Viruses depend on the host cell to provide the energy and biomolecular subunits necessary for production of viral progeny. We have previously reported that human cytomegalovirus (HCMV) infection induces dramatic changes to central carbon metabolism, including glycolysis, the tricarboxylic acid (TCA) cycle, fatty acid biosynthesis, and nucleotide biosynthesis. Here, we explore the mechanisms involved in HCMV-mediated glycolytic activation. We find that HCMV virion binding and tegument protein delivery are insufficient for HCMV-mediated activation of glycolysis. Viral DNA replication and late-gene expression, however, are not required. To narrow down the list of cellular pathways important for HCMV-mediated activation of glycolysis, we utilized pharmaceutical inhibitors to block pathways reported to be both involved in metabolic control and activated by HCMV infection. We find that inhibition of calmodulin-dependent kinase kinase (CaMKK), but not calmodulin-dependent kinase II (CaMKII) or protein kinase A (PKA), blocks HCMV-mediated activation of glycolysis. HCMV infection was also found to target calmodulin-dependent kinase 1 (CaMKK1) expression, increasing the levels of CaMKK1 mRNA and protein. Our results indicate that inhibition of CaMKK has a negligible impact on immediate-early-protein accumulation yet severely attenuates production of HCMV viral progeny, reduces expression of at least one early gene, and blocks viral DNA replication. Inhibition of CaMKK did not affect the glycolytic activation induced by another herpes virus, herpes simplex virus type 1 (HSV-1). Furthermore, inhibition of CaMKK had a much smaller impact on HSV-1 replication than on that of HCMV. These data suggest that the role of CaMKK during the viral life cycle is, in this regard, HCMV specific. Taken together, our results suggest that CaMKK is an important factor for HCMV replication and HCMV-mediated glycolytic activation.

It has long been known that infection with numerous evolutionarily divergent viruses results in a general activation of host cell metabolism (4, 9, 10, 17, 21, 25, 30). Furthermore, this metabolic activation can be clinically helpful. For example, a wide variety of antiviral compounds target specific nucleotide metabolic activities to treat numerous different viral infections, such as those caused by hepatitis B virus, HIV, human cytomegalovirus (HCMV), and herpes simplex virus (HSV) (1, 6, 12, 20). While in some instances these activities have proven to be therapeutically beneficial, the identity of most of the specific metabolic activities induced by viral infection and the mechanisms through which they are activated are unclear. The identification of these activities and their associated mechanisms may highlight novel targets for therapeutic intervention given the viral dependence on the host cell metabolic network for the production of viral progeny.

We have previously found that infection with HCMV induces substantial changes to the host cell metabolic network (22, 23). HCMV is a betaherpesvirus containing a large double-stranded DNA genome (~240-kb) encoding over 200 open reading frames (ORFs). HCMV prevalence is widespread, and infection rarely causes disease in healthy adults. However, in immunsuppressed populations, such as the elderly, transplant recipients, and cancer patients, HCMV is a substantial cause of morbidity (11, 24). HCMV is also a significant cause of birth defects. Congenital HCMV infection occurs in 1 to 2% of all live births (1), with central nervous system damage occurring in the majority of symptomatic newborns (5, 24).

HCMV infection was previously found to increase the cellular glycolytic rate, i.e., glycolytic flux, as well as to increase the activity of a glycolytic rate-determining enzyme, phosphofructokinase (22, 23). The mechanisms through which HCMV infection mediates these changes are currently unclear. Traditional views of metabolic regulation hold that metabolic pathways are largely regulated by the concentrations of allosteric small-molecule effectors on specific rate-limiting enzymes. While these mechanisms of metabolic control undoubtedly still play a role, it is becoming increasingly apparent that metabolic regulation does not rely on allosteric self-regulation alone. Instead, multiple upstream signal transduction networks, for example the phosphatidylinositol 3-kinase (PI3K)/Akt and Ras pathways, play regulatory roles in the control of central carbon and nitrogen metabolism (reviewed in references 8 and 29). As HCMV infection activates numerous signal transduction pathways (reviewed in reference 40), it is possible that viral induction of upstream signal transduction pathways is responsible for downstream metabolic activation.

Here, we have begun to analyze the mechanisms responsible for HCMV-mediated activation of glycolysis. We find that calcium signal transduction is important for HCMV-mediated...
activation of glycolysis. Specifically, pharmacological inhibition of calmodulin-dependent kinase kinase (CaM KK), but not calmodulin-dependent kinase II (CaMKII) or protein kinase A (PKA), blocks HCMV-induced glycolytic activation. HCMV also appears to target calmodulin-dependent kinase I (CaM KK1) expression, as HCMV infection was found to increase its mRNA and protein abundance. Furthermore, inhibition ofCaM KK severely attenuated the production of infectious progeny. Our results suggest that CaM KK is an important cellular factor for both HCMV-mediated activation of glycolysis and HCMV replication.

MATERIALS AND METHODS

Cell culture and virus infection. MRC-5 fibroblasts were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum. Cells were grown to confluence in either 60-mm or 6-well tissue culture plates, resulting in a density of 3.2 × 10^4 cells per cm^2. Once confluent, medium was removed and serum-free medium was added. Cells were maintained in serum-free medium for 24 h before infection.

The HCMV strain used in this study was BAdwt, a bacterial artificial chromosome (BAC) clone of Ad169 (39). HSV-1 KOS (ATCC) was used for all HSV-1 infections. Mock-infected controls were treated with an equal volume of medium containing the same serum concentrations as virus-treated cells. Virus adsorptions were carried out for 90 min at 37°C, after which viral inocula were aspirated and serum-free DMEM was added back. In experiments utilizing UV-irradiated virus, the viral inocula were exposed to 254 nm light at 0 or 1,920 mJ/cm^2 with a model 240 Stratallinker UV cross-linker prior to infection. Production of infectious virus was measured by a standard viral plaque assay. Toxicity associated with STO-609 treatment was analyzed by a Live/Dead assay (Marker Gene Technologies) according to the manufacturer's protocol.

MRC-5s expressing a kinase-dead variant of CaM KK1 [CaM KK1-KD(K157A)] were generated by recombinant retroviral transduction. The CaM KK1-KD(K157A)-pME18s construct, which expresses rat CaM KK, was a kind gift from Thomas Soderling and was previously described (38). CaM KK-KD was PCR amplified from this construct with the primers ATACAGAATTCTAGAGCCGCTCAGTCGCTGATGATCCTATGACGACGTCATCTATCCTGACT. The resulting product was digested with EcoRI and inserted in-frame into a modified retroviral transduction vector, pLPCX-Flag vector, which contains a C-terminal double Flag tag. The pLPCX-Flag vector is modified version of pLPCX (Invitrogen) containing a double Flag tag through ligation of the oligonucleotides AATTCGACTACAA

## RESULTS

### Time course of HCMV-glycolytic induction.

Previously, we have found that at 48 h postinfection (hpi), HCMV activates glycolytic flux and specifically increases the activity of the glycolytic enzyme phosphofructokinase-1 (PFK-1) (22, 23). PFK-1 catalyzes the phosphorylation of fructose-6-phosphate to form fructose-1,6-bisphosphate (FBP) (Fig. 1A, diagram). This reaction is a key regulatory step of glycolysis that is targeted by diverse signal transduction cascades (reviewed in reference 37). To measure glycolytic activity, 13C-labeled glucose can be utilized as an isotope tracer in which the labeling speed of specific metabolite pools, e.g., FBP, serves as a proxy for metabolic flux (23). To determine how HCMV infection affects the rate of glycolysis over a time course of viral infection, we pulsed HCMV-infected fibroblasts with [13C]glucose and measured the rate of accumulation of 13C-labeled FBP by LC−MS−MS. As shown in Fig. 1B, HCMV infection resulted in only a modest increase in the rate of accumulation of 13C-labeled FBP compared to the level for mock-infected cells at 24 h. However, at 48 h and 72 h, HCMV-infected fibroblasts demonstrated a >3-fold increase in the rate of accumulation of 13C-labeled FBP (Fig. 1B). These time points during infection coincide with a period of intense viral DNA replication and are consistent with a scenario in which virally mediated glycolytic
activation provides the increased energy and carbon building blocks for viral replication.

Protein expression, but not DNA replication, is required for HCMV-induced glycolytic activation. The mechanisms responsible for HCMV-mediated glycolytic activation are currently unclear. To determine if the earliest events of viral infection, i.e., virion binding, envelope fusion, and tegument protein delivery, are sufficient to activate glycolytic flux in the absence of DNA replication, we treated mock- or HCMV-infected cells with cycloheximide and assayed for the rate of \(^{13}C\)FDP accumulation as described above. We found that cycloheximide addition completely blocked the HCMV-mediated induction of glycolysis, reducing it to mock infection levels (Fig. 1C). After adsorption, cells were treated with DMSO, an inhibitor of protein synthesis, cycloheximide (10 \(\mu\)g/ml), or an inhibitor of viral DNA replication, PAA (50 \(\mu\)g/ml). At 48 hpi, cells were labeled with \(^{13}C\)glucose containing DMEM for 1 min, quenched with cold methanol, and processed for LC–MS-MS analysis. Values are means ± standard errors (SE) (\(n = 4\)). (D) Serum-starved MRC-5 fibroblasts were mock infected, HCMV infected, or infected with UV-irradiated HCMV (MOI = 3.0). At 48 hpi, cells were labeled with \(^{13}C\)glucose containing DMEM for 1 min, quenched with cold methanol, and processed for LC–MS-MS analysis. Values are means ± SE (\(n = 2\)).

Pharmaceutical inhibition of CaMKK, but not CaMKII or PKA, blocks HCMV-induced glycolytic activation. Numerous HCMV viral factors modulate various cellular signal transduction pathways which, in turn, could affect cellular metabolic activity. For example, HCMV encodes constitutively active G protein-coupled receptors whose activities can result in increased adenylate cyclase activity and calcium mobilization (reviewed in reference 2). This increase in adenylate cyclase activity can, in turn, activate the cyclic AMP (cAMP)-dependent kinase, PKA. Activated PKA can activate glycolysis through CREB-mediated transcriptional induction of glycolytic enzymes, or through activation of PFK-2, which results in direct activation of PFK-1 (Fig. 2A) (reviewed in references 3 and 28). Similarly, activation of calcium-dependent kinases can also result in both CREB activation and activation of specific glycolytic activities (3, 26, 32, 36). To determine if PKA and calcium signaling are important for HCMV-mediated activation of glycolysis, we utilized various inhibitors to target their activity with a subsequent analysis of HCMV-induced glycolytic activation. We found that treatment with HA1004, an inhibitor of PKA (13), had no effect on the rate of accumulation of \(^{13}C\)-labeled FBP in either mock- or HCMV-infected
cells compared to the level for DMSO-treated controls (Fig. 2B). To determine whether calcium signaling may be important for HCMV-mediated activation of glycolysis, we treated cells with STO-609, a specific inhibitor of CaMKK (35), and with KN-62, a CaMKII inhibitor (34). CaMKK is an upstream kinase in the calcium-calmodulin kinase cascade, e.g., phosphorylating and activating CaMKI and CaMKIV (33). CaMKII, however, has been reported to autophosphorylate in response to increased calcium levels, thereby making CaMKII independent of CaMKK activity (33). Treatment with KN-62 had no effect on the rate of $[^{13}C]FBP$ accumulation during HCMV infection, suggesting that HCMV-mediated activation of glycolysis is independent of CaMKII (Fig. 2B). Surprisingly, treatment with the CaMKII inhibitor activated glycolysis in mock-infected cells (Fig. 2B). In contrast to what was observed for inhibitors of CaMKII and PKA, treatment of cells with STO-609 completely blocked the glycolytic activation induced by HCMV infection but had no impact on the glycolytic rate of mock-infected cells (Fig. 2B). Our results indicate that pharmaceutical inhibition of CaMKK blocks HCMV-induced glycolytic activation but that inhibition of CaMKII or PKA does not.

Pharmaceutical inhibition of CaMKK attenuates the production of HCMV viral progeny. Given that pharmaceutical inhibition of CaMKK blocks viral activation of glycolysis, we were interested in determining how CaMKK inhibition affected viral replication. Toward this end, we infected fibroblasts with HCMV (MOI = 3.0), treated them with either DMSO or 5 or 10 μg/ml STO-609, and harvested them at various time points postinfection for analysis of the production of viral progeny. As shown in Fig. 3A, treatment with the CaMKK inhibitor resulted in a dose-dependent decrease in the production of infectious virions at multiple time points of infection. By 120 h postinfection, treatment with 5 and 10 μg/ml STO-609 resulted in $10^{-}$- and $10^{100}$-fold defects, respectively (Fig. 3A). These results suggest that in addition to inhibiting
viral induction of glycolysis, treatment with a CaMKK inhibitor attenuates HCMV replication. In our studies, we had not seen any detectable cell death associated with treatment of mock- or HCMV-infected cells with STO-609; however, we wanted to explore this potential issue further. We treated cells with 10 μg/ml STO-609 and assayed for their viability utilizing the Live/Dead assay, which stains live cells based on their esterase activity (green) and stains the nucleic acids of dead cells (red) based on the breakdown of membrane integrity. A representative field of cells from the Live/Dead staining is shown in Fig. 3B. Over 15 fields for each condition were counted for green versus red staining, with the percentage of viable cells over 95% for each condition, i.e., >95% of cells exhibited green staining and excluded red staining (Fig. 3B). These data indicate that treatment of mock- or HCMV-infected cells with STO-609 does not induce cellular toxicity.

Impact of CaMKK inhibition on the HCMV viral life cycle.
To further investigate the role that CaMKK plays in viral events, Western blot analysis was performed to observe the effect of CaMKK inhibition on the accumulation of immediate-early (IE1), early (UL26), and late (pp28) proteins throughout infection. Fibroblasts were HCMV infected, treated with STO-609, and harvested at 24, 48, and 72 h postinfection and analyzed by Western blotting with antisera specific for IE1, UL26, pp28, and tubulin. Mw, molecular mass. (A) Analysis of the impact of CaMKK inhibition on viral protein accumulation. Serum-starved MRC-5 cells were mock infected or infected with HCMV (MOI = 3). After adsorption, cells were treated with the CaMKK-specific inhibitor STO-609 (5 μg/ml or 10 μg/ml) or DMSO alone. Cells were harvested at 24, 48, and 72 h postinfection and analyzed by Western blotting with antisera specific for IE1, UL26, pp28, and tubulin. Mw, molecular mass. (B) Analysis of the impact of CaMKK inhibition on viral DNA accumulation. Serum-starved MRC-5 cells were mock infected or infected with HCMV (MOI = 3). After adsorption, cells were treated with the CaMKK-specific inhibitor STO-609 (10 μg/ml) or DMSO. Viral DNA was extracted from cells that were harvested at 48, 72, and 96 h postinfection and processed for qPCR analysis of viral DNA accumulation. Values are means ± SE (n = 6). (C) Serum-starved MRC-5 human fibroblasts were HCMV infected (MOI = 3). After adsorption, two sets of cells were treated with STO-609 (10 μg/ml) or DMSO, as indicated on the graph. The last set of MRC-5s was treated with STO-609 (10 μg/ml) at 24 hpi and harvested at 96 hpi with the rest of the treatments. Viral replication was measured by a plaque assay. Values are means ± SE (n = 2). An asterisk indicates that the number of infectious virions were below the limit of detection (~20 PFU/ml). (D) Serum-starved MRC-5 human fibroblasts were mock or HCMV infected (MOI = 3). After adsorption, fresh serum-free medium was placed on the cells. At 24 h postinfection, cells were treated with either DMSO or STO-609. Cells were pulsed at 48 or 72 h postinfection for 1 min with [13C]glucose containing DMEM, quenched with cold methanol, and processed for LC–MS–MS to measure the accumulation of [13C]fructose-1,6-bisphosphate. Values are means ± SE (n = 2).

FIG. 4. Impact of CaMKK pharmaceutical inhibition on viral protein and DNA accumulation. (A) Analysis of the impact of CaMKK inhibition on viral protein accumulation. Serum-starved MRC-5 human fibroblasts were mock infected or infected with HCMV (MOI = 3). After adsorption, cells were treated with the CaMKK-specific inhibitor STO-609 (5 μg/ml or 10 μg/ml) or DMSO alone. Cells were harvested at 24, 48, and 72 h postinfection and analyzed by Western blotting with antisera specific for IE1, UL26, pp28, and tubulin. Mw, molecular mass. (B) Analysis of the impact of CaMKK inhibition on viral DNA accumulation. Serum-starved MRC-5 cells were mock infected or infected with HCMV (MOI = 3). After adsorption, cells were treated with the CaMKK-specific inhibitor STO-609 (10 μg/ml) or DMSO. Viral DNA was extracted from cells that were harvested at 48, 72, and 96 h postinfection and processed for qPCR analysis of viral DNA accumulation. Values are means ± SE (n = 6). (C) Serum-starved MRC-5 human fibroblasts were HCMV infected (MOI = 3). After adsorption, two sets of cells were treated with STO-609 (10 μg/ml) or DMSO, as indicated on the graph. The last set of MRC-5s was treated with STO-609 (10 μg/ml) at 24 hpi and harvested at 96 hpi with the rest of the treatments. Viral replication was measured by a plaque assay. Values are means ± SE (n = 2). An asterisk indicates that the number of infectious virions were below the limit of detection (~20 PFU/ml). (D) Serum-starved MRC-5 human fibroblasts were mock or HCMV infected (MOI = 3). After adsorption, fresh serum-free medium was placed on the cells. At 24 h postinfection, cells were treated with either DMSO or STO-609. Cells were pulsed at 48 or 72 h postinfection for 1 min with [13C]glucose containing DMEM, quenched with cold methanol, and processed for LC–MS–MS to measure the accumulation of [13C]fructose-1,6-bisphosphate. Values are means ± SE (n = 2).
CaMKKI mRNA at 24 and 48 h postinfection. We find that HCMV increases the abundance of CaMKKI protein levels. (A) Analysis of CaMKKI mRNA during HCMV infection by qPCR. Serum-starved MRC-5 human fibroblasts were mock infected or infected with HCMV (MOI = 3). Cellular mRNA isolated from cells harvested at 24, 48, and 72 h postinfection was processed for qPCR using CaMKKI-specific primers. Values are plotted as mean relative abundances ± SE observed after normalization to GAPDH (n = 3). (B) Analysis of CaMKKI protein accumulation over a time course of HCMV infection. Serum-starved MRC-5 human fibroblasts were mock infected or infected with HCMV (MOI = 3). Cells were harvested at 4, 24, 48, and 72 h postinfection and analyzed by Western blotting with two independent CaMKKI-specific antibodies. Tubulin was also blotted for as a loading control. As CaMKK inhibition appeared to affect the viral life cycle between the immediate-early-to-early transition and blocks DNA synthesis. The inhibition of viral DNA synthesis could potentially result from the impact of attenuated early-gene expression that occurs when CaMKK is inhibited.

As CaMKK inhibition appeared to affect the viral life cycle between the immediate-early-to-early transition, we wanted to test if viral replication was sensitive to STO-609 treatment at this junction, i.e., at 24 h postinfection, when DNA synthesis is just beginning. As shown in Fig. 4C, addition of STO-609 at 24 h postinfection largely rescued HCMV viral growth, although there was still an ~1.5-log defect compared to the level for the DMSO-treated control. This suggests that a large proportion of the inhibitory effect of STO-609 treatment on HCMV infection acts prior to 24 h postinfection. Given this result, we were interested to determine how STO-609 treatment at 24 h postinfection affects HCMV-activated glycolysis. As shown in Fig. 4D, treatment of HCMV-infected cells 24 h postinfection did not significantly inhibit the glycolytic activation induced by HCMV infection when assayed at 48 h postinfection. These results suggest that the STO-609 treatment is inhibiting a virally induced event in the first 24 h of infection that is necessary for glycolytic activation. We also assayed how treatment of mock- or HCMV-infected cells at 24 h postinfection affected glycolytic flux at 72 h postinfection. Contrary to what was observed at 48 h postinfection, virally activated glycolytic flux was mostly inhibited at 72 h postinfection when STO-609 was added at 24 h postinfection (Fig. 4D). These results suggest that HCMV-mediated glycolytic activation can largely be reversed when STO-609 is added at later points, i.e., when STO-609 is added at 24 h postinfection, glycolysis is activated at 48 h but is subsequently inhibited by 72 h postinfection. This could reflect a viral or cellular factor responsible for glycolytic activation that continually requires CaMKK activity to maintain activation of glycolysis.

HCMV infection induces the accumulation of CaMKKI mRNA and protein. Our results indicate that CaMKK activity is important both for HCMV-mediated activation of glycolysis and for production of viral progeny. To further investigate how HCMV infection may target CaMKK, we sought to determine how CaMKK expression is affected by HCMV infection. Fibroblasts were mock or HCMV infected and harvested for qPCR and Western blot analysis at 4, 24, 48, and 72 h postinfection. We find that HCMV increases the abundance of CaMKKI mRNA at 24 and 48 h postinfection ~4-fold (Fig. 5A). By 72 h postinfection, the levels of CaMKKI fell to those observed during mock infection (Fig. 5A). Analysis of CaMKKI protein accumulation produced similar results. HCMV-infected fibroblasts exhibited increased levels of CaMKKI protein with respect to mock infection levels at 24 h postinfection (Fig. 5B). The levels of CaMKKI continued to increase from 48 to 72 h postinfection (Fig. 5B). As seen in Fig. 5B, a polyclonal antibody to CaMKKI detected multiple bands that seemed to be induced by HCMV infection. To determine whether these bands could be phosphorylated isoforms of CaMKK, we tested whether they were sensitive to lambda phosphatase treatment. None of the bands showed any migration changes upon lambda phosphatase treatment (data not shown). To further explore the accumulation of CaMKKI during HCMV infection, we employed a second CaMKK specific antibody, a mouse monoclonal antibody. An induction of CaMKKI levels was again seen at roughly 24 to 72 h postinfection (Fig. 5B). Taken together, our qPCR data as well as the Western analysis suggest that HCMV induces CaMKK expression, consistent with the notion that CaMKK is an important cellular factor for HCMV replication.

Expression of a kinase-dead variant of CaMKKI inhibits the production of HCMV viral progeny. The finding that pharmaceutical inhibition of CaMKK attenuates production of HCMV viral progeny suggests that CaMKK is an important cellular factor for HCMV replication. While STO-609 has been demonstrated to be a very specific inhibitor of CaMKK (35), the possibility for off-target effects is always a potential issue with pharmaceutical inhibitors. As an additional means
were infected with HCMV (MOI 0.1). Cells were harvested 10 days postinfection (α-Flag blot) or 48 h postinfection (mouse α-CaMKKα and tubulin blots). The presence of the CaMKK-KD or tubulin was monitored by Western blotting with the indicated antibodies. (B) Impact of CaMKK-KD expression on HCMV viral replication. Serum-starved MRC-5 fibroblasts expressing either CaMKK-KD or an empty vector were infected with HCMV (MOI = 0.1). Cells were harvested at 10 days postinfection, and viral replication was measured by a viral plaque assay. Values are plotted as means ± SE (n = 6). (C) Impact of CaMKK-KD expression on glycolysis in HCMV-infected cells. Serum-starved MRC-5 fibroblasts expressing either CaMKK-KD or an empty vector were infected with HCMV (MOI = 3.0). At 48 h postinfection, cells were labeled for 1 min with [13C]glucose containing DMEM, quenched with cold methanol, and processed for LC–MS-MS to measure the accumulation of [13C]fructose-1,6-bisphosphate. Values are means ± SE (n = 2).

FIG. 6. Impact of expression of a CaMKK kinase-dead allele on HCMV replication. (A) Analysis of Flag–CaMKK-KD protein expression in fibroblasts. MRC-5 fibroblasts were transduced with an empty vector or a vector expressing a Flag-tagged kinase-dead allele of CaMKK (CaMKK-KD). Fibroblasts containing the CaMKK-KD variant or an empty-vector control were mock infected or infected with HCMV (MOI = 0.1). Cells were harvested 10 days postinfection (α-Flag blot) or 48 h postinfection (mouse α-CaMKKα and tubulin blots). The presence of the CaMKK-KD or tubulin was monitored by Western blotting with the indicated antibodies. (B) Impact of CaMKK-KD expression on HCMV viral replication. Serum-starved MRC-5 fibroblasts expressing either CaMKK-KD or an empty vector were infected with HCMV (MOI = 0.1). Cells were harvested at 10 days postinfection, and viral replication was measured by a viral plaque assay. Values are plotted as means ± SE (n = 6). (C) Impact of CaMKK-KD expression on glycolysis in HCMV-infected cells. Serum-starved MRC-5 fibroblasts expressing either CaMKK-KD or an empty vector were infected with HCMV (MOI = 3.0). At 48 h postinfection, cells were labeled for 1 min with [13C]glucose containing DMEM, quenched with cold methanol, and processed for LC–MS-MS to measure the accumulation of [13C]fructose-1,6-bisphosphate. Values are means ± SE (n = 2).

The kinase-dead mutant on viral replication was much lower than that observed upon pharmaceutical inhibition of CaMKK. While for some kinases, their kinase-dead alleles are capable of acting as dominant-negative mutants, others do not. To determine whether expression of CaMKK-KD blocked expression of HCMV-mediated glycolysis, we assayed glycolytic labeling in HCMV-infected vector control-transduced or CaMKK-KD-transduced cells. We found that CaMKK-KD expression resulted in a statistically insignificant reduction in [13C]FBP labeling, indicating that the CaMKK-KD expression was not sufficient to inhibit HCMV-mediated activation of glycolysis (Fig. 6C). Our results indicate that pharmaceutical inhibition of CaMKK has a much stronger impact on HCMV infection and on glycolysis than expression of a CaMKK-KD allele. Nevertheless, the results indicating that a kinase-dead variant of CaMKK significantly inhibits viral replication further support the idea that CaMKK plays an important role during HCMV infection.

Inhibition of CaMKK does not inhibit HSV-mediated glycolytic activation. As all viruses rely on the cellular metabolic network to provide the energy and biomolecular subunits necessary for viral replication, it is possible that viral activation of glycolysis could be a common hallmark of viral infection. To begin to examine this issue, we analyzed the impact of an alphaherpesvirus, herpes simplex virus type 1 (HSV-1), on glycolysis. We pulsed fibroblasts that had been infected with HSV-1 for 12 h with [13C]glucose and measured the rate of FBP accumulation. We find that HSV-1 increases the speed of FBP labeling compared to the level for mock infection (Fig. 7A). However, in contrast to what we observed with HCMV infection, pharmacological inhibition of CaMKK had no impact on the glycolytic activation induced by HSV-1 infection (Fig. 7A). These results suggest that while both HCMV and HSV-1 induce glycolytic activation, HCMV-mediated glycolytic activation is dependent on the CaMKK cascade, whereas HSV-1 mediated activation of glycolysis is not. This indicates that HCMV and HSV-1 employ different strategies to activate glycolysis.

To determine if HSV-1 replication is sensitive to pharmaceutical inhibition of CaMKK, fibroblasts were infected in the presence of DMSO or STO-609 (10 μg/ml) and harvested at 24 h postinfection to determine the production of viral progeny. As shown in Fig. 7B, pharmaceutical inhibition of CaMKK resulted in an ∼10-fold reduction of HSV titers. While this reduction in production of infectious HSV-1 is significant, similar drug concentrations resulted in a >100-fold defect in HCMV titers (Fig. 3A). These results suggest that HSV-1 and HCMV infections differ in their sensitivity to CaMKK inhibition, both in their activation of glycolysis and in their attenuation of viral growth. HCMV growth is more sensitive to inhibition of CaMKK than HSV-1 growth is, which correlates with the increased impact of CaMKK inhibition on glycolysis during HCMV infection relative to that observed during HSV-1 infection.

Inhibition of glycolysis attenuates HCMV and HSV replication. We find that pharmaceutical inhibition of CaMKK attenuates HCMV-induced activation of glycolysis and production of infectious HCMV. If the inhibition of glycolysis is responsible for the growth defect observed when cells are treated with a CaMKK inhibitor, one would predict that treatment with an
Previous reports have indicated that inhibition of glycolysis attenuates the replication of both HCMV and HSV-1 (7, 27). To confirm these findings in our cells and to determine how the potential growth defect associated with glycolytic inhibition compares to that of CaMKK inhibition, we assayed for the potential growth defect associated with glycolytic inhibition (Fig. 7B) and is consistent with our results showing a lack of a role for CaMKK in glycolytic activation during HSV-1 infection.

Inhibitor of glycolysis would attenuate viral growth to an extent similar to that observed upon CaM KK inhibition. Previous reports have indicated that inhibition of glycolysis attenuates the replication of both HCMV and HSV-1 (7, 27). To confirm these findings in our cells and to determine how the potential growth defect associated with glycolytic inhibition compares to that of CaMKK inhibition, we assayed for the production of HCMV and HSV viral progeny in the presence of the glycolytic inhibitor 2-deoxyglucose (2-DOG). Fibroblasts were infected with HCMV or HSV-1 in the presence of 2-DOG (20 mM) and harvested for analysis of viral replication via a plaque assay. As seen in Fig. 8, 2-DOG treatment resulted in a >100-fold defect in infectious HCMV production, similar to the level of attenuation observed upon CaMKK inhibition (Fig. 3A). While only a correlation, the similar levels of viral attenuation associated with glycolytic and CaMKK inhibition are consistent with a scenario in which HCMV-induced CaMKK activity is necessary for activation of glycolysis and subsequent production of wild-type levels of infectious virions. For HSV-1 infection, 2-DOG treatment resulted in a >1,000-fold defect (Fig. 8). This attenuation was much greater than the observed impact on HSV-1 replication upon CaMKK inhibition (Fig. 7B) and is consistent with our results showing a lack of a role for CaMKK in glycolytic activation during HSV-1 infection.

**DISCUSSION**

We have previously reported that HCMV infection induces numerous changes to the metabolic network; among these changes is the activation of glycolysis (22, 23). In this study, we began to elucidate the mechanisms responsible for HCMV-mediated glycolytic activation. We find that viral envelope fusion and tegument protein delivery are not sufficient for glycolytic activation; instead, de novo protein synthesis is necessary for HCMV-mediated glycolytic activation (Fig. 1). While it is currently unclear which viral factors are responsible for the activation of glycolysis, our results indicating that inhibition of viral DNA replication does not affect glycolytic activation suggest that late proteins, whose expression is dependent on DNA replication, are not involved.

We find that inhibition of CaMKK but not PKA or CaM KII ablates HCMV-induced glycolytic activation (Fig. 2). HCMV has been shown to mobilize cellular calcium stores (14), which suggests that HCMV infection should activate calmodulin-dependent kinase cascades. Viral proteins that have been implicated in calcium signaling, e.g., U1,37 (31), could play a role in CaMKK activation and therefore viral induction of glycolysis. Interestingly, inhibitors of CaM KII did not block HCMV-mediated glycolytic activation compared to the level for DMSO-treated infected cells. This would suggest that CaMKII is not important for HCMV-mediated glycolytic activation. However, strict conclusions from this result are difficult since treatment with the CaMKII inhibitor KN-62 resulted in the elevation of glycolysis in mock-infected cells (Fig. 2B). This was surprising given the reports suggesting that CaMKII can activate glycolysis (3, 32). One possibility is that HCMV infection attenuates CaMKII activity, resulting in increased glycolysis. In this scenario, the magnitude of virally mediated glycolytic activation might be insensitive to pharmaceutical inhibition of CaMKII since it is already inhibited by HCMV infection. Another scenario is that CaMKII inhibition elevates the basal levels of glycolysis for both mock- and HCMV-infected cells, potentially to a high enough level that HCMV cannot induce it further. Such scenarios as well as their importance for HCMV infection could be tested through future analysis of CaMKII activity during the HCMV viral life cycle.

An alternative to CaMKII-dependent signaling that is consistent with our results would be CaMKK-mediated activation...
of CaMKIV. CaMKIV can activate CREB, resulting in transcriptional activation of cAMP response element (CRE)-containing genes (3). CREB has been reported to activate several glycolytic enzymes (16, 19), and we have previously reported that viral infection activates the expression of several glycolytic enzymes as well (22). Taken together, these results suggest a potential scenario in which upstream CaMKK-mediated transcriptional activation of glycolytic genes could be responsible for HCMV-mediated activation of glycolysis. Other mechanisms of CaMKK-mediated activation of glycolysis include CaMKK-mediated activation of CaMKI. CaMKI has been reported to phosphorylate phosphofructokinase-2 (PFK-2) (26), a glycolytic control enzyme whose phosphorylation results in glycolytic activation through activation of PFK-1 activity (reviewed in reference 28). Additionally, the AMP-activated kinase is another enzyme that can be activated by CaMKK and is capable of phosphorylating/activating PFK-2 (reviewed in reference 36). These possibilities are not mutually exclusive, and future work will determine their respective roles in HCMV-mediated glycolytic activation.

Our results indicate that pharmaceutical inhibition of CaMKK attenuates viral infection (Fig. 3A). Additionally, expression of a kinase-dead variant of CaMKK also attenuated HCMV infection, albeit to a lesser extent (Fig. 6B). The discrepancy between these two methods of CaMKK inhibition poses a challenge with respect to data interpretation. On one hand, small-molecule pharmaceuticals can have off-target effects which can be controlled for through analysis of secondary methods of inhibition. On the other hand, results involving a kinase-dead variant, i.e., an ATP-binding mutant, are totally dependent on the extent to which the mutant allele actually inhibits wild-type CaMKK activity. Such inhibition requires both substantial overexpression of the dominant-negative mutant and the subsequent dilution of crucial concentration-limited secondary interacting factors that are necessary for wild-type CaMKK activity. With respect to this point, to our knowledge, there have been no reports indicating that expression of a kinase-dead CaMKK variant can attenuate CaMKK’s glycolytic phenotypes. Our results indicate that CaMKK-KD expression did not affect HCMV-mediated glycolysis support this possibility. Regardless, both our pharmaceutical approaches and our genetic-based approaches indicate that targeted inhibition of CaMKK attenuates HCMV replication. Further analysis of players downstream of CaMKK, using additional pharmaceutical and genetic tools, will further illuminate the role of this pathway during HCMV infection.

We find that pharmaceutical inhibition of glycolysis results in a magnitude of viral growth inhibition similar to that observed for pharmaceutical CaMKK inhibition. These results are consistent with a scenario in which HCMV infection is dependent on CaMKK activity for glycolytic activation. It is currently unclear what aspects of glycolysis contribute to viral replication. Non-mutually exclusive possibilities include an increased rate of ATP generation, production of glycosylation moieties for proteins, and production of acetyl-CoA for fatty acid biosynthesis, which we have previously reported to be increased during HCMV infection (23). Another possibility is that the viral induction of glycolysis is necessary to maintain a basal level or floor of pentose-phosphate pathway flux, an activity we find is actually decreased during HCMV infection (23). This could be required so that the virus can maintain pentose production for nucleotide biosynthesis. Further dissection of these metabolic pathways with subsequent analysis of their impact on the metabolic network and on viral replication will begin to answer these questions.

Our data indicate a correlation between CaMKK inhibition, glycolytic inhibition, and viral growth. Despite this correlation between the viral attenuation resulting from the inhibition of CaMKK and glycolysis, the impact on viral replication in the face of CaMKK inhibition may be independent of its impact on virally induced glycolysis. Additionally, at this point we also cannot rule out the possibility that CaMKK inhibition may block HCMV-mediated glycolytic induction by preventing the accumulation of a viral factor whose expression is necessary for glycolytic activation. It does appear, however, that continued CaMKK activity is necessary for maintenance of glycolytic activation, as we find that delayed treatment with STO-609 allows virally mediated glycolysis activation at 48 h postinfection but subsequently inhibits glycolytic activation at 72 h postinfection. Our data also indicate that HCMV infection induces the accumulation of CaMKK mRNA and protein (Fig. 5). While the exact mechanism through which CaMKK contributes to HCMV replication needs to be elucidated, the attenuation of HCMV replication in the face of CaMKK inhibition and the induction of CaMKK expression observed during HCMV infection suggest that CaMKK is targeted by HCMV infection.

Our results indicate that, like HCMV infection, HSV-1 infection induces glycolysis (Fig. 7A). However, HSV-1 mediated induction of glycolysis is independent of CaMKK inhibition (Fig. 7A). Pharmaceutical inhibition of CaMKK also resulted in a reduction in HSV-1 viral replication (Fig. 7B), but to a much smaller extent than that of the observed impact on HCMV replication (Fig. 3A). These results suggest that HSV-1 and HCMV employ different mechanisms to activate cellular glycolytic flux.

In conclusion, CaMKK is an important cellular factor for both HCMV-mediated activation of glycolysis and HCMV replication, and the expression of CaMKK is induced by viral infection. Future work will elucidate the mechanisms through which CaMKK contributes to both virally mediated glycolytic activation and viral replication. Given the dependence of viral replication on these mechanisms, their specific identification could have therapeutic potential.

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REFERENCES

ERRATUM

Inhibition of Calmodulin-Dependent Kinase Kinase Blocks Human Cytomegalovirus-Induced Glycolytic Activation and Severely Attenuates Production of Viral Progeny

Jessica McArdle, Xenia L. Schafer, and Joshua Munger

Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, New York 14642


Page 712, legend to Fig. 7A and 7B: The figure legends for Fig. 7A and 7B were switched, i.e., the legend for panel A describes panel B and vice versa.

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