virus replication analysis (Fig. 2) indicated a 200-fold deficiency in these cells. A logical explanation for this apparent discrepancy is that, similar to what has been reported for HSV-1, HSV-2 UL16 might also be expected to function in the secondary envelopment of cytoplasmic capsids. As HSV-1 UL16 mutants display a 10-fold reduction in virus production compared to parental strains, a similar requirement for HSV-2 UL16 in secondary envelopment downstream of its role in nuclear egress would be expected to result in the overall replication deficiencies observed.

While it is surprising that the requirements for UL16 by HSV-1 and HSV-2 appear to be so different, we hypothesized that this might be the case based on our previously reported findings that the UL16 binding partner UL21 was essential for HSV-2 replication because of a defect in nuclear egress (24). The requirement for both UL16 and UL21 in the nuclear egress of HSV-2 capsids is consistent with the idea that a complex containing both UL16 and UL21 functions in this process. The ability of HSV-1 to tolerate mutations in UL16 and UL21 may suggest that HSV-1 encodes a function lacking in HSV-2 that enables efficient nuclear egress. Importantly, future studies that make direct comparisons between HSV-1 and HSV-2 strains defective in UL16 and UL21 are required to conclusively establish if these proteins perform distinct functions for the different virus species.

How might the absence of UL16 impact nuclear egress? Similar numbers of A, B, and C capsids were recovered from the nuclei of $\Delta 16$ -infected cells (Fig. 5A). However, it is noteworthy that nuclear aggregates of B capsids were observed in some TEM micrographs of $\Delta 16$ -infected cells (see, e.g., Fig. 6D). Thus, we might have expected to observe an enrichment of B capsids in our capsid fractionation experiments. It may be

that capsids present in an aggregate are poorly solubilized and therefore are not resolved on sucrose gradients. Regardless of the possibility that B capsids are under-represented in our analysis, our findings suggest that the absence of UL16 did not significantly impair HSV-2 capsid assembly. Given that the nuclear egress of C capsids was clearly defective in cells infected with Δ 16 (Fig. 6 to 8), we might have also expected to see the accumulation of C capsids in the nuclei of Δ 16-infected cells relative to cells infected with the parental strain. This was not the case (Fig. 5A). These findings may suggest that UL16 participates in the packaging of viral DNA into capsids. Indeed, Oshima and colleagues suggested that UL16 might play a supplemental role in DNA packaging into capsids (22). It is also noteworthy that the deletion of UL21 from the NIA-3 strain of PRV resulted in defects in the cleavage and packaging of viral DNA into capsids (26). However, because substantial levels of C capsids are produced in Δ 16-infected cells, a possible role for UL16 in DNA packaging cannot fully explain the extent of the replication defect observed in its absence.

It is tempting to speculate that UL16 nuclear functions mirror those described for it in the cytoplasm, that is, the promotion of capsid envelopment. In order to transit from the nucleoplasm to the cytoplasm, C capsids must be recruited to the inner nuclear membrane (INM), where they undergo primary envelopment facilitated by the nuclear egress complex comprised of UL31 and UL34 (2). While capsids were evident in the nucleoplasm of Δ 16-infected cells (Fig. 6), accumulation of capsids at the INM was not observed, as has been seen for PRV strains lacking UL25, for example (30). These findings may suggest that UL16 is required for the recruitment of HSV-2 C capsids to the INM. While UL16 has been reported to be a C-capsid-associated tegument protein in both HSV-1 and HSV-2, studies on HSV-1 from the Wills laboratory suggest that UL16 is associated with cytoplasmic capsids but not nuclear capsids (18). An important caveat here is that weak UL16-capsid interactions may not have been able to withstand the conditions required to purify the nuclear capsids that were analyzed. Our examination of Δ 16 capsid composition failed to reveal any obvious differences between the WT and Δ 16 strains (Fig. 5B); however, a more comprehensive analysis of the Δ 16 capsid composition is warranted. For example, the capsid vertex-specific complex, comprised of UL17 and UL25, functions to recruit UL31 to the surface of C capsids, which in turn has been proposed to link the capsid to the INM via interactions with UL34 embedded in the INM (31, 32). Future experiments to determine if UL16 influences the recruitment of UL17, UL25, and UL31 to the capsid surface should help clarify a role for UL16 in this process.

Our understanding of the complexity of nuclear egress continues to expand as new molecules that participate in this critical process are identified. Several recent studies have cataloged additional viral and cellular components that influence the nuclear egress of HSV-1 capsids, including the viral proteins UL47, ICP22, and ICP34.5 and the cellular mitochondrial protein p32 (33–36). The interplay between these factors and UL21 and UL16 in HSV-2-infected cells is unexplored and can form the foundation of future investigations.

MATERIALS AND METHODS

Viruses and cells. HSV-2 mutants were derived from HSV-2 strain 186 BAC pYebac373 (24). Vero cells and 293T cells were acquired from the ATCC. The murine L fibroblast cell line was a kind gift from Frank Tufaro, University of British Columbia. Phoenix-AMPHO cells were generously provided by Craig McCormick, Dalhousie University. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). L16 cells that stably express HSV-2 UL16 were derived from L cells by retroviral transduction using an amphotropic Phoenix-Moloney murine leukemia virus (MMLV) system (37). 293T16 cells that stably express HSV-2 UL16 were derived from 293T cells and isolated in the same manner as that for L16 cells.

Plasmid construction. To construct L16 and 293T16 cells, UL16 sequences were amplified from the HSV-2 186 strain by PCR using forward primer 5'-GACTGAATTCATGCTGGCACAGCGGGCACTCTGG-3' and reverse primer 5'-GACTCTCGAGTTATTTGTAATCGGACGATGAGGCTCTGGCC-3'. The PCR product was digested with EcoRI and XhoI and ligated into similarly digested pBMN-IP (a kind gift of Craig McCormick, Dalhousie University) to yield pBMN-IP-UL16. pBMN-IP-UL16 was transfected into Phoenix-AMPHO cells to produce the retroviruses used to construct L16.

For producing polyclonal antisera against HSV-2 UL16, sequences containing UL16 codons 181 to 372 were amplified by PCR using primers 5'-AATGAATTCATGACCGACACCGACCGGAA-3' and 5'-GATCTC GAGTATTGTAACTCGCTGCTG-3' from a synthetic HSV-2 UL16 gene that had been codon optimized for expression in *Escherichia coli* (Bio Basic). The PCR products were digested with EcoRI and XhoI and inserted into the BamHI and XhoI restriction sites of a pET21a(+)-derived plasmid containing upstream DNA coding for a hexahistidine tag, the protein G B1 domain, and a tobacco etch virus (TEV) protease recognition sequence that was kindly provided by Steven Smith, Queen's University.

HSV-2 UL16 expression plasmid pCI-16 was constructed by amplifying the UL16 gene from HSV-2 strain 186 DNA using forward primer 5'-GACTGAATTCATGCTGGCACAGCGGGCACTCTGG-3' and reverse primer 5'-GACTGTGCACTTATTGTAATCGGACGATGAGGCTCTGGCC-3'. The PCR product was digested with EcoRI and SalI and ligated into similarly digested pCI-neo (Promega).

For producing the Δ 16/mCh-VP26 strain, the top-strand oligonucleotide 5'-CACCGCCGACAACG TCCGGGCGCT-3' was annealed to the bottom-strand oligonucleotide 5'-AAACAGCGCCCGACGTTG TCGGC-3', and the double-stranded product was cloned into guide RNA-Cas9 expression plasmid pX330-U6-Chimeric_BB-CBh-hSpCas9, a gift from Feng Zhang (Addgene plasmid 42230) (38), that had been digested with BbsI to produce pUL35gRNA1.

Protein expression and antiserum production. Recombinant UL16 fusion proteins were expressed in *E. coli* strain Rosetta(DE3). Bacteria were lysed, and inclusion bodies were purified by using the B-Per protein purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Proteins in inclusion bodies were separated on preparative SDS-PAGE gels, and the bands corresponding to the UL16 fusion proteins were excised and sent to Cedarlane Laboratories to immunize chickens for polyclonal antibody production.

Antibodies. Chicken polyclonal antiserum against UL16 was used for Western blotting at a dilution of 1:200, rat polyclonal antiserum against HSV-2 ICP0 (24) was used for indirect immunofluorescence microscopy at a dilution of 1:200, mouse monoclonal antibody against HSV-2 ICP8 (Virusys) was used for indirect immunofluorescence microscopy at a dilution of 1:10,000 and for Western blotting at a dilution of 1:16,000, rat polyclonal antiserum against HSV Us3 (39) was used for Western blotting at a dilution of 1:500, mouse monoclonal antibody against HSV gD (Virusys) was used for indirect immunofluorescence microscopy at a dilution of 1:1,000, rat polyclonal antiserum against UL21 (24) was used for Western blotting at a dilution of 1:3,000, and mouse monoclonal antibody against beta actin (Sigma) was used for Western blotting at a dilution of 1:2,000. Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 568-conjugated goat anti-rat IgG (Invitrogen-Molecular Probes) were used at a dilution of 1:500 for indirect immunofluorescence microscopy. Horseradish peroxidase-conjugated goat anti-mouse IgG, horseradish peroxidase-conjugated goat anti-chicken IgY, and horseradish peroxidase-conjugated rabbit anti-rat IgG (Sigma) were used for Western blotting at dilutions of 1:10,000, 1:30,000, and 1:80,000, respectively.

Construction of recombinant HSV-2 strains. The full-length infectious HSV-2 186 BAC pYebac373 was constructed as described previously (24). The HSV-2 mutant with a knockout of the UL16 gene (Δ 16 BAC) was constructed by the two-step Red-mediated mutagenesis procedure using pYebac373 in *E. coli* GS1783 (24, 40). Primers 5'-CCTCTGCTTTTGGTGCGTCTCCGGTCCCTTCCCCACCACCACTACCAGCGCCGG GTGGAGGATGACGACGATAAGTAGGG-3' and 5'-GCTGGTATACGATGACAGAACGACAGAGGCCACCCGCGC GCTGGTAGGTAGGTTGGTGGGGCAACCAATTAACCAATTCTGATTAG-3' were used to amplify a PCR product from pEP-Kan-S2, a kind gift for Klaus Osterrieder, Freie Universität Berlin, and used to remove most of the UL16 coding sequence, leaving only the last 32 codons. To repair HSV-2 Δ 16, the I-SceI-flanked kanamycin cassette portion of pEP-Kan-S2 was amplified by using primers 5'-GATCGGCG CGCCCAAGGATGACGACGATAAGTAGGG-3' and 5'-GATCGGCGCGCCACAGCGCGTTGGCGGAATCGATGTGG GCCGTGCGGGCGCAGGCTCGAGCGGCCCGCAGTGTGATGG-3' designed to introduce *Ascl* sites (italicized) and the additional UL16-derived sequence (underlined) to facilitate recombination. The PCR product was then introduced into the unique *Ascl* site within the UL16 gene of pCI-16 to generate pTH123. Primers 5'-GGCCCTCTGCTTTTGGTGCGTCTCCGGTCCCTTCCCCACCACCAATGCGACAGCGGGCACTCTGGCGT CCC-3' and 5'-TTATTGTAATCGGACGATGAGGC-3' were used to amplify a PCR product from pTH123. This PCR product was then used for the two-step Red-mediated mutagenesis procedure of pYebac373- Δ 16 to restore the complete UL16 gene (pYebac373- Δ 16R).

Restriction fragment length polymorphism analysis was used to confirm the integrity of each recombinant BAC clone compared to the WT BAC by digestion with EcoRI. Additionally, a PCR fragment that spanned the mutated or repaired region of interest was amplified and sequenced to confirm that the anticipated sequences were present. Virus lacking BAC sequences was reconstituted from pYebac373- Δ 16 and pYebac373- Δ 16R DNA as described previously (24).

mCherry was fused to the N terminus of the capsid protein VP26 in the Δ 16 strain by using clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9-based mutagenesis as described previously for the manipulation of HSV-1 genomes (41). Using WT/mCh-VP26 viral DNA (24) as a template, a PCR product containing 582 nucleotides upstream of mCherry, mCherry-UL35, and 521 nucleotides downstream of the UL35 stop codon was amplified by using forward primer 5'-GATCGAATTCGAGCAGACCA ACGTGATTCTGC-3' and reverse primer 5'-GATCGAATTCGCTTCAGACGCTGCACATCTCC-3'. Genomic DNA prepared from Δ 16 was cotransfected into 293T16 cells stably expressing HSV-2 UL16 along with the PCR product, as described above, and the Cas9 and guide RNA expression plasmid pUL35gRNA1, which mediates the cleavage of the UL35 gene at codon 18. At 120 h posttransfection, cells and medium were harvested, frozen at -80°C , thawed, and sonicated with 10 1-s pulses in a cup-horn sonicator. Serial 10-fold dilutions of this preparation were used to inoculate monolayers of L16 cells growing in 6-well cluster dishes, and plaques were allowed to form in semisolid medium for 48 h. Plaques were screened

for red fluorescent puncta with the aid of a Nikon TE200 inverted epifluorescence microscope. Red fluorescent plaques were picked, and their associated viruses were purified to homogeneity by two rounds of plaque purification. Sequences upstream, downstream, and within the *UL35* locus were determined from isolate $\Delta 16/\text{mCh-VP26}$ to verify that the expected recombination events had taken place.

Indirect immunofluorescence microscopy. Vero cells growing on 25-mm glass coverslips were infected, washed three times at the indicated times postinfection with phosphate-buffered saline (PBS), and then fixed in 4% paraformaldehyde-PBS for 10 min at room temperature. Fixed cells were washed with PBS containing 1% bovine serum albumin (BSA) (PBS-BSA) and permeabilized with PBS-BSA containing 0.1% Triton X-100 for 2 min. Cells were again washed three times in PBS-BSA and primary antiserum, diluted in PBS-BSA, was applied for 45 min at room temperature. Cells were washed with PBS-BSA, and the appropriate Alexa Fluor-conjugated secondary antibody diluted in PBS-BSA was applied for 30 min. Cells were then washed with PBS-BSA. Nuclei were visualized by incubating cells with Hoechst 33342 (Sigma) diluted to 0.5 $\mu\text{g}/\text{ml}$ in PBS. Images were captured by using an Olympus FV1000 confocal laser scanning microscope and FV10 ASW 4.01 software through a 60 $\times$, 1.42-numerical-aperture (NA) oil immersion objective and a digital zoom factor of 4. Composites are representative images that were assembled by using Adobe Photoshop CC.

Capsid preparation. Capsid purification was performed similarly to what was described previously (31). Five 150-mm dishes containing confluent Vero cell monolayers were infected with the WT or $\Delta 16$ virus at an MOI of 10 PFU/cell. At 18 hpi, cells were collected by scraping and pelleted at $1,000 \times g$ for 10 min at 4°C. The supernatants were discarded, and cells were resuspended in 50 ml of cold PBS and pelleted at $1,000 \times g$ for 10 min at 4°C. Cells were lysed on ice for 30 min in 50 ml of cold NP-40 lysis buffer (150 mM NaCl, 10 mM Tris [pH 7.2], 2 mM MgCl_2 , 1% NP-40) containing 5 mM dithiothreitol and protease inhibitors (Roche). Nuclei were collected by centrifugation at $1,000 \times g$ for 10 min at 4°C, resuspended in 3 ml NP-40 lysis buffer, and sonicated with three 1-min pulses in a cup-horn sonicator. Disrupted nuclei were successively passed through 18-, 21-, and 25-gauge syringe needles, 100 U of Benzonase nuclease (Santa Cruz Biotechnology) was added, and the nuclear lysate was incubated at 37°C for 15 min. The nuclear lysate was clarified by centrifugation at $3,000 \times g$ for 10 min at 4°C, and 500 μl of the clarified nuclear lysate was layered onto a 2.5-ml 35% (wt/vol) sucrose cushion prepared in TNE (500 mM NaCl, 20 mM Tris [pH 7.6], 1 mM EDTA) and centrifuged at $38,000 \times g$ for 45 min at 4°C in a Beckman TLA100.3 rotor. Pellets containing nucleocapsids were resuspended in 100 μl of TNE and sonicated briefly, and capsids were layered onto 10-ml 20% to 50% (wt/vol) linear sucrose gradients and centrifuged in a Beckman SW41 rotor at $77,000 \times g$ for 1 h. After centrifugation, A, B, and C capsids were observed as distinct light-scattering bands. Gradients were fractionated from the top of the tube, 1-ml fractions were mixed with 1 ml TNE, and capsids were pelleted by centrifugation at $32,000 \times g$ for 30 min at 4°C in a Beckman TLA100.2 rotor. Pellets were resuspended in 35 μl of 1 $\times$ SDS-PAGE loading buffer, boiled for 5 min, and analyzed by SDS-PAGE and silver staining.

Fluorescence *in situ* hybridization. FISH was performed by using methods similar to those reported previously (42). Briefly, Vero cells plated onto 35-mm glass-bottom dishes (MatTek) were infected with the WT, $\Delta 16$, or $\Delta 16\text{R}$ strain at an MOI of 0.1 and fixed by using cold (4°C) 95% ethanol–5% acetic acid at 25 hpi. A biotinylated probe against the HSV-2 unique short region was produced by using a Biotin DecaLabel DNA labeling kit (Thermo Fisher Scientific). Cells were prehybridized in 1 ml of hybridization buffer (50% formamide, 10% dextran sulfate, 4 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) at 37°C for 30 min. The probe was added to a final concentration of 1 ng/ μl , and the cells were heated to 95°C for 2 min to denature the probe and target DNA. Hybridization was performed in a humidified 37°C chamber overnight. The next day, cells were washed with 2 $\times$ SSC at 60°C for 5 min, followed by washing with 2 $\times$ SSC at room temperature. After a further wash with PBS–1% fetal bovine serum (FBS), cells were incubated with Alexa Fluor 568-conjugated streptavidin (Invitrogen-Molecular Probes) diluted 1:3,000 in PBS–1% FBS. Nuclei were visualized by incubating cells with Hoechst 33342 (Sigma) diluted to 0.5 $\mu\text{g}/\text{ml}$ in PBS. Images were captured by using an Olympus FV1000 confocal laser scanning microscope and FV10 ASW 4.01 software through a 60 $\times$, 1.42-NA oil immersion objective and a digital zoom factor of 4. Composites are representative images that were assembled by using Adobe Photoshop CC. To quantify the results, *x*, *y*, and *z* series of cells were acquired with a *z* step size of 0.5 μm by using an Olympus FV1000 confocal microscope equipped with a 60 $\times$, 1.42-NA oil immersion objective. Three-dimensional reconstructions were used for the quantification of cytoplasmic viral DNA puncta in entire cells.

Electron microscopy. Vero cells growing on Lab-Tek Permanox chamber slides (Sigma) were infected with HSV-2 $\Delta 16$ and $\Delta 16\text{R}$ at an MOI of 5 to ensure that all of the cells were infected and were readily identified under an electron microscope. Cells infected at this high MOI suffer cytopathic effects of infection more acutely than do cells infected at the lower MOIs and therefore were processed for electron microscopy at 16 hpi to ensure that cellular integrity was maintained. Infected cells were washed twice briefly in 0.1 M sodium cacodylate (pH 7.4), fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 30 min at room temperature, and then transferred to 4°C overnight. The following day, cells were washed three times in 0.1 M sodium cacodylate (pH 7.4), postfixed in 1% osmium tetroxide–1.5% potassium ferrocyanide, dehydrated through a series of increasing ethanol concentrations, and embedded in Epon. Thin sections were prepared, and images were collected by using an FEI Osiris S/TEM instrument at the Queen's University Reactor Materials Testing Laboratory.

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