Introduction

DNA viruses have yielded many insights into cell cycle control as well as regulation of cellular processes associated with oncogenesis because they encode regulatory proteins that modulate cell cycle progression and cell death. Members of the papillomavirus, polyomavirus, hepadnavirus, and herpesvirus families all cause persistent/latent infections, and, regardless of any contribution to malignancy, all of these viruses encode functions that dysregulate cellular growth, inhibit immune activation, and modulate inflammation [1]. Lifelong infection with human herpesviruses, including herpes simplex virus (HSV)-1, HSV-2, varicella zoster virus, Epstein-Barr virus, cytomegalovirus (CMV), human herpesvirus 6A, 6B, or 7, and Kaposi sarcoma–associated herpesvirus, is associated with dysregulation of the cell cycle as well as with medically important opportunistic disease [2,3]. The mechanisms of cell cycle dysregulation in herpesvirus replication and proliferative disease remain poorly understood even though these processes underlie pathogenesis.

Human CMV has a dramatic stimulatory impact on permissive cells in culture, as typically demonstrated by infecting human fibroblasts (HFs). Stimulation follows a bimodal pattern, with an initial peak due to the impact virus binding and penetration, and a later peak coinciding with viral DNA synthesis, assembly and release [4–7]. This late phase of infection is also associated with dysregulation of host cell cyclin levels and with disruption of cell cycle progression [8–11] and suggests that these processes are linked. Viral DNA replication and cell cycle dysregulation both depend upon expression of viral immediate-early (IE, or α) and delayed-early (DE, or β) gene products, which precede expression of late genes and release of progeny virus [6]. This pattern also implicates early viral gene products in cell cycle stimulation and dysregulation [5,12] in a manner reminiscent of oncogene-encoding, DNA tumor viruses [13]. Although infected cells exhibit a pattern of protein expression consistent with continued cell cycle progression, normal cellular prereplication complexes fail to form [14], cellular DNA synthesis (S phase) fails to proceed [8–11], and chromosome segregation and cytokinesis are blocked [8–11]. These investigations showed that infected HFs become arrested in a pseudo-G1 state associated with dramatic modulation of p53, pRB, cyclins, and cyclin-dependent kinases (Cdks) in ways that may be critical for viral replication efficiency.

The importance of cell cycle–regulated kinases in CMV replication is consistent with a strong antiviral impact of selective Cdk inhibitors such as roscovitine [15]. Roscovitine, as well as other Cdk inhibitors such as olomucine and flavopiridol, inhibits herpesviruses, as well as other DNA viruses and retroviruses, in a pattern that reveals the underlying importance of host cell protein kinases in viral
Pseudomitosis Is a Common Feature of CMV-Infected Cultures

To determine whether pseudomitosis observed during infection with a previously evaluated CMV strain variant (AD169varATCC) [7] was a universal feature of commonly used CMV strains, we recorded the percentage of pseudomitotic cells induced in HFFs infected at a multiplicity of infection (MOI) of 10 with AD169varATCC, AD169varDE, Toledo, TownevarATCC, TownevarRIT, or TB40/E for 50, 72, and 96 h (Figure 1). Pseudomitotic cells were identified by staining infected cultures for the viral nuclear antigens IE1/IE2 and for the mitotic spindle pole-localizing protein γ-tubulin 1, as described [7]. All strains showed the ability to drive infected cells into pseudomitosis, albeit with different efficiencies and following different temporal patterns. As expected, AD169varATCC induced abnormal numbers of mitotic spindles and misalignment of condensed chromosomal DNA in approximately 35% of infected cells, peaking at 72 h postinfection [7], while all other CMV strains induced pseudomitosis at lower overall levels. Surprisingly, another AD169 strain variant (AD169varDE) induced much lower
peak levels (7% at 72 h postinfection) compared to AD169varATCC. The only known difference between these strain variants is in the UL36 gene product, which has a point mutation that renders the viral inhibitor of caspase 8 activation nonfunctional [43], suggesting that viral genetic determinants such as this anti-apoptotic function may impact this process. All other strains displayed top levels below 5%, and varied in the timing of maximal pseudomitosis levels, with Toledo and TownevarATCC peaking at 72 h postinfection but TownevarRIT and TB40E showing highest levels at 50 h postinfection. Thus, although induction of pseudomitosis is a common property of CMV strains in culture, AD169varATCC exhibits substantially higher levels than the other strains we examined.

Cdk1 Is Necessary and Sufficient to Promote Pseudomitosis during Infection

The levels of endogenous Cdk1 were induced 30% more in AD169varATCC than in AD169varDE infected cells (Figure 2A), consistent with a role for this cyclin-dependent kinase in driving pseudomitosis levels. To determine the extent to which Cdk1 activity alone contributed to the development of pseudomitosis, retrovirus-transduced HFs expressing enhanced green fluorescent protein (GFP), a GFP-tagged, wild-type Cdk1 (Cdk1wt-GFP) or a GFP-tagged, kinase inactive and dominant negative Cdk1 (Cdk1dn-GFP) [31] were infected with AD169varATCC or AD169varDE (MOI of 10). Immunoblot analysis showed that both endogenous (black arrowhead) and recombinant Cdk1 (Cdk1wt-GFP or Cdk1dn-GFP; gray arrowhead) were comparably expressed in infected cells at 72 h postinfection (Figure 2A). In all experimental settings, AD169varATCC induced endogenous Cdk1 to higher levels than AD169varDE, and, independent of the retrovirus construct, transduction reduced endogenous Cdk1 levels by 20% to 30%. Cdk1dn-GFP or Cdk1wt-GFP was also expressed in cells infected with AD169varATCC or AD169varDE, with levels appearing to be substantially higher in AD169varDE-infected cells transduced with either construct. Total (endogenous plus transduced) Cdk1 levels were approximately 80% in AD169varATCC- and 160% in AD169varDE-infected cells compared to nontransduced cells. No difference was observed in the expression of Cdk1wt-GFP or Cdk1dn-GFP in cells infected with either virus; however, expression was twice as high in AD169varDE-infected cells, suggesting a level of variation in total Cdk1 levels that was not explored further.

GFP fluorescence was detectable in 90% to 100% of transduced HFs (Figure 2B) and facilitated differentiation between the round, anucleated pseudomitotic cells and the spindle-shaped, nucleated cells that retained an interphase appearance (Figure 2B, insets). Because of the differences in endogenous and transduced Cdk1 expression levels depending on the strain variant, comparisons were made within a variant, although Cdk1 inhibition or overexpression had similar effects on both AD169varATCC- and AD169varDE-infected HFs when IE1/IE2 antigen–positive, GFP-positive, pseudomitotic cells were counted (Figure 2C). High levels of pseudomitotic cells were observed in AD169varATCC-infected HFs following transduction with Cdk1wt-GFP. By contrast, such cells were rare in cultures that had been transduced with Cdk1dn-GFP. When four independent experiments were analyzed together, the peak levels of pseudomitosis in either AD169varATCC- (28 ± 2%) or AD169varDE- (7 ± 1%) infected cultures were reduced approximately 4-fold in the presence of Cdk1dn-GFP, indicating that Cdk1 activity was necessary for this process. Conversely, transduction of Cdk1wt-GFP, which increased the total cellular pool of active Cdk1, induced pseudomitosis 1.4-fold with AD169varATCC and 2.5-fold with AD169varDE, consistent with expression levels (Figure 2A). Pseudomitosis levels in GFP controls were similar to levels in nontransduced cells, indicating a neutral impact of GFP expression on this process. Thus, Cdk1 appears necessary and sufficient for induction of pseudomitosis in CMV-infected cells, consistent with its role in regulating mitosis in the conventional cell cycle [31].

Cdk1 and Pseudomitosis Are dispensable for Viral Replication

To assess the impact of pseudomitosis on viral yields, CMV (AD169varATCC) released into culture supernatants was titered at 50, 72, 96, and 120 h postinfection, comparing nontransduced HFs to HFs transduced with GFP, Cdk1dn-GFP, or Cdk1wt-GFP (Figure 2D). No differences in virus titers were observed at any time in any of these conditions despite the dramatic impact that modulation of Cdk1 levels had on pseudomitosis. In separate experiments, nontransduced HFs or HFs transduced with GFP, Cdk1dn-GFP, or Cdk1wt-GFP and infected with AD169varATCC or AD169varDE were found to produce similar amounts of virus at 72 h
postinfection (Figure 2E). Thus, the dramatic modulation of pseudomitosis that was observed using these vectors did not contribute to altered virus replication or release. Therefore, pseudomitosis is a reflection of induced Cdk1 activity during infection, but viral replication proceeds with equal efficiency in HFs regardless of Cdk1 activity.

**Viral Replication Depends on the Activity of Cellular Kinases**

Given that Cdk1 activity does not influence CMV replication levels, we evaluated the impact of IMO, a Cdk1/Cdk2/Cdk5/Cdk9 inhibitor [28], on CMV replication (MOI = 10) at 24, 48, 72, 96, 120, and 144 h postinfection. IMO has been used to inhibit wild-type and drug-resistant strains of HIV-1 in culture [29], but its antiviral effects against CMV or other herpesviruses had never been tested. Enzyme inhibition studies suggested IMp is a more potent and specific Cdk1 inhibitor than roscovitine [28,44]. Appearance of progeny virus in culture supernatants was delayed at 5 μM IMO and significantly inhibited at 10 μM IMO regardless of whether AD169varATCC or AD169varDE was used (Figure 3A). DMSO (the IMO solvent) at 1% or 2% blocked this process via a mechanism that has not been explored. IMO at 5 or 10 μM (or DMSO solvent alone up to 2%) in medium replaced daily had no effect on uninfected cell viability or total numbers over a 5-d time course (unpublished data). Numbers of virus-infected HFs started to decline between 72 and 96 h postinfection, but this has been considered a normal consequence of infection [45] and the intensity and rate of cell loss were not increased by treatment. Staining with Hoechst 44432 showed that nuclei were intact and presented no evidence of pseudomitosis or other changes in nuclear or chromatin morphology.

Finally, we investigated the effects of combining specific Cdk1 inhibition and IMO treatment on viral replication. Neither IMO (10 μM) nor DMSO (2%) alone altered levels of viral replication in GFP-, Cdk1wt-GFP–, or Cdk1dn-GFP–transduced HFs above those expected of IMO alone (Figure 3B). Expression of transduced proteins was not altered by IMO or DMSO treatment, although pseudomitosis was completely blocked in all of these conditions (unpublished data). Thus, Cdk1 does not independently contribute to viral replication in any of the conditions we used, while a broader set of cellular kinase activities is important for efficient viral cycle progression.
Contribution of VPK ppUL97 and Cdks to Viral Yields

Treatment of CMV-infected HFs with 10 μM IMO reduced but did not completely block replication levels, a pattern similar to that observed with cells infected with UL97 mutants [32,34,35] or treated with MBV, a potent inhibitor of the UL97-encoded VPK [41,46–48]. Thus, we investigated the combined impact of simultaneously inhibiting cellular Cdks and VPK. To evaluate the contribution of VPK, we titrated progeny virus levels released in the supernatant of confluent AD169varATCC-infected HFs that were treated with IMO alone, MBV alone, or combinations of these two inhibitors. IMO was used at the two previously evaluated concentrations (5 μM and 10 μM) in the absence of MBV or together with MBV at subinhibitory (0.025, 0.1, or 0.4 μM) concentrations. Drug-containing medium was replaced daily, and virus titers were determined at 24, 48, 72, 96, and 144 h postinfection (Figure 4). As expected from work describing the activity of this antiviral drug on HFs [49], addition of MBV alone at 0.025 or 0.1 μM did not inhibit viral levels and addition of MBV at 0.4 μM reduced viral levels only 2- to 3-fold. MBV substantially enhanced the antiviral activity of IMO in a dose- and time-dependent manner. The most dramatic effects on viral replication were achieved in the presence of 5 or 10 μM IMO plus 0.4 μM MBV. Specifically, there was a 500-fold enhancement of antiviral activity when 10 μM IMO was used together with 0.4 μM MBV at 72, 96, and 144 h postinfection. These results highlight the dramatic dependence of CMV replication on the activity of both cellular and viral protein kinases. Furthermore, these results demonstrate the potential therapeutic benefit of combining cellular Cdk and VPK inhibitors to treat CMV infections.

Discussion

Whereas the aberrant process leading to pseudomitosis is dependent on Cdk1 levels, replication of CMV appears to require overlapping contributions of Cdks and VPKs. The synergism of a specific inhibitor of CMV VPK and a broad inhibitor of Cdks best illustrated this interdependence. The CMV VPK inhibitor MBV inhibits viral replication in culture, showing a pattern that is reminiscent of VPK mutants [32,34,35]. Herpesvirus-common VPKs are encoded by all mammalian and avian herpesviruses [33]. In HSV-1 and other alphaherpesviruses that are studied in actively growing cell lines, VPKs have been found to be dispensable for replication even though they impact the phosphorylation status of viral proteins and are important for viral replication in the host. In CMV, UL97-encoded VPK provides a critical replication function in quiescent cells, promoting the efficiency of virion maturation at different steps [32–35]. The variable impact on viral replication levels together with the fact that a number of distinct late replication events are sensitive to the absence or inhibition of VPK is consistent with an overlap in VPK and Cdk activities, an overlap that gives the impression that VPK is dispensable in some herpesviruses such as HSV-1 because these viruses are propagated in actively growing cell lines. Similar to the behavior of UL97 mutant virus, the antiviral effect of MBV is influenced by culture conditions and metabolic status of host cells in culture [49]. By exploring the effects of Cdks and VPK inhibition, we have been able to dissect the impact of protein kinases in virus-induced cell cycle dysregulation as well as viral replication. The dependence of CMV-induced pseudomitosis on elevated cellular Cdk1 activity contrasts with the broader, overlapping role of Cdks and VPK in replication. Future work will focus on investigating the behavior of UL97 mutant virus or MBV-treated wild-type virus in the presence of cellular kinase inhibitors to further dissect the role of individual kinases in host cell modulation and viral replication.

Induction of Cdk1 underlies the higher levels of pseudomitosis observed with AD169varATCC and lower levels observed with AD169varDE, and both may be modulated with predictable results by transduction of dominant negative Cdk1 or wt Cdk1. The viral function(s) that influence the levels and activity of Cdk1 during infection is unknown, and as such, levels of pseudomitosis may reflect viral regulation of a gene product that drives or inhibits this process. Virus strain and/or cell culture conditions clearly influence pseudomitosis, and one viral function that may play a role, based on characterized differences between strain variants used here, is the cell death suppressor viral inhibitor of caspase 8 activation [43]. The precise roles played by virus-
encoded cell death suppressors is starting to emerge [45]; however, interplay between the viral inhibitor of caspase 8 activation and cell cycle dysregulation has not been established. Further understanding will come from the use of additional viral mutants, from the continued study of viral strains with high or low pseudomitotic behavior, and from studies that aim at dissecting the Cdk1-dependent events that are critical to the process. Transduction of wild-type Cdk1 induces levels of pseudomitosis as high as 50%, whereas use of dominant negative Cdk1 reduces levels to below 1% without either treatment altering overall virus yields. These data demonstrate that dysregulation of the cell cycle is associated and compatible with CMV replication but that the virus does not rely on this process for any step in replication. It is likely, though, that this virus might require a range of cellular functions that are made available as a result of driving cells into an M-like state. The efficiency of CMV replication in pseudomitotic cells, they are compatible with capsid assembly and virion maturation proceeding through the envelopment step in the absence of a nuclear membrane [33,50].

In CMV-infected HFs, Cdk1/cyclin B induction occurs very early, by 8 h postinfection, and this induction is maintained throughout the time course of infection. There appear to be specific virus-induced mechanisms, including the accumulation of cyclin B1, the inactivation of negative Cdk1 regulators such as Myt1 and Wee1, and the accumulation of Cdk1-activating proteins like Cdc25 [18], that provide specific signals to sustain this complex. Despite this, the Cdk1/cyclin B complex by itself does not support CMV replication. Cdk1/cyclin B activation and phosphorylation of specific substrates have also been reported during infection with other herpesvirus, such as human herpesvirus 7 [51] and Epstein-Barr virus [52]. In HSV-1–infected cells, Cdk1 activity is increased [53] and is required to ensure optimal expression of specific late viral genes [54] but also does not affect viral yields. Except for the reported role of Cdk2 in CMV replication [15] and some previous evaluation of MBV [49], there has been little reported investigation of Cdks acting alone or together with viral kinases in the replication of other herpesviruses.

All herpesviruses encode protein kinases which appear to specifically phosphorylate the same amino acid target sequences recognized by Cdk1 [55]. Although the physiological significance of this molecular mimicry is still unclear, it has been suggested that the viral protein kinases may facilitate capsid transport across the nuclear membrane by phosphorylating key components of the nuclear lamina that are also Cdk1 substrates. Consistent with this hypothesis, a CMV UL97 mutant virus exhibits a severely impaired ability to traverse the nuclear envelope [32] under culture conditions where critical roles in DNA replication and encapsidation are apparently supported by cellular kinases such as Cdks [32,34,35].

Compensatory, overlapping activities of VPK and major Cdks may be important much earlier in infection than VPK mutants indicate. The induction of Cdks at early times postinfection has been associated with accurate processing and accumulation of IE1, IE2, UL36, and UL37x1 transcripts [14] as well as with the hyperphosphorylation of the RNA polymerase II C-terminus [56], functions that are important well before the late phase of replication and that may also be carried out by VPK, a virion tegument protein that is introduced into cells upon entry. Even though pseudomitosis has brought focus to the late phase of replication, the coordinated function of viral and cellular kinases likely contributes to regulation from the initial stages through the late stages of replication. This has suggested broad potential for antiviral strategies targeting Cdks, whether applied to viruses that require dividing cells, such as adenoviruses and papillomaviruses, or to viruses that replicate in nondividing cells, such as HIV-1 and herpesviruses [57]. Cdk inhibitors that range in specificity (roscovitine, flavopiridol, olomucine,
and IMO) all inhibit CMV replication [14,15] and would be predicted to synergize with MBV in clinically relevant settings.

Here, we demonstrate the potential for therapeutic targeting of viral and cellular kinases using IMO after first showing it has prominent antiviral effects against CMV. This is particularly important considering that indirubins are already successfully used to treat leukemia [58]. In addition, the synergistic antiviral effects of IMO and MBV shown here suggest that a combination of both drugs could be useful to simultaneously target CMV infection as well as cell proliferation related to cancer. This may prove particularly attractive in certain patient populations where both CMV disease and leukemia are threats, such as leukemic patients recovering from blood and marrow allografts.

Materials and Methods

Cells and viruses. Primary HFs and the 293T cell line (a gift of Dr. Jerry Crabtree, Stanford University) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO/Invitrogen, http://www.invitrogen.com) supplemented with 10% Fetal Clone III (HyClone, http://www.hyclone.com), penicillin G (100 U/ml), streptomycin sulfate (100 µg/ml), L-arginine (0.58 mg/ml), L-glutamine (1.08 mg/ml), and L-asparagine (180 µg/ml), here referred to as medium, as described previously [7,45]. Human CMV strain AD169 or ATCC (lot 22) was obtained from American Type Culture Collection (ATCC, http://www.atcc.com), AD169orDE from Dr. M. Mach (Institute für Virologie, Erlangen, Germany), Towne or ATCC from Dr. G. Kemble (MedImmune, Mountain View, California, United States), TowneswR1T1, from M. Stinski (University of Iowa, Iowa City, Iowa, United States), Toledo from Dr. S. Plotkin (Philadelphia, Pennsylvania, United States), and TB40E from Dr. C. Sinzger (Institute für Virologie, Tübingen, Germany). Propagation and purification of all strains were as described [59]. Virus titers were determined by plaque assay on HF monolayers in six- or 12-well tissue culture plates.

HF infection with CMV, IMO, and MBV treatments. HF monolayers were infected at confluency (day 3 postseeding) with purified virions [7] at an MOI of 10. After virus adsorption for 1 h at 37 °C, cells were infected at confluency (day 3 postseeding) with purified United States), Toledo from Dr. S. Plotkin (Philadelphia, Pennsylvania, United States), and TB40/E from Dr. C. Sinzger (Institute für Virologie, Tübingen, Germany). Propagation and purification of all strains were as described [59]. Virus titers were determined by plaque assay on HF monolayers in six- or 12-well tissue culture plates.

HF infection with CMV, IMO, and MBV treatments. HF monolayers were infected at confluency (day 3 postseeding) with purified virions [7] at an MOI of 10. After virus adsorption for 1 h at 37 °C, the inoculum was removed, and the cells were washed twice with medium prior to the addition of fresh medium. For IMO treatment, medium containing 5 µM or 10 µM IMO or containing 1% or 2% DMSO as control was added to cells after the second wash and replaced every 24 h. IMO was purchased from Sigma (http://www.sigmaaldrich.com) and was dissolved in DMSO (Sigma) at a stock concentration of 1 mM MBA (GlaxoSmithKline, http://www.beckmancoulter.com). Pelleted viral particles were suspended in medium, aliquoted, and stored at −80 °C. For retrovirus transduction, confluent HFs were split at a 1:2 ratio on day 0 and again at a 1:4 ratio on day 1. At 24 h after the second seeding, cells were exposed to the first inoculum of retroviral particles, in the presence of 5 µg/ml Polybrene (Sigma). Then, 5 h later, cells were washed with fresh medium and a second inoculum was applied for another 5 h. At 24 h after removal of the second inoculum, cells were tested for GFP, Cdk1wt-GFP, or Cdk1dn-GFP expression before infection with CMV as described above.

Immunofluorescence analyses and imaging. γ-Tubulin 1 and IE1/IE2 double-positive staining was carried out as described [7] before staining with an unconjugated monoclonal antibody against IE1/IE2 (1:800; MAb101; Chemicon, http://www.chemicon.com) followed by a Texas red-conjugated goat anti-mouse antibody (1:100; Vector Laboratories, http://www.vectorlabs.com). Cellular DNA was highlighted with Hoechst 33342 (Molecular Probes, http://www.invitrogen.com). Coverslips were mounted and analyzed as described [7].

Checkerboard titration of MBV-IMO drug combinations. HFs (passage 11) were seeded onto 24-well culture plates and maintained in medium until confluent at a density of 1.9 × 10⁶ cells per well. Wells were inoculated with 2 × 10⁶ PFU (in 0.5 ml) of AD169 or ATCC (MOI = 10). Virus was adsorbed for 1 h at 37 °C in 5% CO₂, and the inoculum was removed and replaced with 0.5 ml of fresh medium containing combinations of MBV at 0, 0.025, 0.1, and 0.4 µM and IMO at 0, 5, and 10 µM in a checkerboard layout. At 24 h after inoculation, the culture supernatant was harvested from each well, mixed with 0.5% sterile milk, and frozen at −80 °C until virus titers were determined by plaque assay. Fresh medium containing freshly made drug combinations was replaced in each culture well until the next collection time point. The checkerboard experiment was performed in triplicate.

Supporting Information

Accession Numbers

The UniProtKB/Swiss-Prot (http://www.pir.uniprot.org) accession numbers for the proteins discussed in this paper are Cdk1 (entry name: CDC2_HUMAN, primary accession number: P06493) and AD169 UL97 (entry name: GCVK_HCMVA, primary accession number: P16788).

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Author contributions. LH, SC, and ESM conceived and designed the experiments. LH and SC performed the experiments. LH and ESM analyzed the data. ESM and SC contributed reagents/materials/analysis tools. LH and ESM wrote the paper.

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