Human Cytomegalovirus UL97 Phosphorylates the Viral Nuclear Egress Complex

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Abstract

Herpesvirus nucleocapsids exit the host cell nucleus in an unusual process known as nuclear egress. The human cytomegalovirus (HCMV) UL97 protein kinase is required for efficient nuclear egress, which can be explained by its phosphorylation of the nuclear lamina component lamin A/C to disrupt the nuclear lamina. We found that a dominant negative lamin A/C mutant complemented the replication defect of a virus lacking UL97 in dividing cells, validating this explanation. However, as complementation was incomplete, we investigated whether the HCMV nuclear egress complex (NEC) subunits UL50 and UL53, which are required for nuclear egress and recruit UL97 to the nuclear rim, are UL97 substrates. Using mass spectrometry we detected UL97-dependent phosphorylation of UL50 residue S216 and UL53-S19 in infected cells. Moreover, UL53-S19 was specifically phosphorylated by UL97 in vitro. Notably, treatment of infected cells with the UL97 inhibitor maribavir or infection with a UL97 mutant led to a punctate rather than a continuous distribution of the NEC at the nuclear rim. Alanine substitutions of both UL50-S216 and UL53-S19 resulted in a punctate distribution of the NEC in infected cells, and decreased virus production and nuclear egress in the absence of maribavir. These results indicate that UL97 phosphorylates the NEC, and suggest that this phosphorylation modulates nuclear egress. Thus, the UL97-NEC interaction appears to recruit UL97 to the nuclear rim both for disruption of nuclear lamina and phosphorylation of the NEC.
Importance

Human cytomegalovirus (HCMV) causes birth defects, and life-threatening diseases in immunocompromised patients. HCMV assembles in the nucleus, and then translocates to the cytoplasm in an unusual process termed nuclear egress, an attractive target for antiviral therapy. A viral enzyme, UL97, is important for nuclear egress. It has been proposed that this is due to its role in disruption of the nuclear lamina, which would otherwise impede nuclear egress. Validating this proposal, we showed that independent disruption of the lamina can overcome a loss of UL97, but only partly, suggesting additional roles for UL97 during nuclear egress. We then found that UL97 phosphorylates the viral nuclear egress complex (NEC), which is essential for nuclear egress, and obtained evidence that this phosphorylation modulates this process. Our results highlight a new role for UL97, the mutual dependence of the viral NEC and UL97 during nuclear egress, and differences among herpesviruses.
Introduction

Herpesviruses replicate and package their DNA genomes into capsids in the nucleus of the host cell. The nucleocapsids are then transported out of the nucleus through an unusual process called nuclear egress. A widely accepted model for nuclear egress entails envelopment and de-envelopment of capsids as they transit the nuclear membranes, (reviewed in (1-3)). Studies of human cytomegalovirus (HCMV) nuclear egress are of particular interest because of the medical importance of HCMV. HCMV, a beta-herpesvirus, causes widespread and lifelong infections that can result in severe diseases in immunocompromised hosts such as transplant recipients and AIDS patients (4, 5), and developmental and hearing disabilities in infants (6). Current anti-HCMV drugs are limited by acute and long-term toxicities, poor oral bioavailability and/or the emergence of resistance, leading to an urgent need for new antiviral strategies (5, 7). The process of HCMV nuclear egress could be an attractive target for development of specific antiviral therapy, especially given its importance for HCMV replication and features that differ from a cellular process that resembles nuclear egress events (8, 9).

An important player in HCMV nuclear egress is UL97, a serine/threonine-specific protein kinase (10, 11). Although UL97 has been implicated in several other steps during the virus replication cycle (10-16), in dividing cells, the magnitude of the nuclear egress defect due to a UL97 null mutation or a UL97 inhibitor is very similar to the magnitude of the virus yield defect (10, 17). In serum-starved, non-dividing cells, part of the virus yield defect can be ascribed to a defect in viral DNA synthesis, stemming from UL97’s role in inactivation of retinoblastoma protein (16-18). UL97 is necessary for the phosphorylation of a variety of viral and cellular proteins in infected cells (11, 16, 18-24),
and is sufficient for phosphorylation of some of these proteins \textit{in vitro} (11, 19, 20, 25, 26). One such substrate is the nuclear lamina component, lamin A/C. UL97 phosphorylates lamin A/C, mimicking the activity of cellular cyclin-dependent kinase-1 (Cdk1) during mitosis (11), and this activity can explain its role in disruption of the nuclear lamina, which is thought to permit access of nucleocapsids to the inner nuclear membrane during nuclear egress (11, 17, 23). However, these data do not directly show that lamin A/C phosphorylation and lamina disruption mediated by UL97 are required for efficient replication. In particular, to our knowledge, no study demonstrating complementation of HCMV replication by facilitating lamina disruption in the absence of UL97 has been reported.

Another important player in HCMV nuclear egress is a two subunit nuclear egress complex (NEC) containing UL50, a nuclear membrane protein, and UL53, which is nucleoplasmic if not bound to UL50 (8, 27, 28). The HCMV NEC is also required for nuclear lamina disruption, and recruits UL97 to the nuclear rim (8), suggesting that this NEC-UL97 association is important for lamin phosphorylation and nuclear lamina dissolution by UL97. Based on work with other herpesviruses, the NEC is also thought to orchestrate primary envelopment and possibly other steps in nuclear egress (29-31).

We wished to investigate if lamina disruption is indeed required for efficient HCMV replication and, if so, whether this is the only role for UL97 during nuclear egress. In other words, are there UL97 substrates other than lamin A/C that are relevant to nuclear egress? To address these questions, we investigated if independent disruption of the nuclear lamina could complement the replication defect observed in the
absence of UL97, whether UL97 might phosphorylate NEC subunits, and if such phosphorylation is relevant for virus replication and nuclear egress. Our analysis validated the importance of UL97 phosphorylation-driven disruption of nuclear lamina, and suggests that UL97 phosphorylation of the NEC is also relevant for efficient nuclear egress during HCMV infection.
Materials and Methods

Generation of recombinant and mutant viruses. HCMV BAC constructs carrying dominant negative lamin A mutants. We engineered sequences encoding a dominant negative (D/N) GFP-lamin A construct lacking the lamin A head and CaaX domains (ΔH/C) (kindly provided by David Gilbert, Florida State University), into derivatives of AD169rv, a bacterial artificial chromosome (BAC) of HCMV strain AD169 (32) that were either wild type (WT) or null for UL97 (Fig.1 and Table 1), using the en-passant two-step red recombination method of Tischer and coworkers (33, 34). For this purpose, a universal transfer plasmid (33) (pGFP-dhead-caax-KanS) was generated by insertion of the selection marker ISce-AphAI (KanaR) (34) into the D/N GFP-lamin A sequence. Following this, PCR primers with sequences to facilitate homologous recombination into the viral genome, (listed in Supplemental Table S1 at https://coen.med.harvard.edu), were used to amplify the D/N GFP-lamin A sequence containing the ISce-AphAI (KanaR) region from the plasmid pGFP-dhead-caax-KanS. The PCR product was gel purified and electroporated into GS1783 cells harboring either WT_E7 AD169rv (16), or WT AD169rv BAC. Kanamycin resistant integrates were resolved by heat-shock and L-(+) arabinose induction of I-SceI, and the resulting BACs were sequenced to confirm the introduced changes. For the WT HCMV construct (WT LMN ΔH/C AD169rv), the mutated GFP-lamin A sequences were cloned under the control of a duplicated UL97 promoter (described previously (16)) in the unique short (US) region of the HCMV genome within an intergenic locus between US9 and US10 by replacing the previously described human papillomavirus E7 gene cloned in that region (16). For the UL97 null (Δ97) HCMV construct (Δ97 LMN ΔH/C AD169rv), the UL97
encoding sequence was replaced with the D/N GFP-lamin A encoding sequences. The bacmids (Table 1) were electroporated into HFF cells, (Hs27, ATCC CRL-1684; American Type Culture Collection; Manassas, VA) as described previously, with plasmids pCGN71 (15, 35), expressing the viral transcriptional transactivator pp71, and pBRep-Cre, to generate the corresponding WT and UL97 null viruses expressing D/N GFP-lamin A, WT LMN ΔH/C and Δ97 LMN ΔH/C, respectively.

**NEC phosphorylation site mutants.** Mutations in UL50 or UL53 coding sequences – UL50-S216A, or UL53-S19A, were engineered into 53-F pBADGFP, an AD169-derived BAC containing a GFP cassette under the control of the major immediate early promoter of HCMV, and a FLAG-encoding sequence at the C-terminus of UL53 coding sequence described previously (8), using the two-step Red recombination method (33, 34), and primers listed in Supplemental Table S1 at https://coen.med.harvard.edu. The resulting viruses were designated S216A 53-F pBADGFP, and S19A 53-F pBADGFP, respectively. The double mutant construct UL50-S216A/ UL53-S19A 53F pBADGFP was generated by introducing the UL53-S19A mutation into S216A 53-F pBADGFP, using the same procedure. The rescued derivative of the double mutant construct, termed, S216AR/ S19AR 53-F pBADGFP, was generated by restoring the original serine residues first in the UL50 coding sequence, to generate the intermediate construct UL50-S19AR/ UL53-S19A pBADGFP. This was followed by restoration of the UL53 coding sequence to generate the final double rescued BAC, S216AR /S19AR 53-F pBADGFP. The double mutant carrying glutamate substitutions of both the UL97 phosphorylation sites (phosphosites) in the NEC, UL50-S216E and UL53-S19E, was
generated using primers listed in Supplemental Table S1 at https://coen.med.harvard.edu, and the procedure described above. This double mutant construct was designated UL50-S216E/UL53-S19E 53F pBADGFP. These bacmids were individually electroporated into HFF cells as described above to generate the single mutant viruses, S216A or S19A 53-F BADGFP, the double alanine mutant virus S216A/S19A 53-F BADGFP (AA), its rescued derivative S216AR/S19AR 53-F BADGFP (RR), and the double glutamate mutant virus S216E/S19E 53-F BADGFP (EE).

**K355Q UL53-FLAG AD169 BAC** The UL97 active site point mutation K355Q was introduced in the UL53-FLAG AD169 BAC construct (53-F AD169-RV, (8)) using the PCR primers listed in Supplemental Table S1 at https://coen.med.harvard.edu and the procedures described above. The resulting bacmid K355Q UL53-FLAG AD169 was electroporated into HFF cells to generate the virus K355Q 53-FLAG AD169-RV (K355Q) (Table 1).

**Immunofluorescence.** Immunofluorescence assays of mock-infected or virus-infected cells to visualize subcellular localization of lamin A/C, UL50, UL53 or UL53-FLAG were performed as described previously (8). All imaging experiments were done at the Nikon Imaging Center at Harvard Medical School using a Nikon Ti with Spinning Disk Confocal Laser microscope at 100X magnification. Images shown were obtained by acquiring sequential optical planes in the z axis using the MetaMorph program. Statistical analysis was performed with GraphPad Prism software.
Viral replication kinetics. HCMV replication following infection at a multiplicity of infection (MOI) of either 0.1 or 1 was assessed as described previously (8). Where indicated, data are presented as the average viral titer ± standard deviation obtained from three independent experiments. Statistical analysis was performed with GraphPad Prism software, where differences in mean log titer values were assessed for significance using one-way ANOVA followed by Sidak’s multiple comparison tests.

Immunoprecipitation of UL53-FLAG from HFF cells. HFF cells were infected with UL53-FLAG AD169-RV (MOI=1) in the presence of 1μM maribavir, MBV (generously provided by John Drach, University of Michigan) in dimethylsulfoxide (DMSO) or in the presence of the vehicle DMSO alone. Immunoprecipitations using anti-FLAG antibody (FLAG-IPs) were performed as described previously (8), except for the following changes to the protocol: Following washing with ice cold phosphate buffered saline (PBS), the infected cells were scraped directly into lysis buffer, incubated on ice at 4°C for 15 min, and the lysate was centrifuged at 10,000 X g for 25 min. This supernatant was then used for the FLAG-IP. After the final wash, the protein was boiled in 2X Laemmli buffer containing protease and phosphatase inhibitors (Thermo Scientific). The eluate was stored in 100 μl aliquots at -80°C. For mass spectrometric (MS) analysis, the eluates were concentrated using the Wessel Flugge protocol (36), and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were visualized using colloidal blue and bands corresponding to UL50, UL53 and UL97 were excised and submitted for MS analysis.
Expression and purification of proteins
To express HCMV UL53 1-292, a previously described expression plasmid encoding residues 1-292 of UL53 in the vector pGEX-6P1 (37) was transformed into *E. coli* BL21 (DE3) CodonPlus RIPL cells (Stratagene). The purification protocol was modified for better yield as described below. The cells were grown in 9 L of Luria Bertani medium and expression was induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside for ~14 hours at 16°C. Cells were lysed by sonication in buffer A (25 mM Tris-HCl pH 7.5, 500 mM NaCl) plus 1 mM phenylmethylsulfonyl fluoride (PMSF), and the lysate was centrifuged at 10,000 X g (Beckman JA-20 rotor) for 60 minutes. The supernatant was loaded onto a glutathione sepharose column, which was washed with 500 ml of buffer A. The glutathione S-transferase (GST) tag was cleaved using PreScission Protease (GE Healthcare), and the eluted protein was further purified using size exclusion chromatography (Superdex 200 10/300 GL;GE) in buffer A plus 1 mM dithiothreitol (DTT). Protein purity was estimated using SDS-PAGE to be about 95%. GST-UL97 (WT) was expressed and purified from insect cells infected with recombinant baculovirus as previously described (11). Protein concentrations were estimated by Bradford assay.

In vitro kinase reactions. For radioactive in vitro kinase assays, 200 ng of GST-UL97 was incubated with 0.65 μg of UL53 1-292 per 20 μL reaction in an optimized kinase buffer (10mM HEPES [pH 8.0], 100mM NaCl, 10 mM MgCl₂, 2 mM DTT, and 200 μM unlabeled ATP) and 0.5 μL of [γ-32P]-ATP (3,000 to 6,000 Ci/mmol) (Perkin Elmer Inc., Waltham, MA) and either 1μM MBV in DMSO or the same volume of DMSO, at 37°C for 2h. For non-radioactive kinase reactions submitted for MS analysis, reactions contained
125 ng of UL97 and 5 µg of UL53 1-292, and either 1µM MBV or the same volume of DMSO. In both cases the reactions were terminated by the addition of concentrated SDS-PAGE loading buffer. Samples were heated at 95°C for 5 min and proteins were resolved by SDS-PAGE. For radioactive samples, gels were dried onto blotting paper under vacuum and incorporated $^{32}$P was assessed using a PhosphorImager (Molecular-Imager FX System; Bio-Rad). For non-radioactive samples, proteins were visualized with colloidal blue post SDS-PAGE. Protein bands corresponding to UL53 and UL97 were excised from the gels and submitted for MS analysis.

**MS analysis.** Samples from infected lysates or *in vitro* kinase assays were analyzed for the identification of phosphorylation sites by LC/tandem MS at the Taplin MS Facility, Harvard Medical School. The relative percentages of phosphorylated and unphosphorylated peptides were estimated in each experiment by the Taplin Facility by measuring the peak intensities of the relevant peptides.

**Electron Microscopy (EM).** HFF cells were seeded at 1 x 10^5 cells/well in a 12 well plate and infected with WT, AA or RR BADGFP viruses in duplicate at an MOI of 1 for 1 h. The cells were fixed at 96 hours post-infection (h.p.i. Processing for transmission EM, image acquisition, and data analysis for statistical significance were performed as described previously (17).
Results

Effects of independent disruption of nuclear lamina on replication of viruses lacking UL97. During infections with a UL97 null mutant virus, there is a marked impairment in phosphorylation of lamin A/C and disruption of the nuclear lamina, and a substantial reduction in nuclear egress, that, in dividing cells, corresponds to the reduction in virus yield (10, 11). This suggested that independent disruption of the nuclear lamina might complement the replication defect of a virus lacking UL97 in dividing cells, which would in turn indicate that lamina disruption by UL97 is indeed important for viral replication. Our initial efforts entailed use of lamin A mutant cells and RNA interference, but these were confounded by poor replication of the WT virus in the mutant cells, and inefficient siRNA-mediated knockdown of lamin A/C, respectively (unpublished results). We then turned to the use of a dominant negative (D/N) lamin A/C mutant carrying deletions of both the lamin A “head” domain and the C-terminal CaaX domain (ΔH/C) in a GFP-lamin expression construct (generously provided by David Gilbert). Expression of this lamin mutant in cells leads to formation of intranuclear aggregates, and results in the disruption of endogenous nuclear lamins (38).

We engineered sequences encoding the ΔH/C GFP-lamin A construct into HCMV either WT or null for UL97, under the control of the UL97 promoter (Fig.1 and Table 1), using en passant two-step red recombination. To determine the effect of ΔH/C lamin A on nuclear lamina during infection, we mock-infected or infected cells (MOI=1) with either WT or UL97 null virus (Δ97), or WT or Δ97 viruses expressing the D/N GFP-lamin A (WT LMN-ΔH/C or Δ97 LMN-ΔH/C). At 72 hours post-infection (h.p.i.) the cells
were stained with an anti-lamin A/C antibody that recognizes the first 18 amino acids of lamin A/C, to allow detection of endogenous lamin A/C, but not the D/N lamin A mutant, which lacks the head domain (amino acids 1-33). As compared to mock-infected cells (Fig. 2A, panels i-iii, Fig. 2B), about 70% of the WT virus-infected cells had deformed nuclei, often exhibiting a kidney shape (Fig 2A panels iv-vi). (This percent of cells is somewhat lower than what we have observed previously (11, 17), likely due to the analyses in earlier reports being conducted at a later time point, 96 h.p.i.). In contrast, as previously observed (11, 17), most cells infected with the Δ97 virus showed less dramatic or no changes in nuclear morphology (Fig 2A panels vii-ix), often retaining an oval shape with a uniform lamin staining around the rim of the nuclei, much like mock-infected cells (Fig 2A panels i-iii). Cells infected with either the WT or the Δ97 virus expressing the GFP-D/N lamin A showed intranuclear GFP aggregates that co-localized with endogenous lamin A/C (Fig. 2A, panels x-xii and xiii-xv, respectively). Notably, a majority of cells infected with Δ97 LMN-ΔH/C displayed distortion and deformation of nuclear shape, and thinning and ruffling of the nuclear lamina, similar to that seen in WT-virus infected cells (Fig. 2A, panels iv-vi and xiii-xv). To quantify the D/N lamin A-dependent changes in nuclear morphology, we surveyed 56-119 nuclei from each infection condition by confocal microscopy, and performed statistical analysis (Fig. 2B). The results showed that expression of ΔH/C lamin A in the absence of UL97 led to a ~2-fold increase in nuclear deformities as compared to cells infected with Δ97, and this increase was significant (Fig. 2B). However, fewer cells infected with Δ97 LMN-ΔH/C displayed nuclear deformities as compared to those infected with WT LMN-ΔH/C (or WT virus only), although these differences were not statistically significant.
Overall these results suggest that expression of D/N lamin A during infection with the Δ97 virus at least partially recapitulates modifications to the nuclear lamina characteristic of WT virus infection (Fig. 2B).

We then sought to determine if this D/N lamin A-mediated disruption of the nuclear envelope offered any replication advantage to a Δ97 virus. Dividing HFF cells were infected (MOI=3) with WT, Δ97, WT LMN-ΔH/C, or Δ97 LMN-ΔH/C viruses. Viral yields were assessed at 96 h.p.i. Similar to previous results (10, 16, 17), Δ97 replicated to ~20-fold lower yields than did WT (Fig. 2C). Expression of the D/N lamin A in a WT background had little effect on virus yield. Thus, the D/N mutant does not merely increase replication of any HCMV non-specifically. However, the Δ97 virus expressing D/N lamin A (Δ97 LMN-ΔH/C) replicated to modestly but significantly higher levels (~2.5 fold) than did Δ97 virus. This result suggests that lamina disruption due to the D/N lamin mutant alleviates the defect in virus replication in the absence of UL97, and thus that disruption of the nuclear lamina by UL97 is important for HCMV replication.

Despite the significant increase in virus replication over UL97 null infection, the Δ97 LMN-ΔH/C virus still replicated to significantly lower levels when compared to its WT counterpart (WT LMN-ΔH/C) or the WT virus (6.5 and 8 fold reduced replication, respectively) (Fig. 2C). This incomplete complementation of the Δ97 defect could arise from insufficient disruption of endogenous lamina by D/N lamin A or because generalized disruption of nuclear lamina by D/N lamin A might differ from localized disruption due to NEC recruitment of UL97 (11, 17, 39). On the other hand, it could also arise because UL97 substrates other than lamin A/C are relevant to nuclear egress and replication in dividing cells.
UL50 and UL53 are phosphorylated in a UL97-dependent manner during HCMV infection. The above results and our recent observations of UL50- and UL53-dependent recruitment of UL97 to the nuclear rim (8) led us to investigate if HCMV UL50 and UL53 are substrates for UL97 during infection. We infected cells with UL53-FLAG AD169-RV (53-F) in the presence or absence of 1µM maribavir (MBV), a selective UL97 kinase inhibitor (40), and performed FLAG immunoprecipitation (FLAG-IP) at 72 h.p.i. The eluate from the FLAG-IP was separated by SDS-PAGE, and bands corresponding to UL50, and UL53 were sent for MS analysis. These experiments were performed three times with similar results. We present results from one representative experiment, summarized in Table 2 and in Supplemental Fig. S1 at https://coen.med.harvard.edu. The coverages for UL50 and UL53 in this experiment were 67% and 69%, respectively. We detected one UL50 residue, Ser216 that was phosphorylated during HCMV infection in the absence of MBV (Table 2). However, in the presence of MBV, peptides containing phosphorylated Ser216 were not found, although the corresponding unphosphorylated peptides could still be readily detected. Three UL53 residues were found to be phosphorylated during infection in the absence of MBV, with Ser19 being more highly phosphorylated than the other two residues, Ser15 and Ser51 (Table 2). MBV effectively eliminated UL53-Ser19 phosphorylation, while Ser15 and Ser51 were phosphorylated even in the presence of MBV (Table 2), suggesting that they may be sites of phosphorylation by cellular protein kinases. We also compared the phosphorylation of UL50 and UL53 during infection with WT FLAG-53 AD169-RV and the kinase-inactive mutant UL97 K355Q FLAG-53 AD169-RV, and again found that UL53-Ser19 and UL50-Ser 216 were phosphorylated in a UL97-
dependent manner (data not shown). In these various MS analyses, detection of sites of UL97 autophosphorylation (41) served as a positive control for UL97 activity, while lack of autophosphorylation served as an indicator of the effectiveness of MBV or the K355Q mutation (data not shown). These results indicate that phosphorylation of UL50 and UL53 on particular residues during HCMV infection requires UL97.

UL97 phosphorylates UL53 on Ser19 in vitro. We next wished to investigate, using purified proteins expressed in heterologous systems, if UL97 is sufficient for phosphorylating the NEC on the residues identified in infected cells. Unfortunately, the form of UL50 that we had previously expressed and purified does not contain Ser216 (37), and efforts to express and purify a longer form were not successful. However, we were able to express and purify a polypeptide containing UL53 residues 1-292 [UL53 (1-292)] and study its phosphorylation by purified GST-UL97 in vitro. In the presence of radiolabeled ATP, both UL97 and UL53 (1-292) became phosphorylated, and this phosphorylation required the presence of UL97 and was inhibited by MBV (Fig. 3A). MS analysis of non-radiolabeled samples revealed that UL53-Ser19 was phosphorylated efficiently, and MBV eliminated this phosphorylation (Table 3). Of note, the sequence downstream of Ser19 conforms to the previously reported UL97 preference for an Arg or Lys residue at P+5 (42). We also observed MBV-sensitive phosphorylation of Ser15, as well as inefficient phosphorylation of peptides containing residue Ser31, and residues Ser35, Thr36, Thr41, and Ser42 in the absence of MBV (Table 3).

Phosphorylation of sites such as Ser15 by UL97 in vitro that have not been found to be
phosphorylated in a UL97-dependent manner in infected cells is not uncommon (11, 20). Phosphorylation of the peptide containing residue Ser31 was not eliminated by MBV (Table 3), which could be due to a contaminating kinase that may have co-purified either with UL53 (1-292) or with UL97 (as previously reported (20)).

Overall, these results, in conjunction with the results from infected cells, show that UL97 is both necessary and sufficient for phosphorylation of at least one NEC residue, UL53-Ser19. We conclude that UL97 phosphorylates the NEC during infection.

UL97 activity is important for continuous distribution of UL50 and UL53 at the nuclear rim. We next investigated whether UL97 affects the distribution of NEC subunits in infected cells. We mock-infected or infected cells with WT AD169-RV, or with viruses where UL97 expression or activity was genetically ablated (Δ97 and K355Q, respectively) (15, 41), and stained for UL50 and UL53 at 72 h.p.i. (Fig. 4A).

While most UL50 and UL53 showed relatively continuous and tight distributions at the nuclear rim of cells infected with WT virus (Fig. 4A, panels i and ii, respectively), infection with Δ97 or K355Q viruses led to a markedly punctate distribution of both UL50 (Fig. 4A, panels iii, and v) and UL53 (Fig. 4A, panels iv and vi, respectively) at the nuclear rim. As observed previously (8), the rabbit antisera against UL50 and UL53 also detected staining in a region adjacent to the nucleus that may correspond to the assembly compartment, but only faint staining was observed using a mouse monoclonal antibody that detects epitope-tagged UL53 (see below). To compare this result with the effect of pharmacological inhibition of UL97 activity, we infected HFF cells with HCMV expressing FLAG-tagged UL53 (UL53-FLAG AD169rv), in the presence or absence of
1μM MBV. In the absence of MBV, (DMSO treated cells), the NEC subunits showed a continuous staining at the nuclear rim (Fig. 4B panels iii and iv). However, MBV treatment led to a punctate appearance of both NEC proteins at the nuclear rim (Fig. 4B panels v and vi). Western blot assays showed that neither the UL97 mutations nor MBV meaningfully decreased levels of UL53 or UL50 (Supplemental Fig. S2A at https://coen.med.harvard.edu, and data not shown, respectively). These observations indicate that the UL97 kinase activity is important for the normal, continuous distribution of the NEC subunits at the nuclear rim.

Substitutions of NEC phosphorylation sites affect NEC localization. UL53-Ser19 is located within a nuclear localization sequence (NLS) of HCMV UL53 (43). UL50-Ser216 lies in a proline-rich region of the protein, which is conserved in and important for the viability of murine cytomegalovirus (MCMV) (44). Additionally both residues are relatively well conserved across beta-herpesvirus homologs of UL53 and UL50 respectively (not shown). We therefore examined the relevance of phosphorylation at these sites by first mutating either UL50-Ser216 or UL53-Ser19 to alanine, on respective UL53-FLAG AD169rv BACs. The NEC localization and replication kinetics of both single mutant viruses (S216A and S19A) were indistinguishable from the parental 53-F AD169 virus (referred to as WT from here on for describing the results) following infection at MOI=0.1 (Fig. S3A and B, respectively, at https://coen.med.harvard.edu). This could mean that each UL97 phosphorylation site (phosphosite) plays a redundant role in viral replication.
We then generated a double mutant virus containing alanine substitutions of both these residues, designated S216A/S19A or AA, and a rescued derivative of AA in which the alanines were restored to serines, designated S216AR/S19AR or RR (Table 1). To test the effects of the double alanine substitution, cells were infected (MOI=1) with the WT virus, mutant virus AA, or rescued virus RR, in the presence or absence of 1μM MBV.

At 72 h.p.i., the infected cells were stained with antibodies against lamin A/C and UL53-FLAG and observed using confocal microscopy. In the absence of MBV, cells infected with the WT and RR viruses typically showed continuous staining of UL53-FLAG, while cells infected with AA typically showed punctate staining (examples in Fig. 5A, panels i, iii, v (a color version of Fig. 5A is Supplemental Figure S4 at https://coen.med.harvard.edu); and Supplemental Figure 2B, panels i, iv, and vii at https://coen.med.harvard.edu). Similar staining was seen when using anti-UL50 antibody (data not shown). We counted the number of cells showing punctate or continuous staining, and performed statistical analysis (Fig. 5B). About 70% of cells infected with AA showed punctate UL53 staining at the nuclear rim, and this was significantly higher than that seen in either WT or RR-infected cells, only 5-10% of which exhibited punctate staining. Put another way, ~3-fold more WT- or RR-infected cells exhibited continuous staining of UL53-FLAG than did AA-infected cells. We also examined cells infected with the viruses in the presence of MBV. Here we found punctate staining for UL53 at the nuclear rim in cells infected with any of the three viruses (Fig. 5A, panels ii, iv, vi). The lamin A/C staining in cells infected with the mutant virus showed thick, intact nuclear lamina in the presence of MBV, and characteristic thinning, ruffling and lamina gaps in the absence of MBV, similar to what is seen in cells
infected with WT (11, 28, 45) and RR-infected cells (Supplemental Fig. 2B, panels ii, v, viii, and xi at https://coen.med.harvard.edu). Thus, alanine substitution of the phosphosites did not obviously affect lamina disruption by HCMV. Western blot assays showed that the AA mutations did not meaningfully decrease levels of UL53 or UL50 (Supplemental Fig. S2A at https://coen.med.harvard.edu, and data not shown, respectively). In summary, the AA mutations cause a punctate distribution of the NEC similar to that induced by a UL97 inhibitor.

**Effect of NEC phosphosite substitutions on virus replication.** We compared the replication kinetics of the alanine double mutant AA to the WT virus (Fig. 6). The alanine double mutant, AA displayed a 4- to 5-fold reduction in virus production as compared to the WT virus from 72 h p.i. onwards during a multiple-cycle infection assay (MOI=0.1). At a higher MOI (1), the mutant virus exhibited a delayed-replication phenotype with 3- to 4-fold reduced viral titers during the course of infection, but WT virus titers at the end of the time course (Fig. 6B), while the rescued derivative, RR, showed replication kinetics indistinguishable from those of the WT virus (Fig. 6B). We conclude that the alanine double mutation impairs viral replication.

**Alanine substitutions of the NEC phosphosites impair nuclear egress.** To investigate if alanine substitutions of both phosphosites lead to defects in nuclear egress, we conducted transmission electron microscopy (EM) on cells infected (MOI=1) with each of the three viruses-WT, AA or RR, on day 4 p.i. (examples of images are shown in Fig. 7A; panel i shows a portion of nucleus and cytoplasm; panel ii, perinuclear
space, and panel iii, a portion of cytoplasm and extracellular space). We analyzed 10 whole-cell sections for each infected sample and counted virus particles in the nucleus, cytoplasm, perinuclear space, or extracellular region in each section, and subjected the data to statistical analysis (Fig. 7B). As previously observed (9, 10, 17), there were relatively few cytoplasmic capsids compared to nuclear capsids in WT-infected cells. The double mutant (AA) did not show any significant differences in the numbers of capsids seen in the nucleus, perinuclear space, and the extracellular space, as compared to those in cells infected with the WT virus or the rescued derivative (RR) (Fig. 7B). However, cells infected with AA showed a modest (3-7-fold), but significant reduction in the mean number of cytoplasmic capsids. Moreover, 40% of the AA-infected cell sections contained no detectable cytoplasmic capsids, while all of the WT- and RR-infected cell sections did. These results, showing a decrease in cytoplasmic capsids without a significant effect on nuclear capsids due to the AA substitutions, resemble the effects of loss of UL97 activity (9, 10, 17). Thus, the mutations decrease nuclear egress, and this largely accounts for the replication defect of the double mutant.

**Effects of glutamate substitutions.** In certain cases, glutamate substitution of serine can mimic serine phosphorylation. We therefore generated a double mutant virus, EE, where both UL50-S216 and UL53-S19 were substituted with glutamate in a virus expressing UL53-FLAG, and assayed this mutant for distribution of the NEC and replication in the absence and presence of MBV. Unexpectedly, in the absence of MBV, a significantly higher percentage of cells infected with the EE mutant (examples in Fig. 5A panel vii, and Fig. S5A panel vii at https://coen.med.harvard.edu ) exhibited punctate UL53 staining at the nuclear rim, compared to WT virus-infected cells (Fig.
However, this percentage (~30%) was significantly lower than that of AA mutant-infected cells (~70%; Fig. 5B). In the presence of MBV, essentially all EE-infected cells exhibited a punctate UL53 distribution (Fig. 5A, panel viii and data not shown). The EE mutant displayed replication kinetics indistinguishable from the WT virus in multiple-cycle growth experiments in the presence or absence of MBV (Fig. S4A and S4B at https://coen.med.harvard.edu). Thus, while the glutamate substitutions did not appear to decrease viral replication in the absence of MBV, they also did not appear to rescue the effects of MBV on localization of the NEC or viral replication (see Discussion below).
Discussion

HCMV viral kinase UL97 is important for virus replication and nuclear egress (11, 16, 17). Its role in nuclear egress has generally been thought to be explained by phosphorylation and disruption of the nuclear lamina barrier. Our results showing that a D/N lamin A/C mutant designed to independently disrupt the nuclear lamina can complement the replication defect of a UL97 mutant, validates this explanation.

These results are similar to those from a previous study with the alphaherpesvirus, herpes simplex virus-1 (HSV-1), where the use of lamin A knockout cells partially restored replication of a virus lacking one of its viral kinases, US3 (46). We have also found that a UL97 null virus expressing WT lamin A replicated less efficiently (5-fold reduced yield) than a control UL97 null virus expressing no exogenous lamin (data not shown). These data taken together indicate that lamin A/C is ordinarily a barrier for viral replication, which was also a conclusion of the previous HSV-1 study (46). These findings with herpesviruses differ from those from studies of a cellular process that resembles nuclear egress in which lamin A appears to be a requirement rather than an impediment (47).

The incomplete complementation of the UL97 replication defect by the D/N lamin mutant was again similar to the results of the HSV-1 study using lamin A knockout cells (46). This incomplete complementation led us to search for other substrates of UL97 that might be relevant to nuclear egress, and we focused on NEC subunits. In HSV-1-infected cells, as we have found here, NEC subunits are phosphorylated, dependent on an active viral kinase (48, 49). With HSV-1, identification of NEC residues
phosphorylated in infected cells was indirect, and entailed testing the effects of substituting potential phosphosites on electrophoretic mobility and/or radiolabeling of the protein (48-50). Here we utilized mass spectrometry to directly identify residues in UL50 and UL53 that are phosphorylated in infected cells dependent upon UL97, and residues in UL53 that are phosphorylated by purified UL97 in vitro. As the same residue on an NEC subunit is directly phosphorylated in vitro and during infection dependent on the same viral kinase, we conclude that UL97 phosphorylates the viral NEC, Alanine substitution of UL97 phosphorylation sites in both UL50 and UL53 affected NEC localization during infection, and reduced viral replication and nuclear egress. These results by themselves suggest that UL97 phosphorylation of the NEC modulates NEC localization and nuclear egress. To see if we could demonstrate this more definitively, we constructed a virus, EE, in which the phosphosites were substituted with glutamate. Consistent with our suggestion, this mutant replicated normally and, in most (but not all) cells, displayed continuous NEC staining at the nuclear rim. However, if glutamates mimic phosphoserines that are formed by UL97 phosphorylation for their activities during infection, then we would have expected EE to exhibit normal NEC localization and replication in the presence of MBV. This was not observed. This result may be due to glutamate not successfully mimicking phosphoserine for NEC function. In particular, there are multiple chemical differences between glutamate and phosphoserine, and potentially important differences between the temporally controlled conversion of serine to phosphoserine (and perhaps vice-versa) during infection and the constant presence of glutamate. A second possibility is that we may have failed to detect other UL97 phosphorylation sites on the NEC that are required for NEC localization and efficient
nuclear egress. If so, failure to substitute those other sites with phosphomimetic residues would be required to rescue the localization and replication defects imposed by MBV. A third possibility is that normal localization of the NEC might also require lamin phosphorylation. In this case, glutamate substitution of the NEC phosphosites would not overcome MBV blockade of phosphorylation of lamin A/C by UL97. A fourth possibility is that the phosphorylation of these NEC residues is irrelevant to their function, and that serine is important for other reasons. We think this possibility is less likely because 1) the alanine substitutions so closely mimic the loss of UL97 for NEC localization and 2) the glutamate substitutions did not affect viral replication and only modestly affected NEC localization. Thus, although the results with the EE mutant do not permit a definitive conclusion, we favor the interpretation that phosphorylation of the NEC by UL97 modulates NEC localization and nuclear egress.

The alanine substitutions resulted in a ~3-fold reduction in the number of cells exhibiting continuous NEC localization, ~3-fold fewer cytoplasmic particles on average, and a ~3-fold defect in virus production at day 4.p.i. This correlation suggests that the NEC localization defect of the AA mutant causes the nuclear egress defect, which in turn is responsible for the virus production defect. As we observed typical lamin A/C thinning and deformities in AA mutant-infected cells, we infer that the substitutions affect a step of nuclear egress other than lamina disruption. This step could be primary envelopment. Alternatively, although we have not observed statistically significant increases in numbers of perinuclear particles, we have seen trends in that direction in AA-mutant infected cells (this study), in MBV-treated cells (9) and certain UL97 mutant-infected cells (17). Thus, it is possible that the nuclear egress defect could be at the
step of de-envelopment. Regardless, although it is difficult to know if replication defects
due to effects on lamina and NEC phosphorylation would be multiplicative, it is
interesting to note that the defect in replication conferred by the AA mutations is not
very different in magnitude from the difference in replication between WT virus and the
*UL97* mutant expressing the D/N lamin A.

In certain alphaherpesviruses (HSV-1, HSV-2, and pseudorabies virus (PrV)), viral-
kinase dependent phosphorylation of NEC subunits regulates the localization of the
NEC subunits, and/or modulates nuclear egress (46, 48, 50, 51), similar to our results
and interpretations. However, there are key differences. These alphaherpesviruses
encode two viral kinases, UL13 and US3. Mutation of the *UL97* homolog, *UL13*, in these
viruses affects virus replication in cell culture (reviewed in (52)), as is the case for UL97.
However, for NEC phosphorylation, UL13 may act indirectly during infection by effects
on US3 (51), which has no HCMV homolog. Mutation or deletion of the *US3* gene
affects NEC localization at the nuclear rim (46, 48, 51, 53), similar to our results with
*UL97*. Nevertheless, although deletion of NEC subunits leads to severe growth defects
in both HSV-1 and PrV, inactivation of US3 either only modestly affects viral replication
(HSV-1 and PrV) (48, 49, 54, 55), or has no effects on viral yields (HSV-2) (56, 57).
Additionally, loss of the US3 kinase in HSV-1 and PrV infections leads to altered
distribution of the NEC at the nuclear rim (similar to what we see in the absence of
*UL97*), an obvious accumulation of perinuclear virions, and the presence of NEC
subunits in these areas of perinuclear virion accumulation as seen by immune-EM,
suggesting that the NEC puncta correspond to these perinuclear accumulations (49, 53,
58). Moreover, in HSV-1, substitutions of NEC residues that reduce US3-dependent
phosphorylation cause the same phenotypes (48, 49). These results led to the
suggestion that HSV-1 US3-mediated phosphorylation of the NEC is important for
fusion of the primary enveloped particle with the outer nuclear membrane (de-
envelopment) (49, 58). However, although PrV US3 is important for de-envelopment
and PrV NEC subunits are phosphoproteins, it is unclear whether PrV US3
phosphorylates both NEC subunits or whether such phosphorylation is involved in de-
envelopment (53). Moreover, it is not clear whether the redistribution of the HCMV NEC
in the absence of UL97 corresponds to an accumulation of perinuclear particles. Thus
while the basic theme of NEC phosphorylation appears to be conserved across
herpesviruses, the molecular details and relevance of this event for virus replication
may differ.

In summary, our results suggest a mutual dependence of the interaction between
the HCMV NEC and viral kinase UL97, for their roles in nuclear egress. The NEC
recruits the viral kinase to the nuclear rim, to help facilitate UL97 phosphorylation of
lamin A/C and the NEC. UL97 phosphorylation of the NEC in turn would modulate NEC
localization and function at the nuclear rim during nuclear egress.
Acknowledgments

We thank David Gilbert for providing the GFP-lamin A constructs, and John Drach for providing maribavir. We are also grateful for the assistance of staff and the availability of equipment at the Taplin Biological Mass Spectrometry Facility for acquisition and analysis of mass spectrometry data, at the Nikon Imaging Center at Harvard Medical School during acquisition and analysis of immunofluorescence data, and at the Electron Microscope core Facility at Harvard Medical School during acquisition and analysis of EM data. This work was supported by NIH grant R01 AI026077 to DMC and JMH.
References


Mou, F., E. Wills, and J. D. Baines. 2009. Phosphorylation of the U(L)31 protein of herpes simplex virus 1 by the U(S)3-encoded kinase regulates localization of the nuclear envelopment complex and egress of nucleocapsids. J. Virol. 83:5181-91.


Table 1. Summary of HCMV BACs used in this study

<table>
<thead>
<tr>
<th>Construct</th>
<th>Genetic Background</th>
<th>Reference for background construct</th>
<th>Change/s introduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT LMN-ΔH/C</td>
<td>WT_E7 AD169-RV</td>
<td>Kamil et. al. 2009</td>
<td>Sequence encoding the dominant negative GFP-lamin A mutant, lacking the lamin A head and CaaX domains, cloned downstream of a duplicated UL97 promoter region in the US9-US10 intergenic region.</td>
</tr>
<tr>
<td>Δ97 LMN-ΔH/C</td>
<td>AD169-RV</td>
<td>Hobom et. al. 2000</td>
<td>Sequence encoding the dominant negative GFP-lamin A mutant, lacking the lamin A head and CaaX domains, cloned to replace the UL97 encoding sequence.</td>
</tr>
<tr>
<td>S216A</td>
<td>53-F AD169-RV</td>
<td>Sharma et. al., 2014</td>
<td>Alanine substitution of UL50 Serine-216 in the UL53-FLAG genetic background.</td>
</tr>
<tr>
<td>S19A</td>
<td>53-F AD169-RV</td>
<td>Sharma et. al., 2014</td>
<td>Alanine substitution of UL53 Serine-19 in the UL53-FLAG genetic background.</td>
</tr>
<tr>
<td>S216A/ S19A</td>
<td>53-F AD169-RV</td>
<td>Sharma et. al., 2014</td>
<td>Double alanine mutant carrying alanine substitutions of UL50 Serine-216 and UL53 Serine-19 in the UL53-FLAG genetic background.</td>
</tr>
<tr>
<td>S216AR/S19AR</td>
<td>AA-53-F AD169-RV</td>
<td>This study</td>
<td>Rescued derivative of the alanine double mutant described above, restoring WT serine encoding sequences to UL53 and UL50.</td>
</tr>
<tr>
<td>S216E/ S19E</td>
<td>53-F AD169-RV</td>
<td>Sharma et. al., 2014</td>
<td>Double mutant carrying glutamate substitutions of UL50 Serine-216 and UL53 Serine-19 in the UL53-FLAG genetic background.</td>
</tr>
<tr>
<td>K355Q 53-F</td>
<td>53-F AD169-RV</td>
<td>Sharma et. al., 2014</td>
<td>UL97 kinase active site mutation; (mutating lysine 355 to glutamine), in the UL53-FLAG AD169v genetic background.</td>
</tr>
</tbody>
</table>
Table 2. Phosphopeptides detected in MS analysis of samples from HCMV-infected cells in the presence or absence of MBV

<table>
<thead>
<tr>
<th>Residue</th>
<th>Phosphorylated Peptide detected in MS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent peptide phosphorylated&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-MBV</td>
</tr>
<tr>
<td>UL50 S216</td>
<td>TASpPPPPPR</td>
<td>100</td>
</tr>
<tr>
<td>UL53 S15</td>
<td>SpALR</td>
<td>17</td>
</tr>
<tr>
<td>UL53 S19</td>
<td>SALRSpLLR</td>
<td>100</td>
</tr>
<tr>
<td>UL53 S51</td>
<td>VASTVNGATSANHGEPPSpPADARPR</td>
<td>2.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Phosphorylated serine: Sp  
<sup>b</sup> Percentage of the phosphorylated peptide compared to the non-phosphorylated peptide was determined from peak intensities  
<sup>c</sup> ND: Phosphorylated peptide was not detected  

MBV: Maribavir
Table 3. Phosphopeptides detected in MS analysis of the *in vitro* kinase assay with GST-UL97 and UL53 (1-292).

<table>
<thead>
<tr>
<th>Residue</th>
<th>Phosphorylated peptide detected in MS(^a)</th>
<th>Percent peptide phosphorylated(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-MBV</td>
</tr>
<tr>
<td>S19</td>
<td>SALRSpLLR</td>
<td>100</td>
</tr>
<tr>
<td>S15/S19</td>
<td>SpALRSpLLR</td>
<td>100</td>
</tr>
<tr>
<td>S31</td>
<td>QRELASpK</td>
<td>11.7</td>
</tr>
<tr>
<td>S35/T36</td>
<td>VASpTvNGATpSpANNHGEPPSPADARPR</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>and T41/S42(^c)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Phosphorylated serine: Sp; Phosphorylated threonine: Tp

\(^b\)Percentage of phosphorylated peptide over non-phosphorylated peptide was determined from peak intensities

\(^c\)The phosphorylated serine/threonine residue could not be positively localized

\(^d\)ND: Phosphorylated peptide was not detected

MBV: Maribavir
**Figure legends**

**FIG 1.** Construction of HCMV expressing dominant negative (D/N) lamin A.  
A. Schematic showing the domain arrangement for full-length lamin A. Lamins contain central α-helical "rod" domains flanked by the head and tail domains, with a CaaX domain at the carboxyl-terminus that is a site for isoprenylation. The D/N mutant used in these studies lacks the head and CaaX domains.  
B. Construction of HCMV AD169 either WT (left) or null for **UL97** (Δ97; right) carrying a D/N GFP-lamin A sequence. On the top line on the left and right, the organization of the HCMV genome is depicted. TRL, terminal repeat long; UL, unique long; IRL/S, internal repeat long and neighboring internal repeat short; US, unique short; TRS, terminal repeat short. Below this, the **US9-US12 region** and the **UL95-UL98 region** of the viral genome are expanded on the left and right, respectively. Open reading frames are shown as white arrows. The D/N GFP-lamin A mutant sequences (ΔH/C; depicted with the shaded arrow for GFP, and the domain structure of the lamin mutant from A.), were introduced between the **US9** and **US10** genes on the AD169 BAC that is WT for **UL97**, downstream of the UL97 promoter region (white rectangle), to generate WT HCMV viruses expressing the D/N lamin A mutant. The Δ97 virus expressing the D/N mutant was generated by replacing the UL97 encoding sequences with D/N GFP lamin A (ΔH/C) sequences.

**FIG 2.** Effects of D/N lamin A mutant during HCMV infection.  
A. Effect on endogenous lamin A/C and nuclear deformities. HFF cells were infected with either WT or Δ97 HCMV, or viruses WT or null for **UL97** expressing D/N GFP-lamin A (WT LMN-ΔH/C or Δ97 LMN-ΔH/C) at an MOI of 1. At 72 h p.i., cells were fixed, and stained for...
endogenous lamin A/C (red) and imaged for GFP (green). Images were acquired using confocal microscopy. B. Quantification of effects on nuclear deformities. Infected cells from confocal microscopy images (n=56-119) were assessed for nuclear deformities and analyzed for significance using Fisher's Exact tests. The p values for comparison are indicated. No label indicates no significant difference. For a family-wise type I error rate of 0.05 for each set of 5 comparisons, a result can only be considered significant when p<0.0102. C. Effect on replication. Dividing HFF cells were infected with the same viruses as in panel A, at an MOI of 3. At 96 h.p.i., supernatants were collected and titrated by plaque assays. The results represent averages ± S.D. of 3 independent experiments. Log titer values were assessed for statistical significance using one-way ANOVA followed by Sidak's multiple comparison tests (5 comparisons). The p values are indicated. No label indicates no significant difference.

FIG 3. UL97 phosphorylates UL53 in vitro. A. UL53 1-292 and GST-UL97, GST-UL97 alone, or UL53 1-292 alone were incubated with radiolabeled ATP either in the presence (MBV) or absence (DMSO) of 1μM MBV, as indicated in the key at the top, and the products resolved by SDS-PAGE. The phosphorimage of the gel (bottom) shows bands corresponding to autophosphorylated GST-UL97 (UL97) and phosphorylated UL53 1-292 (UL53), which were not detected in the presence of MBV. B. GST-UL97 and UL53 (1-292) were incubated with unlabeled ATP in the presence or absence of MBV, as indicated in the key at the top, and the products resolved by SDS-PAGE. The Coomassie-stained gel (bottom) shows bands for UL53 1-292 (UL53) from this reaction, which were submitted for mass spectrometry.
FIG 4. Localization of HCMV UL50 and UL53 in the absence of UL97 kinase. A. HFF cells were infected with WT virus, or with a UL97 null virus (Δ97), or with a virus carrying a kinase inactivating mutation in UL97, K355Q, at an MOI of 1. At 72 h.p.i., cells were fixed and stained for UL50 (red, panels i, iii and v) or UL53 (red, panels ii, iv and vi) and the nucleus was stained with DAPI (blue). B. HFF cells were infected with UL53-FLAG AD169-RV (WT) at an MOI of 1, in the presence or absence (DMSO) of 1μM MBV. At 72 h. p.i., cells were fixed and stained for UL50 (red, panels i, iii, and v) or FLAG (red, panels ii, iv and vi) and the nucleus was stained with DAPI (blue).

FIG 5. Effects of NEC substitutions on NEC distribution. A. HFF cells were infected with WT, AA, RR, or EE 53-F BADGFP (MOI=1), in the absence or presence of 1μM MBV. At 72 h.p.i. cells were fixed and stained with FLAG and the nucleus was stained with DAPI. A color version of this panel is Supplemental Figure S4 at https://coen.med.harvard.edu.

B. Infected cells (n=42-102) were assessed for whether they showed any punctate staining for UL53-FLAG at the nuclear rim (black bars), or a continuous distribution at the nuclear rim (gray bars) in the absence of MBV. The differences between the data were analyzed using Fisher’s exact test. * indicates p<0.0001. No label means no significant difference. For a family-wise type I error rate of 0.05 for each set of 5 comparisons, a result can only be considered significant when p<0.0102.

FIG 6. Effect of phosphorylation site substitutions in UL50 and UL53 on virus replication. A. HFF cells were infected with either WT or a mutant virus carrying alanine
substitutions of both UL53 residue S19 and UL50 residue S216 (AA) at an MOI of 0.1. At the indicated time points, supernatants were collected and titrated for virus. Titers were calculated by averaging plaque counts from triplicate titrations. Because counts from three sets of titrations differed less than twofold for all data points, error bars are not shown. B. HFF cells were infected with WT virus or the double mutant AA, or its rescued derivative, RR, at an MOI of 1. At the indicated time points, supernatants were collected from triplicate wells and titrated for virus. Titers were calculated by averaging plaque counts from triplicate titrations. Because counts from three sets of titrations differed less than twofold for all data points, error bars are not shown.

FIG 7. EM analysis of cells infected with HCMV. A. Representative EM images of HFF cells infected with WT HCMV at an MOI of 1, fixed for EM at day 4 p.i., showing nuclear, cytoplasmic, perinuclear and extracellular capsids (indicated by black arrows). Nuc: Nucleus, Cyt: Cytoplasm. B. HFF cells infected at an MOI of 1 with either of the three viruses-WT, AA or RR, were fixed on day 4 p.i. Ten EM sections that each represented a whole cell were randomly selected and fully photographed in parts with no overlap at a magnification of ×11,000. Viral capsids in the nucleus, perinuclear space, and cytoplasm or outside the cell (extracellular) were counted. Bars represent mean capsid counts. Statistical analysis for each location was performed using Kruskal-Wallis tests with Dunn’s multiple comparisons post-tests. The p values are indicated. No label indicates no significant difference.
<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL97</td>
<td>+ + + + -</td>
<td>+ + + + -</td>
</tr>
<tr>
<td>UL53</td>
<td>+ + - - +</td>
<td>+ + + + -</td>
</tr>
<tr>
<td>MBV</td>
<td>- + - - -</td>
<td>- + - + -</td>
</tr>
<tr>
<td>DMSO</td>
<td>+ - - - -</td>
<td>+ - - - -</td>
</tr>
</tbody>
</table>

**Autoradiograph**
- UL97
- UL53

**Coomassie stained**
- UL53