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Comparison of Effects of Inhibitors of Viral and Cellular Protein Kinases on Human Cytomegalovirus Disruption of Nuclear Lamina and Nuclear Egress

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Human cytomegalovirus (HCMV) kinase UL97 is required for efficient nuclear lamina disruption during nuclear egress. However, cellular protein kinase C (PKC) has been implicated in this process in other systems. Comparing the effects of UL97 and cellular kinase inhibitors on HCMV nuclear egress confirms a role for UL97 in lamina disruption and nuclear egress. A pan-PKC inhibitor did not affect lamina disruption but did reduce the number of cytoplasmic capsids more than the number of nuclear capsids.

Transit of herpesvirus capsids from the nucleus to the cytoplasm (nuclear egress) involves phosphorylation-driven disruption of the nuclear lamina underlying the inner nuclear membrane (reviewed in references 1 to 3). Lamina disruption in herpes simplex virus 1 (HSV-1) and murine cytomegalovirus correlates with the recruitment of cellular protein kinase C (PKC) isoforms by the viral nuclear egress complex (NEC) (4, 5). During HSV-1 infection, the PKC inhibitor bisindolylmaleimide 1 (Bim-1) reduced cytoplasmic capsid numbers with little effect on nuclear capsid numbers, suggesting a role for PKC in nuclear egress (6). Also, in a cellular process akin to herpesvirus nuclear egress, rearrangement of nuclear lamins requires an isoform of PKC (7). However, during human cytomegalovirus (HCMV) infection, the NEC recruits the viral kinase UL97, not PKC, to the nuclear rim (8). Moreover, UL97 is required for efficient lamin A/C phosphorylation and lamina disruption during nuclear egress (9–11). Nevertheless, a textbook view is that in HCMV nuclear egress, host PKC functions interchangeably with UL97 in the NEC for phosphorylation-driven disruption of the nuclear lamina (12). A role for PKC or other cellular kinases in these processes would be consistent with HCMV replication proceeding, albeit inefficiently, in the absence of UL97 (11, 13). Additionally, both UL97 and cellular cyclin-dependent kinase 1 (Cdk-1, which dissolves nuclear lamina during mitosis) phosphorylate lamin A/C residue Ser22, and Ser22 phosphorylation increases somewhat during HCMV infection in the absence of UL97 (9, 11).

To compare the roles of viral and cellular kinases during lamina disruption and nuclear egress, we utilized inhibitors of UL97, PKC, and Cdk-1 at concentrations that exert substantial effects in herpesvirus systems without major cytotoxicity (6, 14, 15) (see Fig. S1 at https://coen.med.harvard.edu), i.e., the UL97 inhibitor maribavir (MBV) (16) at 1 μM; the PKC isoform α, β_1, β_2, γ, δ, and ε inhibitor Bim-1 (17) at 10 μM; and the Cdk-1, Cdk-2, and Cdk-5 inhibitor roscovitine (Rosc) (18) at 15 μM (6, 14, 15). Neither Bim-1 nor Rosc inhibited UL97 autophosphorylation activity *in vitro* (see Fig. S2 at https://coen.med.harvard.edu). Each inhibitor or a vehicle control (0.1% dimethyl sulfoxide [DMSO]) was added to serum-fed (dividing) mock-infected or HCMV strain AD169-infected cells at 48 h postinfection (hpi) to limit the inhibition of steps prior to nuclear egress. At 72 hpi, we stained cells for lamin A/C and the viral DNA polymerase subunit UL44. Replication compartment formation (UL44 staining) had progressed comparably across the infected samples (Fig. 1A). In vehicle-treated infected cells, lamin A/C staining exhibited a characteristic deformed shape, which is a marker of lamina disruption (9, 19, 20). There was a significant reduction in these nuclear deformities in MBV-treated infected cells (Fig. 1B), similar to when MBV is present throughout infection (9). However, MBV treatment did not significantly reduce the frequency of nuclear deformities in mock-infected cells (6% in both MBV-treated and vehicle-treated samples). Bim-1 or Rosc treatment did not result in significant differences from untreated HCMV-infected cells (Fig. 1B) or mock-infected cells (data not shown). These results confirm the importance of UL97 in lamina disruption during HCMV nuclear egress but provide no evidence of a role for PKC or Cdk-1 in this process.

In parallel, we measured viral titers at 96 hpi with MBV, Bim-1, and Rosc added at 48 hpi. All three inhibitors led to significant reductions in viral titers as follows: MBV, 10-fold; Bim-1, 100-fold; Rosc, 30-fold (Fig. 2, left). Thus, the lack of effect of Bim-1 or Rosc on lamina disruption was not due to a lack of activity. We assessed the effects of these compounds on viral protein expression (Fig. 2, right) as described previously (8, 11). MBV exerted little, if any, effect on the levels of the proteins assayed. Unexpectedly, while Bim-1 and Rosc did not reduce levels of UL44 and pp28, they did reduce levels of UL97 and UL50 2- to 5-fold, suggesting a role for PKC and Cdk in the expression of these proteins. Nevertheless, the reductions in UL97 and UL50 levels were substantially smaller than what one would expect to explain the effects of these compounds on viral titers, and they were not sufficient to affect lamina disruption (Fig. 1B).

We then compared nuclear egress in the presence or absence of kinase inhibitors from 48 to 96 hpi by using electron microscopy as described previously (11). MBV led to significant reductions (~10-fold) in the number of cytoplasmic capsids without de-

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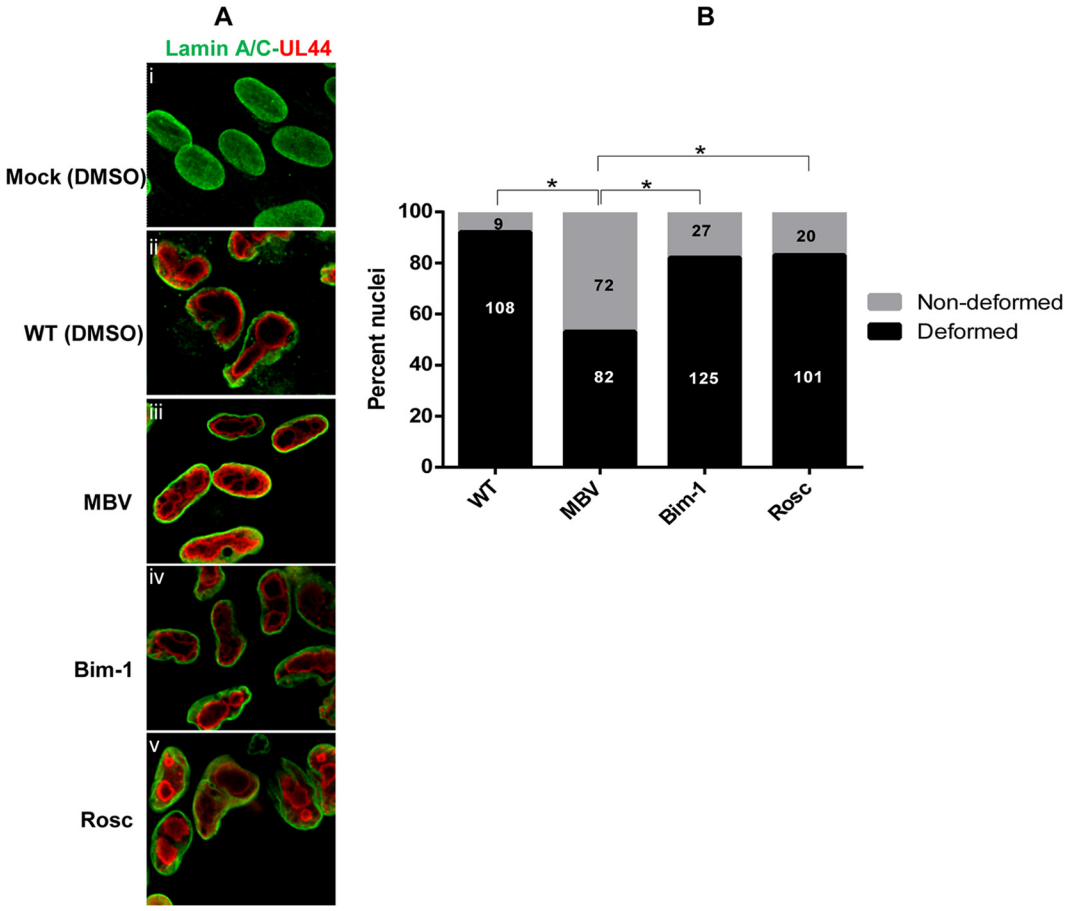

FIG 1 Effects of kinase inhibitors on nuclear lamina morphology. (A) Human foreskin fibroblasts were mock infected or infected with wild-type (WT) HCMV AD169rv (multiplicity of infection = 1). At 48 hpi, cells were treated with DMSO or with the viral or cellular kinase inhibitor MBV, Bim-1, or Rosc. Cells were fixed and stained for lamin A/C (green) and UL44 (red) at 72 hpi. Images were acquired by confocal microscopy and are presented as median planes from Z-stacks. (B) Mock-infected or virus-infected cells from the confocal microscopy images (n = 117 to 154 per condition) were assessed for nuclear lamina deformities and analyzed for significance with Fisher's exact tests. For a family-wise type I error rate of 0.05 in a set of six comparisons, a result can be considered significant only when the P value is <0.0085. *, $P < 0.0001$. No asterisk indicates no significant difference.

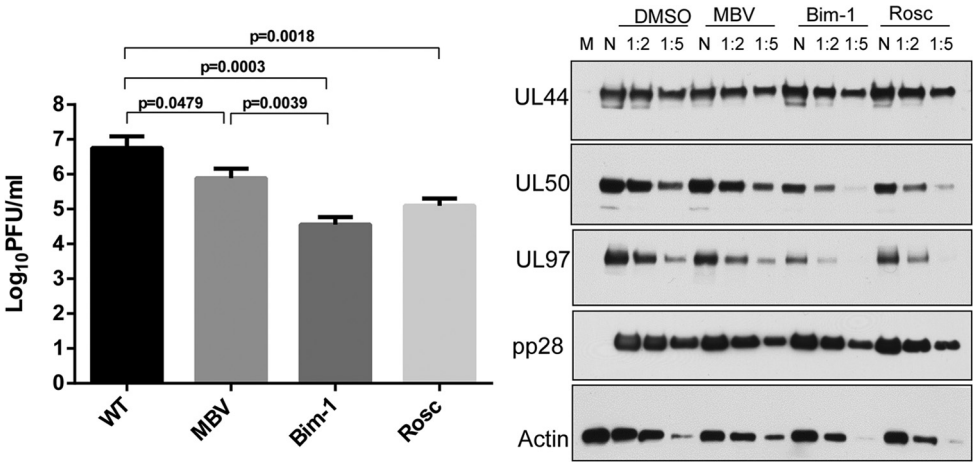

FIG 2 (Left) Effects of viral or cellular kinase inhibitors on HCMV replication as determined by plaque assays with supernatants from cells infected with wild-type (WT) HCMV AD169rv (multiplicity of infection = 1) in the absence or presence of MBV, Bim-1, or Rosc (added at 48 hpi) at 96 hpi. Mean log titers (with error bars displaying standard errors of the means) from three independent experiments were assessed for statistically significant differences by one-way analysis of variance, followed by Sidak's multiple-comparison tests (five comparisons). The P values obtained are shown. No label indicates no significant difference. (Right) Effects of viral or cellular kinase inhibitors on viral protein expression. Lysates were obtained from mock-infected (lane M) or wild-type HCMV-infected cells (in the absence or presence of the kinase inhibitors shown at the top) from a parallel setup at 96 hpi. The undiluted lysates (lanes N) or serial dilutions (2-fold [1:2] or 5-fold [1:5]) were separated by SDS-PAGE, which was followed by Western blotting with antibodies against UL44, UL50, UL97, and pp28, as well as a loading control (β-actin), as indicated to the left.

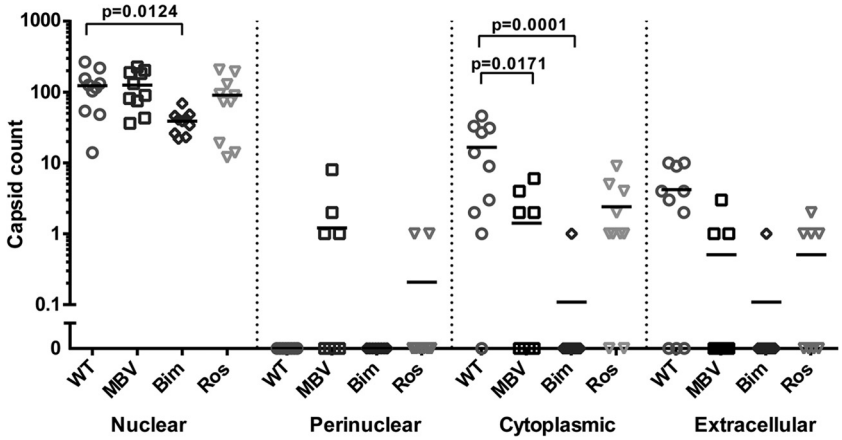

FIG 3 Effects of viral or cellular kinase inhibitors on HCMV nuclear egress. Human foreskin fibroblasts infected with wild-type (WT) HCMV AD169rv (multiplicity of infection = 1) in the absence (WT) or presence of MBV, Bim-1, or Rosc (added at 48 hpi) were fixed for electron microscopy at 96 hpi. Viral capsids in the nucleus, perinuclear space, or cytoplasm or outside the cell (extracellular) were counted in 10 electron microscopy sections that each represented a whole cell. Statistical analysis for each location was performed by using Kruskal-Wallis tests with Dunn's multiple-comparison posttests. The *P* value in each case is shown. No label indicates no significant difference.

creasing the number of nuclear capsids (Fig. 3), consistent with the importance of UL97 for nuclear egress (10, 11). Bim-1 led to significant reductions (~100-fold) in cytoplasmic capsid numbers, similar to results obtained with Bim-1 in the HSV-1 system (6), but also significant reductions (3-fold) in nuclear capsid numbers (Fig. 3). Rosc treatment did not cause any significant alterations in capsid numbers consistent with a role for Cdk after nuclear egress (21, 22).

We also scored for each of the three capsid forms (A, B, and C) (23) in the nucleus by analyzing the data by using Kruskal-Wallis tests with Dunn's multiple-comparison posttests (Table 1). MBV led to no more than modest reductions in the A and C forms (2.5- and 2-fold, respectively), which were not significant ($P = 0.4314$ and 0.7291). Bim-1 led to reductions in all three forms of capsids, with a drastic (32-fold) and significant ($P = 0.0007$) effect on C capsids. Since C capsids contain viral DNA, their reduction likely makes a major contribution to the severe effect of Bim-1 on viral titers. Rosc did not significantly affect the numbers of any of the nuclear capsid forms.

In summary, our results show that HCMV UL97 and PKC are not interchangeable. UL97 is important for lamina disruption and nuclear egress. PKC appears to be important for capsid formation and accumulation in both the nucleus and the cytoplasm. As Bim-1 led to a more drastic reduction in cytoplasmic capsid numbers than in nuclear capsid numbers, PKC may also be important for nuclear egress. If so, PKC could act indirectly by promoting capsid formation and expression of UL97 and UL50. Alternatively, PKC may act directly during nuclear egress, but if it does, its role is evidently not disruption of the nuclear lamina. This is consistent with HCMV not inducing changes in the staining pattern of lamin B (8) (see Fig. S3A and B at https://coen.med.harvard.edu), which is an important substrate of PKC (24, 25). Thus, functions for PKC in HCMV-infected cells differ from those attributed to this kinase during nuclear egress in other systems (4, 5, 7).

TABLE 1 Nuclear capsid forms in the presence or absence of kinase inhibitors[a]

Condition	No. of whole-cell sections scored	No. (%) of capsids of form:		
		A	B	C
Vehicle	10	67 (5.4)	880 (71.3)	288 (23.3)
MBV	10	27 (2.2)	1,079 (86)	148 (11.8)
Bim-1	10	11 (2.8)	370 (94.8)	9 (2.4)
Rosc	10	84 (9.2)	644 (70)	185 (20.8)

[a] Statistical analysis for each comparison was performed by using Kruskal-Wallis tests with Dunn's multiple-comparison posttests. The *P* values are provided in the text.

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