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Herpes Simplex Virus 1 Induces Phosphorylation and Reorganization of Lamin A/C through the $\gamma_1$34.5 Protein That Facilitates Nuclear Egress

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ABSTRACT

Herpes simplex virus 1 (HSV-1) remodels nuclear membranes during virus egress. Although the UL31 and UL34 proteins control nucleocapsid transit in infected cells, the molecular interactions required for their function are unclear. Here we report that the $\gamma_1$34.5 gene product of HSV-1 facilitates nucleocapsid release to the cytoplasm through bridging the UL31/UL34 complex, cellular p32, and protein kinase C. Unlike wild-type virus, an HSV mutant devoid of $\gamma_1$34.5 or its amino terminus is crippled for viral growth and release. This is attributable to a defect in virus nuclear egress. In infected cells, wild-type virus recruits protein kinase C to the nuclear membrane and triggers its activation, whereas the $\gamma_1$34.5 mutants fail to exert such an effect. Accordingly, the $\gamma_1$34.5 mutants are unable to induce phosphorylation and reorganization of lamin A/C. When expressed in host cells $\gamma_1$34.5 targets p32 and protein kinase C. Meanwhile, it communicates with the UL31/UL34 complex through UL31. Deletion of the amino terminus from $\gamma_1$34.5 disrupts its activity. These results suggest that disintegration of the nuclear lamina mediated by $\gamma_1$34.5 promotes HSV replication.

IMPORTANCE

HSV nuclear egress is a key step that determines the outcome of viral infection. While the nuclear egress complex mediates capsid transit across the nuclear membrane, the regulatory components are not clearly defined in virus-infected cells. We report that the $\gamma_1$34.5 gene product, a virulence factor of HSV-1, facilitates nuclear egress cooperatively with cellular p32, protein kinase C, and the nuclear egress complex. This work highlights a viral mechanism that may contribute to the pathogenesis of HSV infection.

Herpes simplex virus 1 (HSV-1) replicates and packages its DNA in the cell nucleus. Once assembled, the nucleocapsids traverse the nucleoplasm and cross the nuclear lamina. The capsids bud through the nuclear membranes in a two-step process called envelopment and de-envelopment (1). In this process, the nuclear egress complex, consisting of UL31 and UL34, mediates vesiculation of the inner nuclear membrane and results in enveloped virions in the perinuclear space. Primary virions fuse with the outer nuclear membrane, which releases the capsids to the cytoplasm for further maturation (2). Accumulating evidence suggests that additional proteins, including Us3, ICP22, UL47, gB, and gH, coordinate with the UL31/34 complex to facilitate nuclear egress in infected cells (3–6).

The nuclear lamina is a dense meshwork underlying the inner nuclear membrane (7). It is composed primarily of type V intermediate filament proteins, lamin A/C and lamin B. Besides providing structural support to the nucleus, the nuclear lamina potentially presents a barrier to the transit of virus capsids. A number of studies suggest that herpesviruses alter the nuclear lamina to promote nuclear egress (8–11). For example, HSV-1 activates protein kinase C (PKC) isoforms and induces phosphorylation of lamin B, which is dependent on the UL31/UL34 complex (12). UL31 and UL34 also bind to lamin A/C and lamin B, which interrupts lamin-lamin interaction and perforates the lamina (8, 10). On the other hand, Us3, a serine/threonine kinase of HSV-1, phosphorylates lamin A/C to dissolve the nuclear lamina (3). Remarkably, isoforms of PKC also participate in nuclear envelope budding or breakdown of host cells that occurs in ribonucleoprotein export, mitosis, and apoptosis (13–18). These observations illustrate that the remodeling of the nuclear envelope is an evolutionarily conserved event. Nevertheless, the regulatory network remains largely unclear.

Previous studies suggest that the $\gamma_1$34.5 protein of HSV-1 facilitates nuclear egress (19). Deletion of the $\gamma_1$34.5 gene results in an accumulation of nucleocapsids and subsequent reduction in infectious virus. The $\gamma_1$34.5 gene encodes a virulence factor with an amino-terminal domain, linker (ATP) repeats, and a carboxyl-terminal domain (20, 21). When expressed, the $\gamma_1$34.5 protein shuttles between the nucleus and cytoplasm, presumably to perform distinct functions (22, 23). It is well established that $\gamma_1$34.5 acts as a regulatory subunit of protein phosphatase 1 to promote protein synthesis in HSV-infected cells (24, 25). Moreover, $\gamma_1$34.5

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negatively modulates TANK binding kinase 1 and I-κB kinase, which inhibits the expression of cytokines, and dendritic cell maturation (26–29). HSV $\gamma_1$34.5 also inhibits autophagy through binding to beclin-1 (30). Additionally, the $\gamma_1$34.5 protein mediates nuclear egress independently of the interferon response (31). This involves the host protein p32, also known as gC1qR, which promotes HSV nuclear egress (32, 33).

This study was undertaken to investigate the mechanism of $\gamma_1$34.5 action. Here we report that the $\gamma_1$34.5 protein facilitates HSV nuclear egress through its amino-terminal domain. We show that this functional module is crucial to reorganize the nuclear lamina, translocate PKC to the nuclear membrane, and activate its activity. Furthermore, we provide evidence that while $\gamma_1$34.5 binds p32 and PKC, it also interacts with the UL31/UL34 complex in infected cells. These results suggest that regulation of lamin phosphorylation by $\gamma_1$34.5 is a mechanism to promote HSV replication.

MATERIALS AND METHODS

Cells and viruses. HeLa and Vero cells were originally obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). HSV-1(F) is a prototype HSV-1 strain used in this study (34). In recombinant virus R3616, a 1-kb fragment from the coding region of the $\gamma_1$34.5 gene was deleted (21). In H1001, the sequences of the $\gamma_1$34.5 gene encoding amino acids 1 to 146 were deleted, and in H1002 the deleted region was repaired with wild-type $\gamma_1$34.5 (29).

Reagents. The plasmids FLAG-$\gamma_1$34.5, FLAG-Δ146, and FLAG-N159 were described previously (35). The FLAG-UL31 plasmid was constructed by inserting a PCR-amplified fragment into the BamHI and EcoRI sites of pCMV-tag2B. The FLAG-UL34 plasmid was constructed by inserting a PCR-amplified fragment into the EcoRV and NotI sites of pCDH-FLAG. Mouse anti-human p32 antibody (sc-271200), anti-p32 antibody (sc-23885), rabbit anti-human p32 antibody (sc-48795), mouse anti-porcine lamin A/C antibody (sc-7292), rabbit anti-human PKCδ (C-20) antibody (sc-937), anti-phospho-PKCδ (A-8) antibody (sc-377560), anti-phospho-PKCδ (H-9) antibody (sc-374613), agarose conjugated with protein A/G (sc-2003), donkey anti-rabbit IgG-FITC (sc-2090), donkey anti-rabbit IgG-R (sc-2095), donkey anti-mouse IgG–fluorescein isothiocyanate (FITC) (sc-2099), bovine anti-chicken IgY-TR antibody (sc-3914), bovine anti-chicken IgY–horseradish peroxidase (HRP) (sc-2497), goat anti-mouse IgG–HRP (sc-2005), goat anti-rabbit IgG–HRP (sc-2004), rottlerin, and bisindolylmaleimide I (BIM I) were purchased from Santa Cruz Biotechnology. Anti-phospho-lamin A/C (Ser22) antibody (CST2026), and anti-histone H3 (3H1) antibody (CST9717) were purchased from Cell Signaling Technology. Anti-γ-tubulin and anti-FLAG–HRP antibodies were purchased from Sigma. Anti-UL31 rabbit antibody and anti-UL34 hen antibody were described previously (36).

Immunoblotting. Cells were harvested, washed with phosphate-buffered saline (PBS), and lysed with ice-cold immune precipitation assay buffer (50 mM Tries-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, and protease inhibitor cocktail) for 30 min on ice. After centrifugation, supernatants were mixed with disruption buffer (50 mM Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 0.1% bromophenol blue, 10% glycerol, and 100 nM β-mercaptoethanol) and boiled. The proteins were separated by 12% or 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and incubated with primary and secondary antibodies. Protein bands were detected by enhanced chemiluminescence (26).

Immunoprecipitation analysis. To examine protein interactions, transfected or infected HeLa cells were harvested and lysed with ice-cold immune precipitation assay buffer on ice. After centrifugation, cell extracts were incubated with the indicated antibodies and agarose conjugated with protein A/G at 4°C. The immobilized protein beads were subjected to immunoblotting analysis (32).

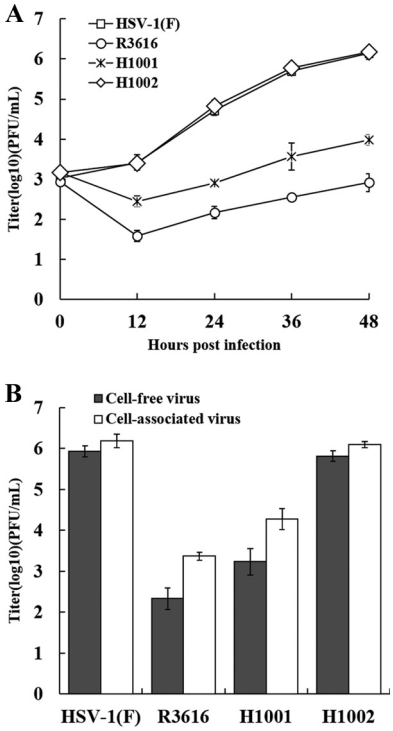

FIG 1 Viral replication is dependent on the amino terminus of $\gamma_1$34.5. (A) Monolayers of HeLa cells were infected with wild-type HSV-1(F), R3616 (the $\gamma_1$34.5 null mutant), H1001 (the $\gamma_1$34.5 mutant devoid of amino acids 1 to 146), or H1002 (the repaired virus with wild-type $\gamma_1$34.5) at 0.05 PFU per cell and incubated at 37°C. At the indicated time points postinfection, the total virus yields were determined on Vero cells. (B) HeLa cells were infected as described above. At 48 h postinfection, cell-associated viruses and viruses in the supernatant were collected separately and titrated on Vero cells. Data represent the mean values from three independent experiments with the standard deviations.

Virus growth assay. HeLa cells were infected with different viruses at the indicated multiplicity of infection. After adsorption for 2 h, the monolayers were overlaid with DMEM (plus 1% FBS) and incubated at 37°C. Cells were harvested at different hours postinfection, and viruses, released by three cycles of freezing and thawing, were titrated on Vero cells (31).

Electron microscopy analysis. HeLa cells were infected with viruses at 5 PFU per cell. At 16 h postinfection, samples were fixed in 2.5% glutaraldehyde with 100 mM PBS (pH 7.4), treated with 1% osmium tetroxide in phosphate buffer, dehydrated in ethanol, and embedded in LX112 resin (Ladd Research Industries). Samples were removed from the petri dishes and remounted on aluminum stubs. Ultrathin sections were cut with a Leica Ultracut UCT, placed on 200-mesh copper grids, and stained with uranyl acetate and lead citrate. Grids were viewed with a Jeol 1220 transmission electron microscope at 80 kV. Images were taken at various magnifications with a digital charge-coupled device (CCD) camera (Gigital Micrograph software; Gatan Inc.).

Confocal microscopy. Cells were washed with PBS, fixed with ice-cold 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 in PBS, and incubated with primary antibodies at 4°C overnight. Cells were then reacted with fluorescence-conjugated secondary antibodies for 2 h at room temperature. After washing with PBS, cells were mounted and visualized with a Zeiss LSM 710 Meta confocal microscope.

RESULTS

The amino-terminal domain of $\gamma_1$34.5 is important to viral growth. To better define the function of $\gamma_1$34.5, we analyzed the kinetics of viral growth in HeLa cells infected with $\gamma_1$34.5 variants. As illustrated in Fig. 1A, wild-type HSV-1(F) replicated efficiently.

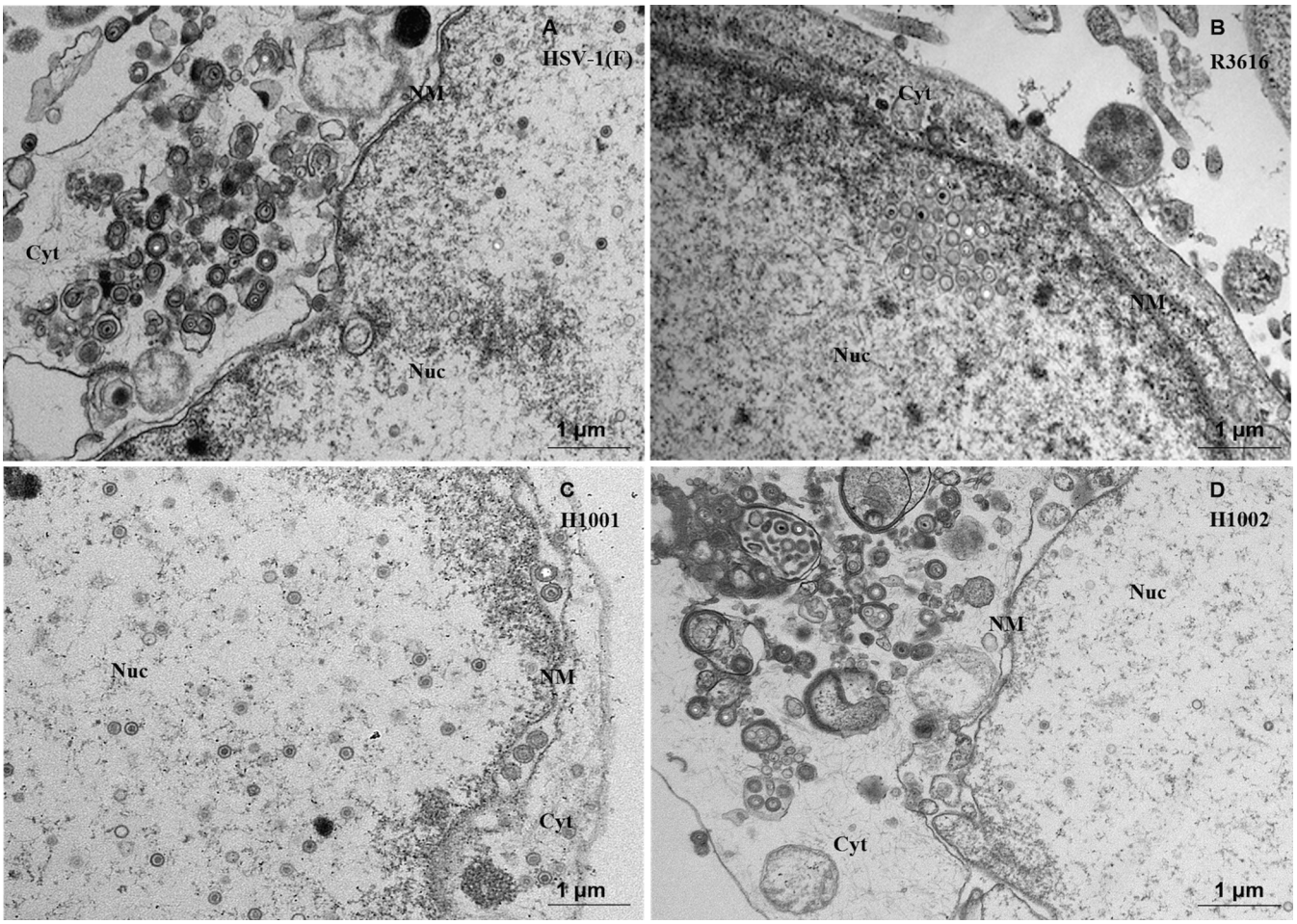

FIG 2 Effect of $\gamma_1$34.5 variants on nuclear egress. Confluent monolayers of HeLa cell were infected with HSV-1(F), R3616, H1001, or H1002 at 5 PFU per cell. At 16 h postinfection, cells were harvested and processed for electron microscopy analysis as described in Materials and Methods. Abbreviations: Nuc, nucleus; Cyt, cytoplasm; NM, nuclear membrane. Scale bars are shown in each panel.

As infection progressed, the viral yield increased to a titer of 1×10^6 PFU/ml at 48 h after infection. Under these conditions, R3616, the $\gamma_1$34.5 null mutant, barely replicated. H1001, which lacks amino acids 1 to 146, replicated modestly, reaching a titer of 1×10^4 PFU/ml. This mutant replicated to levels about 100-fold lower than those for the wild-type virus but 10-fold higher than those for the $\gamma_1$34.5 null mutant. We assayed for cell-associated and cell-free viruses in infected cells. Figure 1B shows that HSV-1(F) and repaired mutant H1002 produced comparable levels of cell-associated and cell-free viruses. Compared to HSV-1(F), R3616 or H1001 exhibited about a 10-fold reduction in cell free viruses. These phenotypes were also seen in mouse 3T6 cells (data not shown).

Deletion of the amino-terminal domain from $\gamma_1$34.5 impairs nuclear egress. Next, we examined nuclear egress by transmission electron microscopy. As presented in Fig. 2, HSV-1(F) infection produced a large fraction of virions in the cytoplasm and on the cell surface (Fig. 2A) in addition to nucleocapsids. R3616 infection produced virus particles that were mainly in the nucleus (Fig. 2B). Only a few were present in the cytoplasm or extracellular space. H1001 exhibited a similar phenotype, with viral particles predominantly confined to the nucleus. On the other hand, H1002 behaved more like wild-type virus. This pattern was evident when enumerated from 10 cells infected with each virus, as summarized in Table 1. In HSV-1(F)-infected cells, 44% of virus particles were in the nucleus, 3% in the perinuclear region, and 52% in the cytoplasm. In R3616-infected cells, 88% of virus particles were in the nucleus, 3% in the perinuclear region, and 9% in the cytoplasm. Similarly, in cells infected with H1001, 64% of virus particles were in the nucleus, 7% in the perinuclear region, and only 29% in the cytoplasm. In H1002-infected cells, viral particles were distributed

TABLE 1 Subcellular localization of viral particles in infected cells

Virus	No. (%) of viral particles in:			Total particles counted/10 cells[a]
	Nucleus	Perinuclear space	Cytoplasm	
HSV-1(F)	422 (44)	30 (3)	497 (52)	949
R3616	102 (88)	3 (3)	11 (9)	116
H1001	206 (64)	20 (7)	94 (29)	320
H1002	431 (47)	41 (4)	445 (49)	917

[a] The number of viral particles in the nucleus, perinuclear space, and cytoplasm were counted in electron micrographs of 10 randomly sampled HeLa cells infected with the indicated virus.

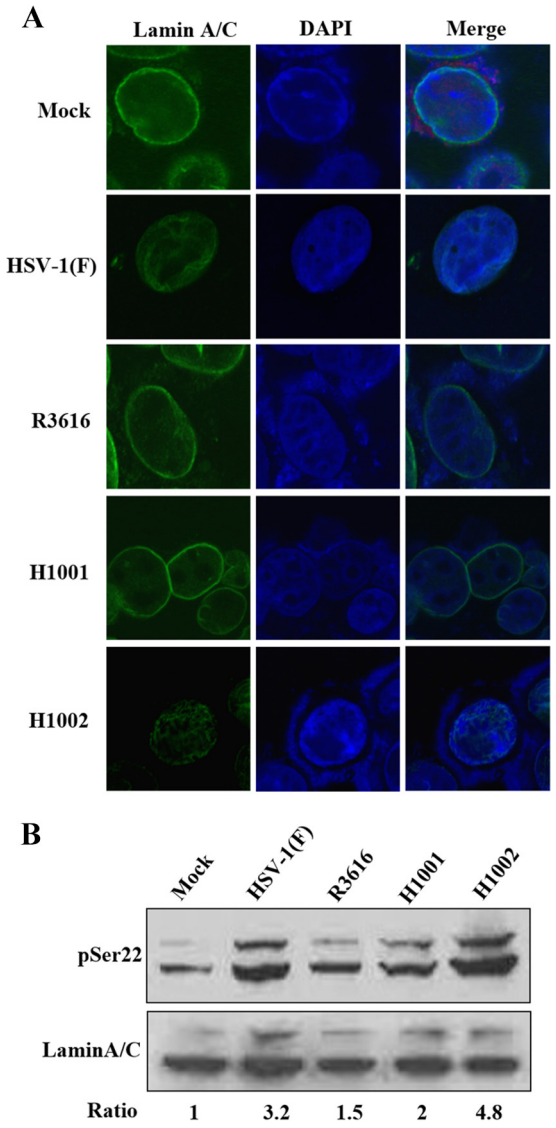

FIG 3 (A) Effect of $\gamma_1$34.5 variants on reorganization of lamin A/C. HeLa cells were mock infected or infected with HSV-1(F), R3616, H1001, or H1002 (5 PFU/cell). At 16 h postinfection, cells were processed and stained with DAPI (4′,6′-diamidino-2-phenylindole) and anti-lamin A/C antibody. Cells were visualized, and images were captured with confocal microscopy. (B) Effect of $\gamma_1$34.5 variants on phosphorylation of lamin A/C. HeLa cells were infected as for panel A. Lysates of cells were prepared and processed for Western blot analysis with antibodies against lamin A/C and phosphorylated lamin A/C (serine 22), respectively. Phosphorylated lamin A/C (serine 22) and lamin A/C were quantitated using Image J software. Phosphorylation of lamin A/C is presented as a ratio to lamin A/C, with normalization to mock infection.

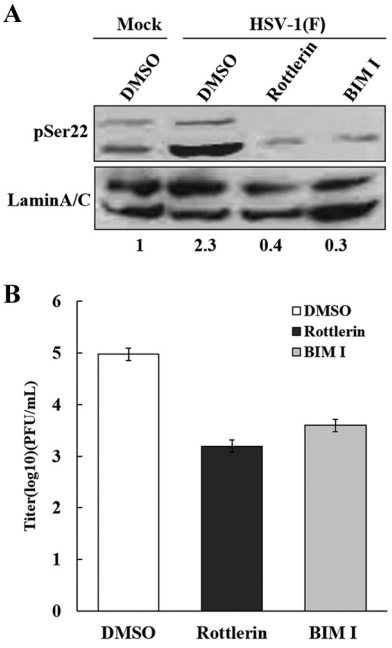

FIG 4 PKC activity is required for virus replication and lamin phosphorylation. (A) HeLa cells were mock infected or infected with HSV-1(F) (5 PFU/cell). Cells, treated with dimethyl sulfoxide (DMSO), rottlerin, and BIM I, were harvested at 16 h postinfection and subjected to Western blot analysis with antibodies against phosphorylated lamin A/C (serine 22) and lamin A/C. Phosphorylation of lamin A/C (serine 22) is presented as a ratio to lamin A/C, with normalization to mock infection. (B) HeLa cells were infected with HSV-1(F) at 0.05 PFU per cell. At 5 h postinfection, cells were treated with DMSO, rottlerin (10 μM), and BIM I (10 μM), and viral yields were determined at 24 h postinfection. Data represent the mean values from three independent experiments with the standard deviations.

evenly. These data suggest that $\gamma_1$34.5 mediates nuclear egress, which relies on the amino-terminal domain.

The $\gamma_1$34.5 protein mediates distortion of the nuclear lamina, which involves the amino terminus. HSV nuclear egress disrupts the nuclear lamina, which results from phosphorylation of lamin A/C and lamin B (8, 37). To investigate the role of $\gamma_1$34.5, HeLa cells were mock infected or infected with $\gamma_1$34.5 variants. Cells were stained with anti-lamin A/C antibody and visualized by confocal microscopy. As indicated in Fig. 3A, lamin A/C densely lined along with the nuclear rim in mock-infected cells. Infection with HSV-1(F) changed the appearance of lamin A/C, with a fraction dispersed into the nucleoplasm. However, in cells infected with the mutant devoid of $\gamma_1$34.5 (R3616) or its amino terminus (H1001), such alteration was not seen. As expected, infection with H1002, with a repaired amino terminus of $\gamma_1$34.5, induced reorganization of lamin A/C.

As reorganization of lamin is accompanied by phosphorylation (11–13), we assessed the effect of $\gamma_1$34.5 variants on lamin A/C in infected HeLa cells. Figure 3B shows that in mock-infected cells, lamin A/C was minimally phosphorylated at serine 22. At 16 h postinfection, HSV-1(F) or H1002 induced two prominent bands, representing phosphorylated lamin A/C. The relative phosphorylation increased by 3- to 4-fold compared to that in mock infection. Nevertheless, phosphorylated bands were modestly induced upon infection with R3616 or H1001, with the ratio of phosphorylation increased by 1- to 2-fold. This weaker phosphorylation suggests that the amino terminus of $\gamma_1$34.5 is required. We conclude that lamin reorganization and its phosphorylation rely on the amino terminus of $\gamma_1$34.5.

The $\gamma_1$34.5 protein directs translocation and activation of PKC. Protein kinase C (PKC) mediates the remodeling of nuclear membranes (11–13). Accordingly, treatment of HeLa cells with rottlerin and bisindolylmaleimide I (BIM I), inhibitors of protein kinase C, precluded phosphorylation of lamin A/C induced by wild-type virus (Fig. 4A). This paralleled with a 20- to 80-fold reduction in viral production (Fig. 4B), although cell viability was

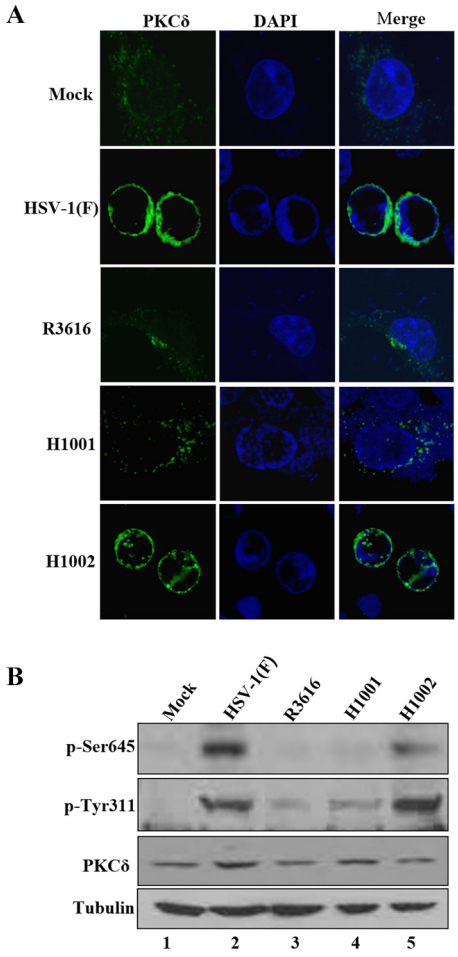

FIG 5 The $\gamma_1$34.5 protein mediates the recruitment and activation of PKCδ in infected cells. (A) HeLa cells were mock infected or infected with the indicated viruses (5 PFU/cell). At 16 h postinfection, cells were stained with DAPI and anti-PKCδ antibody. Cells are then visualized with confocal microscopy. (B) HeLa cells were mock infected or infected with indicated viruses as for panel A. Cell lysates were prepared and analyzed by Western blotting with antibodies against PKCδ, phosphorylated PKCδ (Ser645), phosphorylated PKCδ (Tyr311), and tubulin.

unaffected (data not shown). To investigate whether $\gamma_1$34.5 regulates protein kinase C, we focused on subcellular localization of PKCδ in HeLa cells mock infected or infected with viruses (Fig. 5A). Cells were reacted with anti-PKCδ antibody and analyzed by confocal microscopy. In mock-infected cells, PKCδ localized to the cytoplasm, with faint punctates distributed diffusely. Infection with wild-type HSV-1(F) or H1002 resulted in translocation, where PKCδ concentrated at the nuclear rim. However, in cells infected with R3616 or H1001, PKCδ remained dispersed in the cytoplasm. A similar pattern was seen with PKCα (data not shown), suggesting that $\gamma_1$34.5 recruits PKC to the nuclear membrane, which requires its amino-terminal domain.

To evaluate the status of PKC activation, we examined its phosphorylation in response to HSV infection. HeLa cells were mock infected or infected with $\gamma_1$34.5 variants. At 16 h postinfection, cells were processed for immunoblotting analysis with antibodies against PKCδ and phosphorylated PKCδ. As shown in Fig. 5B, expression of PKCδ was detectable in all cells. Infection with HSV-1(F) or H1002 induced phosphorylation of serine 645 and tyrosine 311 on PKCδ. This was not observed in mock-infected cells. Similarly, little phosphorylation was seen in cells infected with R3616 or H1001. These results suggest that HSV-1 induces PKCδ activation, which is dependent on $\gamma_1$34.5 or its amino-terminal domain.

The $\gamma_1$34.5 protein associates with PKC and p32 through its amino-terminal domain. To investigate the mechanism of $\gamma_1$34.5 action, we assessed the relationship of $\gamma_1$34.5, PKC, and cellular p32, which is linked to the reorganization of lamin A/C in HSV-1 infection (32). HeLa cells were infected with $\gamma_1$34.5 variants and subjected to immunoprecipitation analyses. With anti-$\gamma_1$34.5 antibody (Fig. 6A), $\gamma_1$34.5 pulled down PKCδ and p32 in cells infected with HSV-1(F) or H1002 (lanes 1 and 4). This interaction was not detectable in cells infected with R3616 or H1001 (lanes 2 and 3). In a reverse experiment with anti-p32 antibody (Fig. 6B), p32 captured PKCδ in the presence of wild-type $\gamma_1$34.5 (lanes 1 and 4). Deletion of $\gamma_1$34.5 or its amino terminus abrogated this interaction (lanes 2 and 3).

To explore whether $\gamma_1$34.5 forms a complex with PKC and p32 in the absence of other viral protein, HeLa cells, transfected with plasmid vector or $\gamma_1$34.5 variants, were subjected to immunoprecipitation with anti-$\gamma_1$34.5 antibody. As shown Fig. 6C, wild-type $\gamma_1$34.5 bound to endogenous PKCδ and p32 (lane 2). In contrast, the mutant lacking the amino terminus lost its binding activity (lane 3). The $\gamma_1$34.5 mutant with deletion of the carboxyl terminus retained its ability to interact with PKCδ and p32 (lane 4), suggesting that the amino-terminal domain of $\gamma_1$34.5 interacts with PKCδ and p32. These experimental results are consistent with a model where $\gamma_1$34.5 bridges PKCδ and p32, forming a complex that mediates reorganization of the nuclear lamina.

The $\gamma_1$34.5 protein communicates with the nuclear egress complex in HSV-infected cells. The UL31/UL34 complex mediates nucleocapsid docking, reorganization of the nuclear lamina, and remodeling of nuclear envelope (1). Deletion of UL31 or UL34 from HSV-1 impedes viral nuclear egress (37, 38). Because $\gamma_1$34.5 facilitates viral egress from the nucleus, we asked whether it associates with the UL31/UL34 complex upon viral infection. Accordingly, HeLa cells were infected with wild-type virus and $\gamma_1$34.5 variants. At 16 h postinfection, cells were processed for immunoprecipitation with anti-$\gamma_1$34.5 antibody (Fig. 7A). In cells infected with wild-type HSV-1(F), $\gamma_1$34.5 associated with UL31 and UL34. However, in cells infected with H001, which lacks the amino terminus of $\gamma_1$34.5, such interaction disappeared. Infection with H1002 restored the interaction of $\gamma_1$34.5 with UL31 and UL34. Further analysis showed that PCKδ and p32 were present in the UL31/UL34 complex. These results suggest that $\gamma_1$34.5 forms a multiprotein complex that mediates HSV nuclear egress.

To determine whether $\gamma_1$34.5 affects the localization of UL31 and UL34 in viral infection, HeLa cells were mock infected or infected with viruses. Cells were stained with antibody against UL31 or UL34 and visualized by confocal microscopy (Fig. 7B). Consistent with previous findings, UL31 and UL34 localized to the nuclear rim in cells infected with wild-type HSV-1(F). In cells infected with H1001 or H1002, UL31 and UL34 similarly distributed to the nuclear membrane, suggesting that $\gamma_1$34.5 does not regulate subcellular localization of the UL31/UL34 complex in HSV-infected cells.

To delineate whether $\gamma_1$34.5 alone binds to UL31 or UL34,

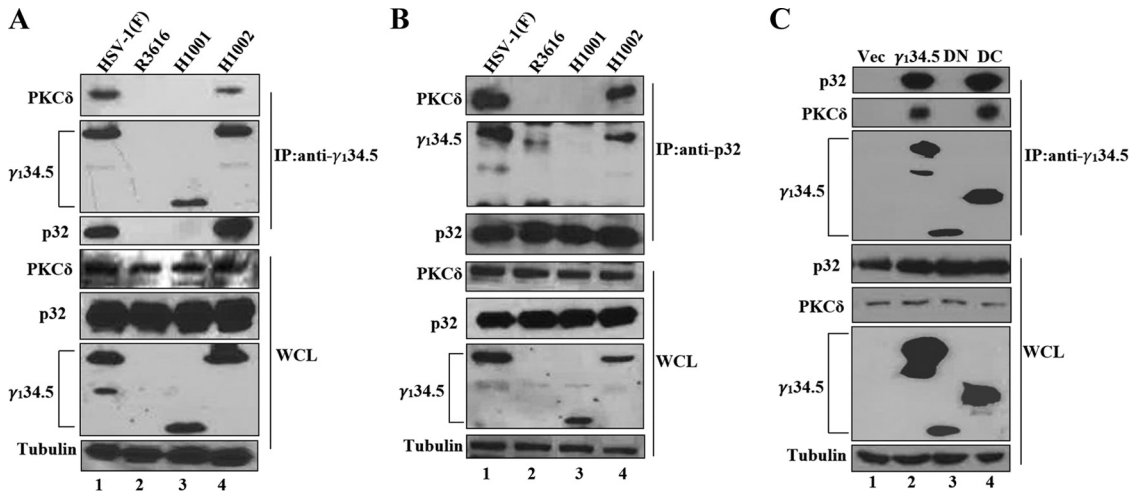

FIG 6 The amino terminus of $\gamma_1$34.5 is required to form a complex with PKCδ and p32. (A) HeLa cells were infected with the indicated viruses at 5 PFU per cell. At 16 h postinfection, cells were harvested and subjected to immunoprecipitation (IP) with anti-$\gamma_1$34.5 antibody. Precipitated proteins and whole-cell lysates (WCL) were processed for Western blot analysis with antibodies against PKCδ, p32, $\gamma_1$34.5, and tubulin. Tubulin was used as a loading control. (B) HeLa cells were infected as for panel A and processed for immunoprecipitation with anti-p32 antibody, which was followed by Western blotting with antibodies against PKCδ, p32, $\gamma_1$34.5, and tubulin. (C) HeLa cells were transfected with a vector plasmid or a plasmid expressing wild-type $\gamma_1$34.5 or the $\gamma_1$34.5 mutant devoid of amino acids 1 to 146 (DN) or amino acids 147 to 263 (DC). At 36 h after transfection, cells were processed for immunoprecipitation with anti-$\gamma_1$34.5 antibody. Precipitated proteins and whole-cell lysates were probed with antibodies against p32, PKCδ, $\gamma_1$34.5, and tubulin.

HeLa cells were transfected with $\gamma_1$34.5 along UL31 or UL34. Samples were then subjected to immunoprecipitation with anti-$\gamma_1$34.5 antibody. As illustrated in Fig. 8A, wild-type $\gamma_1$34.5 interacted with UL31 but not UL34. Furthermore, the $\gamma_1$34.5 mutant that lacks the carboxyl terminus bound to UL31 (Fig. 8B). However, the $\gamma_1$34.5 mutant devoid of its amino terminus lost its activity (Fig. 8B). Therefore, when expressed, the $\gamma_1$34.5 protein binds to UL31 via its amino-terminal domain.

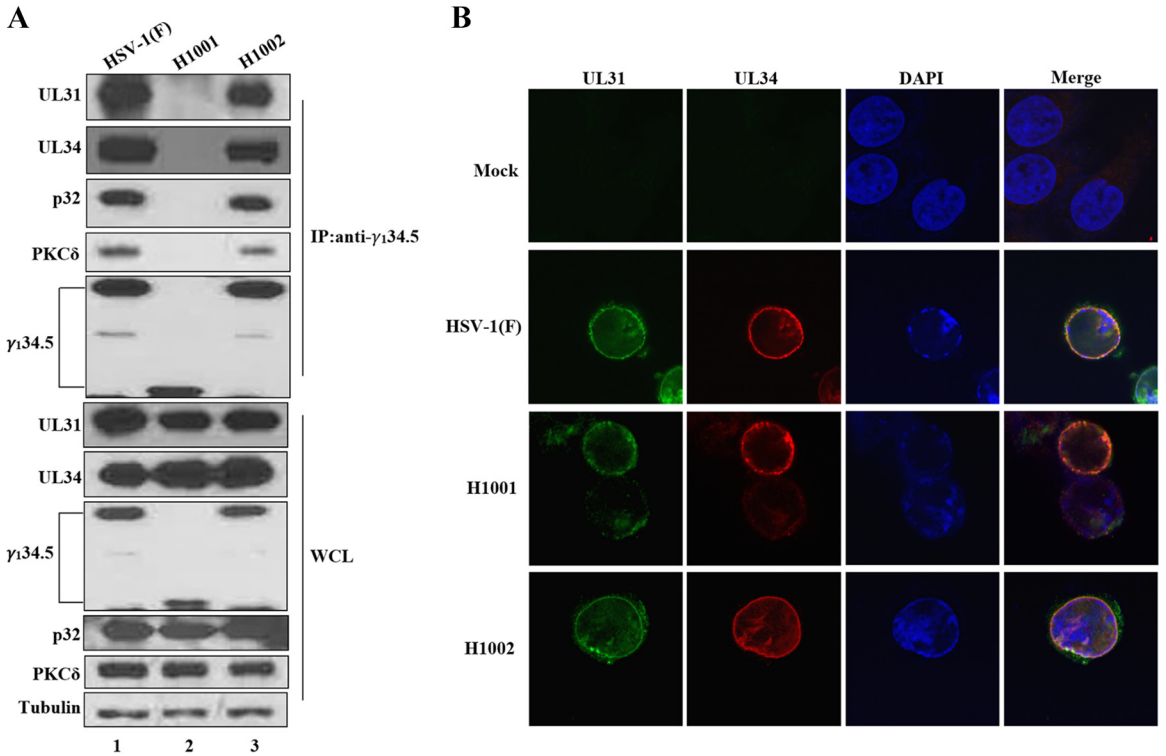

FIG 7 (A) The $\gamma_1$34.5 protein forms a complex with UL31, UL34, PKC, and p32 in HSV-infected cells. Monolayer HeLa cells were mock infected or infected with HSV-1(F), H1001, or H1002 (5 PFU/cell). At 16 h postinfection, lysates of cells were immunoprecipitated with anti-$\gamma_1$34.5 antibody. Precipitated proteins and whole-cell lysates (WCL) were processed for immunoblotting with antibodies against PKCδ, p32, UL31, UL34, $\gamma_1$34.5, and tubulin. (B) UL31 and UL34 localize to the nuclear membrane independently of $\gamma_1$34.5. HeLa cells were mock infected or infected with the indicated viruses (5 PFU/cell). At 16 h postinfection, cells were stained with DAPI and antibodies against UL31 and UL34. Samples were then visualized by confocal microscopy.

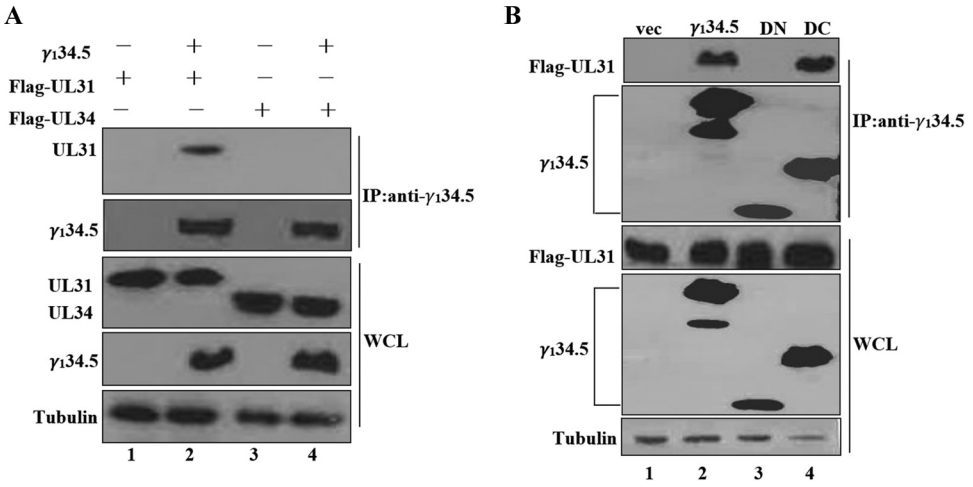

FIG 8 (A) The $\gamma_1$34.5 protein interacts with UL31. Monolayers of HeLa cells were transfected with $\gamma_1$34.5 along with a plasmid expressing UL31 or UL34. At 36 h after transfection, cells were processed for immunoprecipitation with anti-$\gamma_1$34.5 antibody. Precipitated proteins and whole-cell lysates were probed with antibodies against FLAG, $\gamma_1$34.5, and tubulin. (B) The amino terminus of $\gamma_1$34.5 interacts with UL31. HeLa cells were transfected with a vector plasmid or a plasmid expressing wild-type $\gamma_1$34.5 or the $\gamma_1$34.5 mutant devoid of amino acids 1 to 146 (DN) or amino acids 147 to 263 (DC). Cells were then processed for immunoprecipitation with anti-$\gamma_1$34.5 antibody. This was followed by Western blotting with antibodies against FLAG, $\gamma_1$34.5, and tubulin as for panel A.

DISCUSSION

Several lines of evidence suggest that nucleocapsid budding of herpesviruses alters the architecture of nuclear envelope (8, 11). Relevant to this is local disintegration of the nuclear lamina, which is necessary to engage capsids to the nuclear membrane and for subsequent release. In the present work, we show that HSV-1 mediates reorganization of lamin A/C through the amino terminus of the $\gamma_1$34.5 protein. This parallels with efficient release of capsids from the nucleus and viral growth. In particular, the $\gamma_1$34.5 protein engages with the UL31/UL34 complex and activates protein kinase C in infected cells. These observations suggest a model in which HSV $\gamma_1$34.5 is a mediator of nuclear egress in HSV-infected cells (Fig. 9).

The $\gamma_1$34.5 protein is essential to promote HSV virulence (21). In addition to inhibition of autophagy and interferon responses, it facilitates viral nuclear egress (19, 26, 30). We noted that an HSV mutant lacking the amino terminus of $\gamma_1$34.5 was severely impaired in viral growth compared to wild-type virus. While this phenotype may reflect a compounding effect, it is partly attributable to aberrant nuclear egress. Restoration of wild-type $\gamma_1$34.5 reversed the phenotype, suggesting that the region encompassing amino acids 1 to 146 is functionally important. Two lines of evidence support this interpretation. First, in cells infected with the amino-terminal mutant of $\gamma_1$34.5, the relative yield of cell-free virus was reduced. Second, electron microscopy examination suggests that this $\gamma_1$34.5 mutant was confined mainly to the nucleus upon infection. A simple explanation is that a block or reduction in the rate of nucleocapsid transit may account for defective nuclear egress. Therefore, concerted activities of $\gamma_1$34.5 likely contribute to efficient viral replication. We favor the notion that the amino terminus of $\gamma_1$34.5 may act as a functional module that mediates the budding machinery in HSV infection.

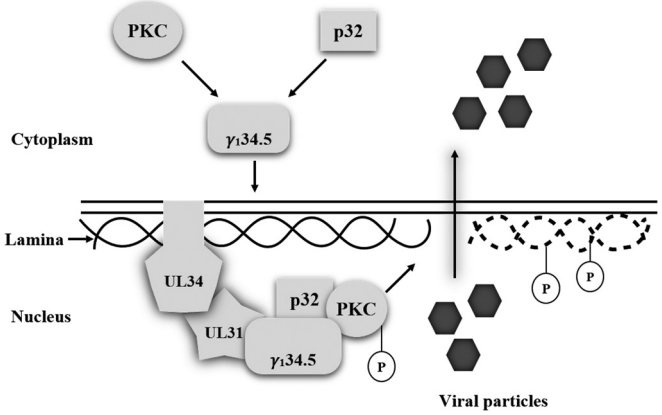

FIG 9 A model of $\gamma_1$34.5-mediated nuclear egress. Upon HSV-1 infection, the $\gamma_1$34.5 protein binds and redirects PKC and p32 to the nuclear membrane where the UL31/UL34 complex resides. Formation of a multiprotein complex activates PKC, which dephosphorylates and subsequently disintegrates the nuclear lamina for nucleocapsid egress.

Our work suggests a role of $\gamma_1$34.5 in remodeling the nuclear envelope in virus-infected cells. Several studies indicate that herpesvirus infection dismantles the nuclear lamina through phosphorylation of lamin A/C and lamin B (3, 11, 12), which permits capsid access for primary envelopment. The data presented in this work suggest that such changes are linked to the amino terminus of $\gamma_1$34.5. In its absence, neither reorganization nor phosphorylation of lamin A/C occurred. In HSV-infected cells, protein kinase C is believed to phosphorylate lamin A/C and lamin B (12, 32). In addition, Us3 phosphorylates lamin A/C (3). Therefore, the $\gamma_1$34.5 protein may regulate a kinase(s) that phosphorylates lamin A/C and locally dissolves the lamina. Indeed, the $\gamma_1$34.5 protein mediated the activation of PKCδ in infected cells. Deletion of $\gamma_1$34.5 or its amino terminus precluded the translocation of protein kinase Cδ to the nuclear membrane and its activation. Previous studies suggest that chemical inhibition of PKC impairs nuclear egress (39). In line with this, we observed that bisindolylmaleimide I or rottlerin inhibited phosphorylation of lamin A/C. Given that BIM I is a pan-PKC small-molecule inhibitor (40), multiple kinases may contribute to lamin phosphorylation. Al-

though it was not tested, our results do not exclude the possibility that $\gamma_1$34.5 may target an additional kinase(s).

In HSV-infected cells, the $\gamma_1$34.5 protein forms complex with p32 and protein kinase C through its amino-terminal domain. Deletion of the amino terminus disrupted the interaction of p32 and PKCδ. p32, a mitochondrial protein, travels to nuclear membrane upon HSV infection (32). While incompletely characterized, p32 is required for nuclear egress of HSV-1 and human cytomegalovirus (HCMV) (32, 33, 41). It has been shown that knockdown of p32 prevents HSV-induced reorganization of lamin A/C (32). Given a link of PKC to the nuclear envelope budding, it is logical to postulate that the $\gamma_1$34.5 complex regulates reorganization of the nuclear lamina. At this point, the precise role of $\gamma_1$34.5 is unknown. A plausible hypothesis is that $\gamma_1$34.5 bridges p32 and PKC via its amino-terminal domain, where p32 may modulate PKC or a distinct kinase(s). Alternatively, HSV $\gamma_1$34.5 may simply work in the cytoplasm as a trafficking protein that delivers p32 and PKC to the nuclear membrane. In this special context, it is noteworthy that $\gamma_1$34.5 shuttles between the nucleus and cytoplasm (22, 23).

In addition, the $\gamma_1$34.5 protein interacts with the UL31/UL34 complex in HSV-infected cells. Removal of the amino terminus of $\gamma_1$34.5 abrogated such interaction and crippled viral nuclear egress. These experimental data suggest a model in which $\gamma_1$34.5 may facilitate nuclear egress through cooperation with UL31 and UL34. The UL31/UL34 complex is the workhorse that mediates nuclear egress (1). In infected cells, UL31 and UL34 colocalize to the nuclear rim and bind to lamin A/C and lamin B (8, 36). This is suggested to modulate disintegration of the nuclear lamina. Although the UL31/UL34 complex is necessary to induce the recruitment of PKC isoforms to the nuclear membrane, a direct interaction is not detectable (12). In this respect, we suspect that the UL31/UL34 complex may function in a $\gamma_1$34.5-dependent mechanism. An attractive possibility is that $\gamma_1$34.5 recruits PKC in the cytoplasm, which is directed to the UL31/UL34 complex in the nuclear membrane. In this regard, UL31 probably serves as a contact site. Together with p32, $\gamma_1$34.5 may activate PKC, which leads to lamina disassembly and subsequent nuclear egress.

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