Mechanism of Nuclear Lamina Disruption and the Role of pUS3 in Herpes Simplex Virus 1 Nuclear Egress

Masoudeh Masoud Bahnamiri,^a Richard J. Roller^a

^aDepartment of Microbiology and Immunology, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

ABSTRACT Herpes simplex virus (HSV) capsid envelopment at the nuclear membrane is coordinated by nuclear egress complex (NEC) proteins pUL34 and pUL31 and is accompanied by alteration of the nuclear architecture and local disruption of the nuclear lamina. Here, we examined the role of capsid envelopment in the changes of the nuclear architecture by characterizing HSV-1 recombinants that do not form capsids. Typical changes in nuclear architecture and disruption of the lamina were observed in the absence of capsids, suggesting that disruption of the nuclear lamina occurs prior to capsid envelopment. Surprisingly, in the absence of capsid envelopment, lamin A/C becomes concentrated at the nuclear envelope in a pUL34-independent and cell type-specific manner, suggesting that ongoing nuclear egress may be required for the dispersal of lamins observed in wild-type virus infection. Mutation of the virus-encoded protein kinase pUS3 on a wild-type virus background has been shown to cause accumulation of perinuclear enveloped capsids, formation of NEC aggregates, and exacerbated lamina disruption. We observed that mutation of US3 in the absence of capsids results in identical NEC aggregation and lamina disruption phenotypes, suggesting that they do not result from accumulation of perinuclear virions. Transmission electron microscopy analysis revealed that, in the absence of capsids, NEC aggregates correspond to multi-folded nuclear membrane structures, suggesting that pUS3 may control NEC self-association and membrane deformation. To determine the significance of the pUS3 nuclear egress function for virus growth, the replication of single and double UL34 and US3 mutants was measured, showing that the significance of pUS3 nuclear egress function is cell type specific.

IMPORTANCE The nuclear lamina is an important player in infection by viruses that replicate in the nucleus. Herpesviruses alter the structure of the nuclear lamina to facilitate transport of capsids from the nucleus to the cytoplasm and use both viral and cellular effectors to disrupt the protein-protein interactions that maintain the lamina. Here, we explore the role of capsid envelopment and the virus-encoded protein kinase pUS3 in disruption of the lamina structure. We show that capsid envelopment is not necessary for the lamina disruption or for US3 mutant phenotypes, including exaggerated lamina disruption, that accompany nuclear egress. These results clarify the mechanisms behind alteration of the nuclear lamina structure and support a function for pUS3 in regulating the aggregation state of the nuclear egress machinery.

KEYWORDS US3, herpes simplex virus, nuclear egress, nuclear lamina, protein kinases, virus assembly

Herpesviruses, including herpes simplex virus 1 (HSV-1), express and replicate their genomes in the nucleus of the infected cell (1). The major capsid protein, called VP5, is essential in capsid assembly inside the nucleus (2). Capsids are also assembled and filled with DNA in the nucleus, but assembly of the mature virion takes place by envelopment into cytoplasmic membranes, necessitating movement of the nucleocapsid across the two membranes of the nuclear envelope. This is accomplished by a

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Address correspondence to Richard J. Roller, richard-roller@uiowa.edu.

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process of sequential envelopment at the inner nuclear membrane (INM) and deenvelopment at the outer nuclear membrane (ONM) called nuclear egress (reviewed in reference 3). Nuclear egress is mediated by two conserved viral proteins, pUL31 and pUL34, that form a heterodimer called the nuclear egress complex (NEC), which is concentrated on the INM (4–15). Nucleocapsids accumulate inside the nucleus of either pUL31-null or pUL34-null virus-infected cells, and enveloped virions are rarely detectable in the perinuclear space (6, 16–19).

Envelopment of capsids at the INM requires that capsids have access to dock at the INM and that there be a sufficient area of freely deformable membrane to enclose the capsid (20). The nuclear lamina presents an obstacle to meeting both of these requirements. The nuclear lamina is a filamentous meshwork of four type V intermediate filament proteins called nuclear lamins (reviewed in references 21 and 22). The A-type lamins (lamins A and C) and the B-type lamins (lamins B1 and B2) each form filamentous, cross-linked networks that overlap each other (23). The lamin meshworks are tethered to the INM by association with integral membrane lamina-associated proteins (LAPs), including emerin and lamin B receptor (LBR) (reviewed in references 24 to 26). The lamina is multifunctional; it provides both structural support and rigidity for the nuclear membrane and plays a critical role in regulation of gene expression (27). While the lamina meshwork does have some gaps large enough to provide capsid access to the INM, most gaps are smaller than the diameter of a capsid, and thus the lamina likely inhibits capsid docking (23). Also, the connection between the lamina meshwork and the INM by association with LAPs inhibits the free deformation and curvature of the INM that are necessary for capsid envelopment.

To achieve efficient viral replication, herpesviruses recruit cellular and viral protein kinases to the nuclear periphery upon infection, to disrupt the lamina by phosphorylation of lamins and LAPs (10, 28–35). HSV-1 infection has been shown to result in (i) phosphorylation of lamin A/C and emerin, (ii) exposure of lamin epitopes, and (iii) disconnection of lamins and LAPs, as shown by increased mobility of emerin and LBR (28, 36–38). One result of HSV-1 infection-mediated lamina disruption is deformation and wrinkling of the nuclear envelope, including bleb formation on the nuclear surface (28, 34, 39, 40).

One interesting feature of HSV-1 infection-mediated lamina disruption is that it does not result in wholesale dissolution of the nuclear lamina. Instead, lamins are redistributed in such a way that small gaps and limited areas of thinning and thickening of the lamin layer appear, suggesting that disconnection of lamin subunits from the network is local rather than global (28, 34, 40). One hypothesis that accounts for nuclear lamina local disruption is that it is the result of capsid envelopment. Recruitment of kinases might be triggered by the approach or docking of capsids at the INM, resulting in observable changes in nuclear architecture, including redistribution of lamins and changes in nuclear shape, as a net result of many capsid envelopment events. Additionally, the reduction in INM surface area and the consequent increase in ONM surface due to envelopment/deenvelopment might cause nuclear envelope convolution, resulting in reorganization of the lamina. It was shown previously that ectopic coexpression of HSV-1 pUL31 and pUL34 caused mislocalization of LAP2 and resulted in diminished levels and some redistribution of lamin A/C, suggesting that some changes might be envelopment independent (40). These effects seen in transfected cells differed from what was observed in infected cells, however, leaving the question of whether the NEC has envelopment-independent effects in infected cells unanswered.

The changes in lamina structure and nuclear shape so far reported have been shown to be dependent on expression of the NEC (11, 40), suggesting that pUL31 and pUL34 recruit the necessary kinases, and in fact the HSV-1 NEC has been shown to recruit protein kinase C isoforms, both directly and indirectly (30, 33, 35). HSV also encodes a protein kinase pUS3 that has been shown to mediate phosphorylation of both lamins and emerin (28, 36, 38, 41). Paradoxically, however, deletion of pUS3 or mutation that disrupts its catalytic activity results in an exacerbated lamina disruption phenotype in which large gaps form in the lamin network (28). pUS3 has additional specific functions in nuclear egress, including promotion of nucleocapsid deenvelopment

FIG 1 Schematic diagrams and expression analysis of VP5-null recombinant virus. (A) Schematic diagram of the WT HSV-1(F) BAC-derived genome (line 1) and of the VP5-null recombinant virus construct (line 2). Line 1 shows the expanded region of the UL19 locus with its neighboring genes (UL18 and UL20) with respect to the unique long (UL) and unique short (US) sequences, which are flanked by inverted repeats (e.g., TRL plus IRL and IRS plus TRS) in the WT virus. Line 2 shows the VP5-null virus, which has complete deletion of the coding sequence for the UL19 gene. (B and C) Digital images of immunoblots for the indicated proteins in lysates of VP5-complementing cells (B) or Vero cells (C) infected for 18 h with 5 PFU/cell of BAC-derived WT HSV-1(F) and VP5-null viruses. In panel B, ICP27 serves as a marker of equivalent infection and actin as a loading control. In panel C, pUL34 serves as a marker of equivalent infection and loading.

at the ONM and regulation of NEC localization, that are mediated by phosphorylation of pUL31 (28, 41–46). In nuclei of cells infected with either US3 mutants or mutants with mutations of UL31 that alter the US3 phosphorylation sites, perinuclear enveloped virions (PEVs) accumulate in areas of dilation of the perinuclear space, and the NEC mislocalizes in large puncta on the nuclear envelope. The NEC puncta are thought to correspond to the accumulations of PEVs in the perinuclear space, since the NEC is highly concentrated in the envelope of the PEV (47). Interestingly, in US3 mutant-infected cells, the NEC puncta are localized within the large gaps that form in the lamina, suggesting a mechanistic connection between these two phenotypes (28). One economical hypothesis to account for all of these US3 mutation phenotypes is that inhibition of capsid deenvelopment results in accumulation of PEVs, which, in turn, results in displacement of the lamin network by expansion of the dilated perinuclear space. This hypothesis predicts that formation of NEC puncta and exacerbated lamina disruption both depend on ongoing capsid envelopment.

Here, we use recombinant mutant viruses lacking the gene for the major capsid protein, VP5, to determine the role of capsid envelopment in the disruption of nuclear architecture on both wild-type (WT) and US3 mutant backgrounds. We show that the changes in nuclear shape and A-type lamin distribution that occur in WT virus infection arise even when capsids are absent, suggesting that these changes take place not as a consequence of capsid envelopment but rather as preparation for it. We also make the surprising observation that US3 mutant phenotypes do not depend on the presence of capsids, and we observe that mutation of US3 in the absence of capsids results in the formation of folded nuclear membrane structures.

RESULTS

Capsid envelopment is not necessary for disruption of nuclear architecture. In order to determine whether capsid envelopment is necessary to disrupt nuclear envelope architecture and the distribution of lamin A/C, we eliminated capsid envelopment by deletion of the entire coding sequence of the UL19 gene (Fig. 1A). The UL19 locus codes for the major capsid protein (VP5), which is essential in capsid assembly (2). The

FIG 2 Capsid envelopment is not necessary for disruption of nuclear architecture and lamin A/C localization. (A to D, F to I, and K to N) Representative digital confocal images of uninfected and infected cells. Vero cells (A to D), HaCaT cells (F to I), or SH-SY5Y cells (K to N) were either left uninfected (A, F, and K) or infected with the UL34-null (B, G, and L), HSV-1 (C, H, and M), or VP5-null (D, I, and N) virus. Cells were fixed at 16 hpi (Vero and HaCaT cells) or 9 hpi (SH-SY5Y cells) and were stained using antibodies directed against lamin A/C (green) and pUL34 (red). Representative images of one of at least three independent experiments are shown. Scale bars = 5 μ m. (E, J, and O) Nuclear contour ratios for lamin A/C on Vero (E), HaCaT (J), and SH-SY5Y (O) cells. The ratios of the area and the perimeter of 50 nuclei were used to calculate the nuclear contour ratio for each condition in each cell type. Statistical significance was determined by ANOVA using the Tukey method for multiple comparisons. n.s., not significant; *, $P \leq 0.05$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

VP5-null virus was propagated on cells that express VP5 in *trans* under the control of its own promoter/regulatory sequences. These complementing cells expressed VP5 in response to infection at levels comparable to those seen in WT virus-infected cells (Fig. 1B). As expected, the VP5-null recombinant expressed no detectable VP5 in infected noncomplementing cells (Fig. 1C).

The VP5-null virus was used to infect Vero cells to observe lamin A/C localization and nuclear membrane deformation via confocal microscopy (Fig. 2A to D). Consistent with previous reports, both mock-infected and UL34-null virus-infected cells showed an even distribution of lamin A/C in the nuclear envelope and an oval nuclear shape (Fig. 2A and B). Interestingly, similar disruption of the nuclear lamin A/C network occurred in WT virus-infected cells (Fig. 2C) and VP5-null virus-infected cells (Fig. 2D), as evidenced by thickening and thinning of the lamin A/C layer, and the same nuclear envelope deformation was observed, suggesting that capsid envelopment is not necessary to disrupt nuclear architecture and lamina meshwork. Quantitation of the degree of nuclear shape distortion using nuclear contour ratios (20) showed no significant differences between WT and VP5-null viruses (Fig. 2E).

FIG 3 Cell type-specific recruitment of lamin A/C to the nuclear envelope in HSV-1 infection. (A to D) Representative digital confocal images of lamin A/C localization in the nuclei of HaCaT cells infected with the indicated viruses. Scale bars = 5 μ m. For each nucleus, a cross-section line was drawn (examples are shown in each panel) for determination of fluorescence intensity. (E) Fluorescence intensity of lamin A/C through the cross section in HaCaT cells. Each point is an average of the intensity at that distance from the edge of the nucleus for 20 cell nuclei. (F) Graph of the ratio of fluorescence intensity at the edge of the nucleus to fluorescence intensity in the middle of the nucleus in HaCaT cells. Each point represents one cell. (G) Same as in panel F but determined in SH-SY5Y cells. Statistical significance was determined by one-way ANOVA using the Tukey method for multiple comparisons. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Each graph shows one of at least three independent experiments.

Since Vero cells do not always accurately reflect the behavior of human cells that are important for virus pathogenesis, a human keratinocyte cell line (HaCaT) and a human neuronal cell line (SH-SY5Y) were also examined (Fig. 2F to O). In both cell types, disruption of lamin A/C and distortion of the nuclear contour occurred to the same degree in VP5-null virus-infected cells (Fig. 2I and N) and WT virus-infected cells (Fig. 2H and M). In contrast to UL34-null virus-infected Vero cells, UL34-null virus-infected HaCaT and SH-SY5Y cells both showed some degree of nuclear distortion (Fig. 2G and L), compared to mock-infected cells (Fig. 2F and K), even though the degree of distortion was significantly less than that seen in WT and VP5-null virus-infected cells (Fig. 2H, I, M, and N). This is surprising, because previous studies suggested that disruption of the nuclear lamina and deformation of the nuclear envelope are UL34 dependent (28). Our observations suggest that there is a cell type-dependent, UL34-independent pathway for nuclear architecture change and disruption of the lamina in HSV-1-infected cells.

Lamin A/C concentrated at the nuclear envelope in HSV-1 infection in a cell type-specific manner. Although HSV-1 has been reported to disrupt and to disperse lamins during infection, we observed enhanced concentration of lamin A/C to the nuclear rim in infected HaCaT and SH-SY5Y cells but not Vero cells (Fig. 2B to N). This effect was most pronounced in UL34-null and VP5-null virus-infected nuclei (Fig. 3A to D). To quantify this observation, we measured and plotted the intensity of lamin A/C fluorescence along a cross-sectional profile of infected cell nuclei (Fig. 3E). The relative intensity of staining at the edges of the nucleus, compared to that in the middle, was greater for UL34-null and VP5-null virus-infected cells than for either uninfected cells

FIG 4 Lamin A/C recruitment to the nuclear membrane occurs independent of HSV-1 DNA replication. (A) Digital images of immunoblots for the indicated proteins in lysates of HaCaT cells infected for 18 h with 5 PFU/cell of BAC-derived WT HSV-1(F). ICP27 serves as a marker of both equivalent infection and immediate early gene control, and actin serves as a loading control. pUL34 serves as a marker of early late gene control. (B to F) Representative digital confocal images of uninfected and infected HaCaT cells. Cells were either left untreated (C) or treated with PAA (B and D to F). Cells were fixed at 16 hpi and stained using antibodies directed against lamin A/C. Representative images of one of at least three independent experiments are shown. HSV-1(F) not treated with PAA serves as a control to assay the efficiency of the PAA treatment. The PAA concentration was 0.3 μ g/ml. Scale bars = 5 μ m. (G) Graph of the ratio of fluorescence intensity at the edge of the nucleus to fluorescence intensity in the middle of the nucleus in HaCaT cells. Each point represents one cell. Statistical significance was determined by one-way ANOVA using the Tukey method for multiple comparisons. n.s., not significant; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. Each graph shows one of at least three independent experiments.

or WT virus-infected cells. The ratio of lamin A/C staining at the edge of the nucleus to that in the middle for 20 nuclei under each condition was significantly higher in UL34-null and VP5-null virus-infected cells than in uninfected or WT virus-infected cells in both HaCaT (Fig. 3F) and SH-SY5Y (Fig. 3G) backgrounds. This result suggests that there is a UL34-independent mechanism for reorganization of lamin A/C in HaCaT and SH-SY5Y cells and that this effect is most clearly seen in infected cells that are not undergoing active capsid nuclear egress.

Lamin A/C recruitment to the nuclear membrane is not dependent on HSV-1 DNA replication. HSV-1 assembly and egress proteins are often late gene products; therefore, we hypothesized that the viral function that causes UL34-independent redistribution of lamin A/C in HaCaT and SH-SY5Y cells also involves a late gene product. To test this hypothesis, DNA replication and subsequent late gene expression were inhibited by treatment with the HSV-1 DNA polymerase inhibitor phosphonoacetic acid (PAA). As expected, infected HaCaT cells that were treated with PAA showed no signal for the late protein US11, while untreated cells showed a strong US11 band (Fig. 4A), indicating that PAA treatment effectively inhibited true late gene expression. Interestingly, lamin A/C redistribution to the nuclear rim in UL34-null virus-infected cells occurred to similar extents in PAA-treated and untreated cells (compare Fig. 3B and Fig. 4E), suggesting that it does not require normal levels of late gene expression. Consistently, quantification analysis of lamin A/C localization to the nuclear envelope of WT HSV-1-infected, PAA-treated nuclei demonstrated similar lamin A/C redistribution patterns, compared with UL34-null and

FIG 5 Schematic diagrams and expression analysis of VP5-null/US3_{K220A} recombinant virus. (A) Schematic diagrams of the WT HSV-1 BAC-derived genome (line 1) and of the VP5-null/US3_{K220A} recombinant virus construct (line 2). Line 2 shows the VP5-null/US3_{K220A} virus, which has complete deletion of the coding sequence for the UL19 gene and a single amino acid substitution of the US3 invariant lysine to alanine. (B) Digital images of immunoblots for the indicated proteins in lysates of Vero cells that had been infected for 18 h with 5 PFU/cell of BAC-derived WT HSV-1(F) and VP5-null/US3_{K220A} viruses. Note that the mock-infected cells contain a background band that comigrates with pUS3. In panel B, pUL34 serves as a marker of equivalent infection and loading.

VP5-null virus-infected cells (Fig. 3B, 3D, 4E, and 4F). While we cannot exclude a role for low-level leaky late gene expression, overall our data suggest that cell type-specific redistribution of lamin A/C in HSV-1-infected cells occurs before the nuclear egress step, as a result of either immediate early or early gene function(s).

Formation of NEC puncta and enhanced lamina disruption do not depend on capsid envelopment. Deletion or elimination of the catalytic activity of the US3 protein has been shown to cause several dramatic changes in the nuclear egress process, including (i) accumulation of PEVs in dilations of the nuclear envelope, (ii) redistribution of the NEC into punctate structures in the nuclear envelope, and (iii) formation of large gaps in the lamin A/C network that correspond to the sites of NEC puncta (28). As noted earlier, one economical way to account for all three of these changes is to hypothesize that all of them result in inhibition of capsid deenvelopment at the ONM. According to this hypothesis, the accumulations of PEVs that result from inefficient deenvelopment correspond to the NEC puncta, and the expansion of the nuclear envelope dilations that contain these virions physically displaces the lamins. This hypothesis predicts that these effects of US3 mutation are all dependent on ongoing capsid envelopment to generate the accumulations of enveloped virions. To test this prediction, we made two independently constructed and isolated VP5-null/US3_{K220A} recombinant viruses (Fig. 5A). As expected, the VP5-null/US3_{K220A} recombinant expressed normal amounts of pUS3 but no detectable VP5 in infected noncomplementing cells (Fig. 5B). In these experiments, anti-pUS3 antibody reproducibly reacted with a protein in uninfected cells that comigrates with pUS3. We also regularly observe the same signal from cells infected with US3-null virus (data not shown). Infected cells, however, reproducibly demonstrated a much stronger signal at this position.

The VP5-null/US3_{K220A} viruses were used to infect Vero, HaCaT, and SH-SY5Y cells at a multiplicity of infection (MOI) of 5, and cells were fixed at 16 h, immunostained for lamin A/C and UL34, and observed by confocal microscopy (Fig. 6). Consistent with previous reports (28), US3_{K220A} virus-infected cells of all three types showed pUL34 localization in punctate structures and large holes in the lamin A/C meshwork, giving a “cobweb” appearance in the nucleus. Interestingly, the same phenotypes were

FIG 6 NEC aggregates in punctate structure in US3 catalytically dead infected cells in the absence of capsid. Shown are digital confocal images representing the localization of UL34 and lamin A/C in infected Vero cells (A to D), HaCaT cells (E to H), or SH-SY5Y cells (I to L). Green indicates pUL34, and red indicates lamin A/C. Cells were infected with HSV-1(F) (A, E, and I), US3_{K220A} (B, F, and J), or VP5-null/US3_{K220A} (C, D, G, H, K, and L) for 16 h at an MOI of 5. Cells were stained for UL34 (A to F) and lamin A/C (A to C), fixed at 16 hpi, and stained using antibodies directed against lamin A/C and pUL34. Representative images of one of at least three independent experiments are shown. Scale bars = 5 μ m.

observed in cells infected with both isolates of the VP5-null/US3_{K220A} double mutant virus (Fig. 6C, D, G, H, K, and L), demonstrating that ongoing capsid envelopment is not required for the appearance of characteristic US3 mutant phenotypes and suggesting that NEC aggregation and enhanced lamina disruption are independent of the inhibition of capsid deenvelopment. In HSV-1-infected cells, most pUL34 is found associated with the nuclear envelope, but it is not unusual to observe a faint pUL34 signal associated with cytoplasmic membranes. Surprisingly, we also observed that, in HaCaT cells infected with either WT or mutant viruses, more pUL34 is found in the cytoplasm than in Vero or SH-SY5Y cells (Fig. 6E to H).

VP5-null/US3_{K220A} virus-infected cells form multilayer membrane structures at the nuclear periphery. In order to characterize the NEC puncta that are formed in the absence of capsid envelopment, Vero cells were infected with WT, VP5-null, US3_{K220A}, or VP5-null/US3_{K220A} virus at an MOI of 5 for 18 h and prepared for analysis by transmission electron microscopy (TEM) (Fig. 7). Consistent with previous reports, TEM images of WT HSV-1-infected cells showed convoluted nuclear membranes and production of numerous intranuclear capsids and extracellular virions (Fig. 7A). Consistent with confocal microscopy analysis (Fig. 2), VP5-null recombinant virus-infected cells also showed convolution of the nuclear envelope but, as expected, no capsids were observed (Fig. 7B). As reported previously (44), cells infected with US3_{K220A} recombinant virus showed enveloped virions accumulated within intranuclear vesicles (Fig. 7C). In addition, we noted enveloped virions accumulated within extranuclear vesicles (Fig. 7C, black arrow). Interestingly, VP5-null/US3_{K220A} recombinant virus-infected cells contained many multimembranous nuclear inclusions and extrusions with at least four (Fig. 7D to H, yellow and red arrows) and in some cases as many as eight (Fig. 7F, red arrow with asterisk) densely staining membrane layers. Additionally, higher magnification analysis revealed that the multimembrane structures are nuclear envelope derived (Fig. 7D to H, asterisks), and it seems likely that those that appear unconnected might have connections that are out of the plane of section. We refer to these nuclear envelope-derived folded structures as “karmellae,” a word previously used to describe folded-over stacks and concentric rings of membranes (48).

The dense staining of the membranes of the karmellae suggested the presence of high protein concentrations, and we hypothesized that these represent the aggregations of the NEC in the nuclear membrane observed in immunofluorescence images (Fig. 6) and that the function of pUS3 is to prevent self-association of the NEC. It was

FIG 7 VP5-null/US3_{K220A} recombinant virus-infected cells show intranuclear and extranuclear karmellae. Digital micrographs show Vero cells infected with WT (A), VP5-null (B), US3_{K220A} (C), or VP5-null/US3_{K220A} (D to H) virus at an MOI of 5 for 18 h. Infected Vero cells were fixed with glutaraldehyde and processed for TEM. (A) Typical morphology of WT HSV-1 infection, with a convoluted nuclear envelope and enveloped virions accumulated on the cell surface. (B) Typical morphology of VP5-null virus-infected Vero cells, with no encapsidated virions in the nucleus, in the cytoplasm, or on the cell surface. (C) Typical morphology of US3_{K220A} virus infection, with vesicles of enveloped virions (black arrows). (D to H) Representative images of both inclusion and extrusion karmellae. The arrows point to at least four thicknesses of lipid bilayer membranes that are folded-over stacks and form concentric rings. (F and G) 3× magnified images of the boxed areas indicated in panel D. (H) 3× magnified image of the boxed area indicated in panel E. Red and yellow arrows indicate extrusion and intrusion karmellae, respectively. A star next to an arrow indicates that the karmella has a visible connection to the nuclear envelope. N, nucleus; C, cytoplasm.

reported previously that coexpression of pUL31 and pUL34 in the absence of other viral proteins leads to the formation of NEC puncta at the nuclear rim (11), and we determined whether coexpression of pUS3 might inhibit their formation. Vero cells were cotransfected with tagged hemagglutinin (HA)-UL34, enhanced green fluorescent protein (EGFP)-UL31, and FLAG-US3 plasmid constructs and fixed 48 h posttransfection, followed by staining for HA and FLAG (Fig. 8). As reported previously, coexpression of pUL31 and pUL34 in the absence of pUS3 resulted in punctate distribution of the NEC on the nuclear rim (Fig. 8A to D). In contrast, coexpression of pUS3 along with pUL34 and pUL31 resulted in smooth and even distribution of the NEC at the nuclear rim (Fig. 8E to H), similar to that seen in WT virus-infected cells.

US3 function in nuclear egress is quantitatively important to viral growth in a cell type-specific manner. pUS3 controls apoptosis and many other cellular functions in addition to nuclear egress (49). In order to characterize the specific quantitative significance of the pUS3 NEC-dependent nuclear egress function, an epistasis analysis was performed. The single-step growth (SSG) properties of UL34-null, US3_{K220A}, and UL34-null/US3_{K220A} double mutant viruses were compared (Fig. 9B to D). If most or all of the pUS3 enhancement of SSG was due to its function in facilitating NEC-dependent nuclear egress, then the double mutant virus would be no more impaired than a single mutant UL34-null virus. HaCaT (Fig. 9B), SH-SY5Y (Fig. 9C), and Vero (Fig. 9D) cells were infected at an MOI 5 and, at 16 or 18 h postinfection (hpi), viral infectivity in the culture was measured by plaque assay on UL34-complementing cell lines (CX cells). SSG

FIG 8 pUS3 phosphorylation of NEC components prevents self-aggregation of NEC. Shown are digital confocal images representing the localization of HA-UL34 and EGFP-UL31 transiently coexpressed proteins in Vero cells without (A to D) or with (E to H) coexpression of US3-FLAG. Cells were fixed at 48 h posttransfection and were stained using antibodies directed against HA and FLAG. Green indicates EGFP-UL31, blue indicates HA-UL34, and red indicates US3-FLAG. Representative images of one of three independent experiments are shown. Scale bars = 5 μ m.

analyses in HaCaT and SH-SY5Y cells demonstrated a large growth defect phenotype, with the US3 mutant in both cell lines showing $\sim$ 100-fold reduced growth, compared to the WT virus (Fig. 9B and C). Additionally, in these cell lines, mutation of US3 on a UL34-null background resulted in a significant $\sim$ 10-fold further reduction in SSG. This reduction was not as large as that seen when US3 is mutated on a WT background, indicating that the growth defect due to US3 mutation results from defects in both NEC-dependent nuclear egress and other functions of pUS3 (49). In Vero cells, however, we observed an $\sim$ 10-fold growth defect for the US3 mutant virus, compared to the WT virus, consistent with previous reports, whereas mutation of US3 on a UL34-null background resulted in $\sim$ 2- to 3-fold reduction in growth, compared to the UL34 null parent. This suggested that most or all of the US3 mutant growth defect in Vero cells was due to US3 function in NEC-dependent nuclear egress.

DISCUSSION

The nuclear lamina is responsible for maintenance of regular nuclear shape and for resistance of the nucleus to stress-induced distortion (21, 22). The changes in nuclear shape that regularly accompany HSV infection have been proposed to be a consequence of disruption of the structure of the nuclear lamina, including phosphorylation-induced dissociation of lamin subunits from each other and from their tethering interactions with INM-embedded LAPs such as emerin and LBR (28, 30, 33, 36–39, 50). Despite recruitment of viral and cellular kinases to the nuclear envelope in HSV-1 nuclear egress, the nuclear lamina is not mostly dissociated, as happens during nuclear envelope dissolution during mitosis (51, 52). Rather, smaller areas of thinning and thickening of the lamina have been observed. This suggests that lamina disruption is local rather than general and might be associated with, and triggered by, capsid envelopment. Larger-scale changes in nuclear shape might thus be the result of the aggregate effects of many capsid envelopment events. Here, we show that capsid envelopment is not necessary for disruption of nuclear shape or rearrangement of lamin A/C. As immunofluorescence, nuclear contour (Fig. 2), and TEM (Fig. 7B) analyses showed, VP5-null virus-infected nuclei demonstrated a convoluted nuclear phenotype and lamin A/C redistribution, demonstrating that disruption of the nuclear lamina is not a result of capsid envelopment but instead is likely a preparation step for the HSV-1 nucleocapsid to undergo nuclear egress.

FIG 9 pUS3 nuclear egress function and viral growth. (A) Schematic diagrams of the WT HSV-1 BAC-derived genome (line 1) and the UL34-null/US3_{K220A} recombinant virus construct (line 2). Line 2 shows the UL34-null/US3_{K220A} virus, which has complete deletion of the coding sequence for the UL34 gene and a single amino acid substitution of the US3 invariant lysine to alanine. (B to D) SSG analyses of HaCaT (B), SH-SY5Y (C), or Vero (D) cells that were infected with WT, US3_{K220A}, UL34-null, or UL34-null/US3_{K220A} virus at an MOI of 5. At 16 hpi, cells were frozen, thawed, and titered on UL34-complementing cells (CX cells). One of two independently performed experiments with three biological replicates is shown. The error bars in each graph are represented as means with ranges. Statistical significance was determined by one-way ANOVA using the Tukey method for multiple comparisons performed on logarithmically converted data. n.s., not significant; *, $P \leq 0.05$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. Each graph shows one of at least three independent experiments.

Immunofluorescence analysis revealed the surprising result that lamin A/C becomes concentrated at the nuclear envelope during HSV-1 infection in a cell type-specific manner (Fig. 2G to I and L to N and Fig. 3). Nuclear lamins are found both at the nuclear rim and in the nucleoplasm in many interphase cells (53). How the balance between these two populations of lamins is determined is not well understood, but maintenance of an intranuclear lamin population can be affected both by lamin post-translational modification and by changes in the abundance or localization of intranuclear lamin-binding proteins like LAP2 α (53). Here, we show that this balance is altered during HSV-1 infection to favor nuclear rim localization of lamin A/C in HaCaT and SH-SY5Y cells but not Vero cells. This effect was most evident in UL34-null and VP5-null virus-infected nuclei (Fig. 3A to D) that were not undergoing active capsid envelopment, suggesting the possibility that ongoing nuclear egress may result in some dispersal of lamins. This result was additionally surprising because previously characterized infection-induced changes in lamina distribution have been found to be dependent on expression of the NEC (54, 55). Our observations and analyses, however, reveal that there is a cell type-dependent, UL34-independent pathway for alteration of lamin A/C localization in HSV-1-infected cells. Although we have not identified the HSV-1 gene(s) responsible for localization of lamins to the nuclear periphery, the observation that this localization does not depend on viral DNA replication suggests that the factors responsible are expressed during the immediate early or early phases of infection. Immediate early and early proteins are responsible for establishment and expansion of nuclear subcompartments in which virus genome transcription and replication take place (40, 56, 57). These replication compartments begin as punctate structures and expand during infection to occupy most of the nucleoplasm. One possibility is that lamins are

progressively excluded from the interior of the nucleus as a result of replication compartment expansion. Further study will be required to elucidate the underlying mechanisms.

Previous studies demonstrated contrasting roles for pUS3 in alteration of the nuclear lamina. pUS3 mediates phosphorylation of both lamin A/C and the LAP emerlin, suggesting a role in disruption of the interactions that stabilize the lamina (36, 38). On the other hand, deletion of the US3 coding sequence or alteration of its sequence to eliminate protein kinase activity has been shown to exacerbate disruption of the lamina, as evidenced by the appearance of large gaps in the lamin A/C and lamin B networks, which suggests that it might negatively regulate lamina disruption (12, 28, 44). Here, we tested the hypothesis that the apparent negative regulatory activity of pUS3 is due to the accumulation of enveloped virions that results from a deenvelopment defect that is also caused by mutations in pUS3 (12, 44). This hypothesis would also account for the formation of NEC puncta, as reflected in the local accumulation of NEC-containing PEVs. We showed, however, that NEC aggregation and enhanced lamina disruption were both observed in cells infected with the US3_{K220A} mutant even in the absence of capsids (Fig. 6), demonstrating that ongoing capsid envelopment is not required for appearance of these characteristic US3 mutant phenotypes.

The appearance of NEC puncta in the absence of pUS3 activity in infected cells, coupled with the inhibition of formation of NEC puncta in transfected cells that also express pUS3, suggests an alternative unifying model, namely, that the US3-dependent nuclear egress phenotypes all result from pUS3 inhibition of NEC self-association. Previous studies showed that, in the absence of pUS3, cellular protein kinases are recruited to the nuclear envelope and colocalize there with NEC aggregates (30, 33). Thus, NEC aggregates might be kinase recruitment “hot spots” that cause extensive local dissolution of the lamin A/C network. Formation of discrete NEC aggregates might also explain the observation that trapping of perinuclear PEVs in US3 mutant virus-infected cells occurs at discrete spots, rather than being distributed randomly over the nuclear envelope, since capsid docking and envelopment events would also be concentrated at sites of NEC aggregation.

pUS3 phosphorylates both pUL31 and pUL34 (43, 44, 58). Failure to phosphorylate pUL34 has not been associated with changes in NEC localization or function (44). Failure to phosphorylate pUL31 at its pUS3 kinase motif, however, results in deenvelopment defect and NEC localization phenotypes similar to those seen in US3 mutants (11, 43), suggesting that phosphorylation of pUL31 controls the self-association status of NEC heterodimers. Membrane curvature for nuclear capsid envelopment is thought to be driven by energetically favorable self-association of NEC heterodimers into hexameric arrays (4, 59, 60). Successful deenvelopment can occur only if these energetically favorable interactions are reversed upon fusion of the viral envelope with the ONM. Therefore, failure to negatively regulate this type of NEC self-association might easily account for most or all of the nuclear egress phenotypes associated with US3 mutants.

Ectopic coexpression of pUL31 and pUL34 was shown previously to result in formation of puncta that superficially resemble those seen in infected cells in the absence of pUS3 (11, 40). TEM analysis, however, shows that these are remarkably different structures. The puncta formed by ectopic expression of the NEC correspond to accumulations of single-membrane vesicles between the INM and the ONM that resemble capsidless perinuclear virions, whereas the structures formed with the VP5-null/US3_{K220A} virus are nuclear envelope-derived multimembranous nuclear inclusions and extrusions called karmellae. Several considerations suggest that these karmellae correspond to areas of highly concentrated NEC. First, the existence of discrete areas of karmella formation is consistent with the distribution of NEC puncta on the nuclear membrane. Second, the highly electron-dense connections between the membrane stacks strongly resemble the electron-dense NEC layer that underlies the envelope in primary enveloped virions. Finally, tightly apposed double-membrane structures were observed previously in US3-null virus-infected nuclear membranes, with the apposing faces of the

observed double membranes consisting of two membrane layers bridged by NEC complexes (47). While the relationship between these double-membrane structures and the karmellae observed here is not certain, it seems likely that the karmellae arise by stacking of the double membranes.

The formation of multilayer nuclear membrane structures suggests two mechanisms operating on the INM and ONM at the sites of karmella formation. First, the free bending of the INM required for the observed membrane curvature and folding is consistent with substantial disruption of the nuclear lamina. Second, the close apposition of the membrane layers in the karmellae likely requires a mechanism for association between faces of the same membrane. Interestingly, an interaction of exactly the sort required for this face-to-face stacking was observed in the crystals used for solving the HSV-1 NEC heterodimer structure (59). In those crystals, sheets of NEC hexamers were stacked such that predicted membrane-distal surfaces of pUL31 interacted with each other. Such pUL31-pUL31 interactions might promote the folding and stacking of membranes at sites of NEC aggregation in US3 mutant-infected cells.

The ultrastructural consequences of pUS3 mutation are striking and might be expected to have a substantial effect on production of infectious virus. Deletion or mutation of pUS3, however, has been reported to have relatively minor effects on viral SSG in cultured cells, and this effect must be the sum of the many functions that pUS3 has been shown to have in infected cells (61). However, our findings in HaCaT and SH-SY5Y cells, demonstrating ~100-fold reduced growth of the US3 mutant, compared to the WT virus, strongly suggest that there is cell type specificity to the growth phenotype of US3_{K220A}. It should be noted, however, that the pUS3 consensus phosphorylation site is similar to that for cellular protein kinases, including protein kinase A and Akt (62, 63), and mutation of the pUS3 phosphorylation sites in pUL31 severely inhibits virus growth (43). Therefore, it is likely that the partial effect of US3 mutations reflects the presence of compensating kinases in the infected cells studied so far. As the US3_{K220A} growth defect is much stronger in HaCaT and SH-SY5Y cells, it is quite possible that compensating kinases are either absent or expressed at lower levels in HaCaT and SH-SY5Y cells. By assessing the effect of US3 mutation on a UL34-null versus WT background, we were able to specifically assess the importance of pUS3 function in NEC-mediated nuclear egress. The observation that a catalytically inactivating US3 mutation has a quantitatively different effect on virus growth when NEC-mediated nuclear egress has been eliminated suggests that its function in nuclear egress makes only a partial contribution to virus production in the cell types studied. HSV-1 infects multiple cell types to cause disease in humans, and the quantitative significance of pUS3 nuclear egress function may vary in different human cells *in situ*.

MATERIALS AND METHODS

Cell cultures. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% bovine calf serum and penicillin-streptomycin. HaCaT cells (a kind gift from David Johnson) were maintained in DMEM supplemented with 10% fetal bovine serum. SH-SY5Y cells (a kind gift from Stevens Lewis at the University of Iowa) were maintained in Opti-MEM supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin. A humidified incubator at 37°C with 5% CO₂ was used to culture cells.

Construction of recombinant viruses. The HSV-1(F) bacterial artificial chromosome (BAC) described by Tanaka et al. (64) (a kind gift from Yasushi Kawaguchi at the University of Tokyo) was used for construction of all of the recombinant viruses. The recombinant virus with a point mutation at residue 220 in US3 was constructed using gentamicin resistance cassette insertion and excision, as described previously (65). The mutagenesis insertion DNA fragment was designed as two fragments and amplified by PCR as follows: fragment 1: forward primer, 5'-GACAGCAGCCACCCAGATTACCCCAACGGTAATCGTGGCGGGGGTGGTACGGATCCTAGGGATAACAGGG-3'; reverse primer, 5'-GGCTCGTGCTCGTGACACCCCGCCGACGATTACCCGTCTAGAGCGCGCGGTG-3'; fragment 2: forward primer, 5'-GACAGCAGCCACCCAGATTACCC-3'; reverse primer, 5'-GCAGTCGCGCTCGTGGCTCGTGCTCGTGACCA-3'. In the next step, another PCR was performed to assemble these two fragments together with a pair of outer primers, as follows: forward primer, 5'-GAACTGGACGCCATGGACAGGG-3'; reverse primer: 5'-CCCAGCGGGTTCAGGCG-3'. All of the recombinant viruses were rescued by transfecting BAC DNA into Vero or Vero-derived complementing cells. HSV-1(F) BAC-derived virus containing deletions in the UL34 gene sequence were described previously and were amplified on UL34-complementing cells (65). The HSV-1(F) BAC-derived VP5-null virus was generated by a complete deletion of the UL19 gene sequence.

The VP5-null/US3_{K220A} double mutant recombinant virus was generated by creating a point mutation at residue 220 in US3 using HSV-1(F) BAC VP5-null recombinant virus as a backbone. To generate UL34-null/US3_{K220A} virus, a point mutation at residue 220 in US3 was constructed using HSV-1(F) BAC UL34-null recombinant virus.

Plasmids and cell lines. A plasmid for creation of a VP5-complementing cell line was created in two steps. First, pRR1123 was created by ligating the 5.62-kb BglIII fragment of the HSV-1(F) genome that contains the UL19 gene into the BamHI site of the vector pGEM-3Z(F⁺). The entire insert sequence was then removed from pRR1123 by cleavage with EcoRI and XbaI and was cloned between the MfeI and XbaI sites of pcDNA3 to generate pRR1377, in which expression of UL19 is driven by its own promoter/regulatory sequences.

The VP5-complementing cell line was constructed as described previously (65), with some modifications. Vero cells were transfected with pRR1377 and then selected for stable transfection with G418. Individual cell clones were isolated and tested for formation of plaques after infection with VP5-null virus (a gift from Prashant Desai). One of the clones that formed large plaques after infection was chosen for further use. A pcDNA3 plasmid carrying FLAG-tagged US3 was described previously (66).

A pcDNA3 plasmid carrying N-terminally HA-tagged UL34 (pRR1385) was constructed by amplification of the UL34 coding sequence from the plasmid pRR1072Rep (65) using primers 5'-GATCAAGCTTCCATGTACCCATACGATGTTCCAGATTACGCTGCGGACTGGGCAAGCCC-3' and 5'-CTAGTCTAGATTATAGGCGCGCGCCAGC-3', digestion of the resulting PCR product with HindIII and AflII, and ligation into HindIII/AflII-digested pRR1238 (65).

A plasmid expressing C-terminally EGFP-tagged UL31 (pRR1404) was constructed by amplification of the UL31 coding sequence with primers 5'-GCAGTACATCAAGTGATCAACATGTTCTGTGACCCCATGTGCG-3' and 5'-GCGACCGGTAGCGGCGGAGAACTCGTCG-3' and insertion into the pEGFP-C1 vector by Gibson assembly.

Western blotting. Vero and VP5-complementing cells in 6-well culture plates were either mock infected or infected with HSV-1(BAC), VP5-null, or VP5-null/US3_{K220A} virus strains, at an MOI of 5. At 18 hpi, infected cells were washed in phosphate-buffered saline (PBS) and lysed by the addition of RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and sonication. Cell lysates were separated by 10% SDS-PAGE, blotted onto nitrocellulose membranes, and probed as described previously (19) using chicken polyclonal anti-UL34 (1:250) (19), mouse monoclonal anti-HSV-1 infected cell culture polypeptide 27 (ICP27) (1:500; Virusys Corp.), mouse monoclonal anti-HSV-1 VP5 (Biodesign International), rabbit polyclonal anti-HSV-1 US3 (a kind gift from B. Roizman), and mouse monoclonal antiactin (Sigma-Aldrich).

Immunofluorescence assay. Vero, HaCaT, or SH-SY5Y cells were infected with virus strains at an MOI of 5, and at 16 hpi they were fixed with 4% formaldehyde for 20 min, followed by a PBS wash. Cells were permeabilized and blocked by incubation in immunofluorescence buffer containing 1×X PBS, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1% egg albumin, and 0.01% Na₂S₂O₃. Cells were incubated with primary antibodies as follows: chicken polyclonal anti-UL34 and mouse monoclonal anti-lamin A/C (Santa Cruz Biotechnology). Confocal microscopy was performed with a Leica DFC7000T confocal microscope using Leica software. Fiji ImageJ software was used to quantify localization signals. Images shown are representative of experiments performed at least three times.

Cotransfection assay. Vero cells in 24-well culture plates were transfected with EGFP-tagged UL31, HA-tagged UL34, and FLAG-tagged US3 plasmid constructs by use of X-tremeGENE HP DNA transfection reagent (Millipore-Sigma), according to the manufacturer's instructions. Two days posttransfection, transfected cells were fixed with 4% formaldehyde for 20 min, followed by a PBS wash. Subsequently, cells were permeabilized and blocked by incubation in immunofluorescence buffer containing 1×X PBS, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1% egg albumin, and 0.01% Na₂S₂O₃, followed by incubation with primary antibodies as follows: goat polyclonal anti-HA (1:500; GenScript) and mouse monoclonal anti-FLAG (1:500; Sigma-Aldrich). Confocal microscopy was performed with a Leica DFC7000T confocal microscope using Leica software. Images shown are representative of experiments performed at least three times.

TEM. Confluent monolayers of Vero cells were infected with HSV-1(F) BAC, US3_{K220A}, VP5-null, and two isolates of VP5-null/US3_{K220A} virus strains at an MOI of 5, and at 18 hpi they were fixed by incubation with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h. Following fixation in 1% osmium tetroxide, cells were washed in cacodylate buffer and embedded in Spurr's resin. Cells were cut into 95-nm sections, which were mounted on grids and stained with uranyl acetate and lead citrate for examination with a JEOL 1250 transmission electron microscope.

SSG measurement. Replication of the HSV-1(F) BAC, UL34-null, US3_{K220A}, and UL34-null/US3_{K220A} virus strains on Vero, HaCaT, or SH-SY5Y cells was measured as described previously (19). Viral replication was statistically analyzed using analysis of variance (ANOVA). *P* values of <0.05 were considered statistically significant.

Nuclear morphology analysis. Nuclear contour ratios were calculated using cross-sectional images of nuclei of Vero, HaCaT, or SH-SY5Y cells infected with HSV-1(F) BAC, UL34-null, or VP5-null strains at an MOI of 5 for 16 h. The cells were fixed to perform a lamin A/C immunofluorescence assay. ImageJ was used to measure the nuclear cross-sectional area and perimeter. The nuclear contour ratio was calculated using the formula $(4\pi \text{ area})/\text{perimeter}^2$, as described previously (67). Fifty nuclei from each sample were used to measure the nuclear contour ratio for each experiment. Graphs shown are representative of experiments performed at least three times.

Measurement of lamin recruitment to the nuclear perimeter. To measure the intensity of lamin A/C at the nuclear rim, a cross-sectional profile of lamin A/C fluorescence of infected cell nuclei was

plotted using Fiji ImageJ software. The ratio of lamin A/C staining at the edge of the nucleus to that in the middle was measured for 20 nuclei under each condition. Statistical significance was determined by ANOVA. Graphs shown are representative of experiments performed at least three times.

PAA treatment experiment and analyses. (i) PAA immunofluorescence assay. At time zero, HaCaT cells were treated with 0.3 μ g/ml PAA and infected with virus at an MOI of 5. After 90 minutes, the virus inoculum was removed and replaced with fresh medium containing PAA. At 16 hpi, cells were fixed, blocked, and incubated with primary antibodies as follows: rabbit polyclonal anti-gE (1:500; a kind gift from Harvey Friedman) and mouse monoclonal anti-lamin A/C (Santa Cruz Biotechnology). Confocal microscopy was performed, and images shown are representative of experiments performed at least three times. Quantitative analysis and measurement of lamin recruitment to the nuclear perimeter are described above.

(ii) PAA immunoblot assay. PAA treatment was assessed via Western blotting of HaCaT cells that had been either mock infected or infected with HSV-1(F) BAC virus at an MOI of 5 at time zero. At 2 hpi, 0.3 μ g/ml PAA was added. Cells were harvested at 18 hpi, followed by protein separation and transfer to membranes as described above. Membranes were then incubated for 90 min with primary antibodies as follows: chicken polyclonal anti-UL34 (1:250) (19), mouse monoclonal anti-HSV-1 ICP27 (1:500; Virusys Corp.), mouse monoclonal anti-HSV-1 US11 (1:1,000) (68), and mouse monoclonal antiactin (1:500; Sigma-Aldrich). The membranes were then incubated with the respective alkaline phosphatase-conjugated secondary antibodies for 60 min and developed in Western blotting development solution as described previously.

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