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VIRUS-CELL INTERACTIONS

Glycolysis, Glutaminolysis, and Fatty Acid Synthesis Are Required for Distinct Stages of Kaposi's Sarcoma-Associated Herpesvirus Lytic Replication

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ABSTRACT Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma (KS). KSHV infection induces and requires multiple metabolic pathways, including the glycolysis, glutaminolysis, and fatty acid synthesis (FAS) pathways, for the survival of latently infected endothelial cells. To determine the metabolic requirements for productive KSHV infection, we induced lytic replication in the presence of inhibitors of different metabolic pathways. We found that glycolysis, glutaminolysis, and FAS are all required for maximal KSHV virus production and that these pathways appear to participate in virus production at different stages of the viral life cycle. Glycolysis and glutaminolysis, but not FAS, inhibit viral genome replication and, interestingly, are required for different early steps of lytic gene expression. Glycolysis is necessary for early gene transcription, while glutaminolysis is necessary for early gene translation but not transcription. Inhibition of FAS resulted in decreased production of extracellular virions but did not reduce intracellular genome levels or block intracellular virion production. However, in the presence of FAS inhibitors, the intracellular virions are noninfectious, indicating that FAS is required for virion assembly or maturation. KS tumors support both latent and lytic KSHV replication. Previous work has shown that multiple cellular metabolic pathways are required for latency, and we now show that these metabolic pathways are required for efficient lytic replication, providing novel therapeutic avenues for KS tumors.

IMPORTANCE KSHV is the etiologic agent of Kaposi's sarcoma, the most common tumor of AIDS patients. KS spindle cells, the main tumor cells, all contain KSHV, mostly in the latent state, during which there is limited viral gene expression. However, a percentage of spindle cells support lytic replication and production of virus and these cells are thought to contribute to overall tumor formation. Our previous findings showed that latently infected cells are sensitive to inhibitors of cellular metabolic pathways, including glycolysis, glutaminolysis, and fatty acid synthesis. Here we found that these same inhibitors block the production of infectious virus from lytically infected cells, each at a different stage of viral replication. Therefore, inhibition of specific cellular metabolic pathways can both eliminate latently infected cells and block lytic replication, thereby inhibiting infection of new cells. Inhibition of metabolic pathways provides novel therapeutic approaches for KS tumors.

KEYWORDS HHV-8, Kaposi's sarcoma-associated herpesvirus, fatty acid synthesis, glutaminolysis, glycolysis, herpesvirus, lytic replication, metabolism, virus assembly

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Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), a human gammaherpesvirus, is the infectious agent of KS, the most common tumor of AIDS patients worldwide. KS frequently occurs in HIV-negative patients in sub-Saharan Africa, making it among the

most common cancers in parts of this region (1–3). The main tumor cell of KS is the spindle cell. Spindle cells express markers of endothelium and are generally thought to be of endothelial cell origin (2, 4). KSHV is present in the KS spindle cells, where it is predominantly in the latent state, expressing only a few viral genes (4–6). However, approximately 5% of the spindle cells express lytic genes and are thought to be undergoing lytic replication (5). Therefore, KS tumor cells support both latent infection and lytic infection, and studies have suggested that both latent and lytic viral replicative cycles of KSHV likely contribute to KS tumor development (2, 4).

Previous work from our laboratory explored alterations in host cell metabolism during latent KSHV infection of endothelial cells. Three of the major pathways of central carbon metabolism, glycolysis, fatty acid synthesis (FAS), and glutaminolysis, are commonly activated in cancer cells. A global metabolomics analysis of KSHV-infected cells indicated that endothelial host cell metabolism, including the induction of many anabolic pathways, is significantly altered during latent KSHV infection (7). Our work, and work of others, has demonstrated that latent KSHV infection of endothelial cells, similarly to infection of cancer cells, induces central carbon metabolism, including glycolysis, fatty acid synthesis, and glutaminolysis (7–10). We have also shown that inhibition of each of these three metabolic pathways leads to greatly increased cell death in the latently infected endothelial cells but not in matched uninfected cells (7, 8, 10). Therefore, the induction of metabolic pathways critical for cancer cell biology is required for the survival of latently infected endothelial cells. KSHV is also associated with primary effusion lymphomas (PEL), a rare B-cell lymphoma of AIDS patients. PEL cells also have increased glycolysis and FAS and require both glycolysis and FAS for their survival (11). More recently, it was shown that the presence of the KSHV latent microRNA (miRNA) cluster is sufficient to induce glycolysis and reduce oxidative phosphorylation, supporting previous data showing that latent KSHV infection induces the Warburg effect (12).

Host metabolic requirements for maximal virus production have been examined in RNA viruses, including dengue virus, hepatitis C virus, and poliovirus, and in DNA viruses, including human cytomegalovirus (HCMV), herpes simplex virus 1 (HSV-1), adenovirus (Ad), and vaccinia virus (13–19). Interestingly, while similarities exist, these studies found that virus-specific metabolic signatures are established during infection. While central carbon metabolic pathways are altered by several of these viruses, significant differences in carbon utilization have been identified. For example, HCMV has been found by several groups to alter and require glycolysis, glutaminolysis, and FAS for maximal virus production (15, 16, 19–23). Surprisingly, HCMV, but not HSV-1, has been shown to induce glycolytic flux. HCMV increased the levels of the tricarboxylic acid (TCA) metabolites fueling FAS, while HSV-1 increased the level of flux to the TCA cycle to feed pyrimidine synthesis (19). Recent work has shown that a model adenovirus (Ad5) depends on both glycolysis and glutamine metabolism for its replication, and the viral protein E4ORF1, which interacts with cellular Myc, has been implicated in modulating these cellular metabolic pathways during infection (17, 18). In contrast, vaccinia virus replication depends on glutamine metabolism, while glucose metabolism is completely dispensable for virus production (13).

While a number of metabolic pathways required for latent KSHV infection have been determined, little is known about the host cell metabolic requirements during lytic KSHV replication. Here we demonstrate that KSHV lytic replication also depends on the major central carbon metabolic pathways. Inhibition of glycolysis, glutaminolysis, and fatty acid synthesis reduces KSHV virus production in both endothelial lytic systems and the iSLK cell-inducible KSHV system. Furthermore, inhibition of glycolysis, glutamine metabolism, and FAS blocks lytic replication at different stages of virus production. Glycolysis and glutamine metabolism contribute to virus production at distinct stages prior to viral genome replication. However, there were few to no changes in the levels of intracellular KSHV genomes or the levels of late genes in the presence of fatty acid synthesis inhibitors, indicating that neither KSHV genome replication nor late gene expression was inhibited. However, the presence of fatty acid synthesis inhibitors led to

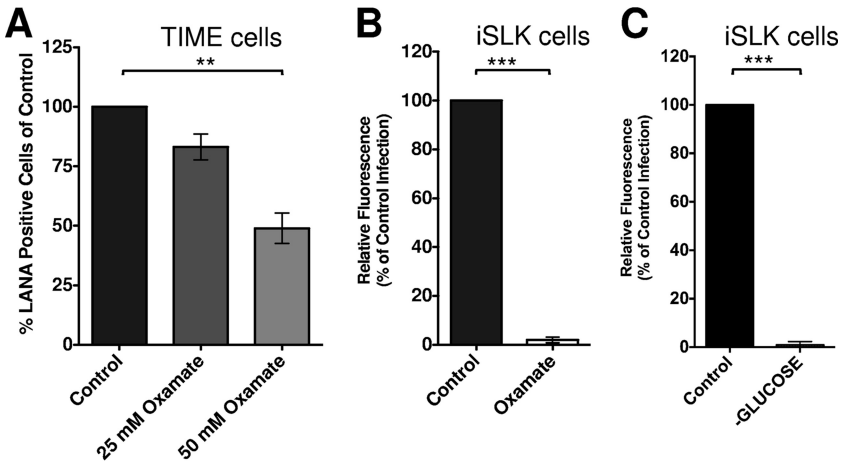

FIG 1 Glycolysis is required for maximal KSHV virion production. (A) TIME cells were infected with KSHV and then superinfected with an RTA-expressing adenovirus. Cells were overlaid with media with or without the glycolytic inhibitor oxamate at 25 mM and 50 mM. At 48 h postinfection, supernatants were collected and titers were determined on fresh TIME cells. Titer levels were measured by IFA for LANA-expressing cells. Data shown represent the average percentages of LANA-expressing titer cells determined in at least 3 biological replicate experiments. (B and C) iSLK cells were chemically induced with doxycycline and sodium butyrate in the presence or absence of oxamate (50 mM) (B) or glucose (C) in the media. After 24 h, supernatant was harvested and titers were determined on TIME cells. Wild-type (WT) KSHV BAC16 expresses GFP; therefore, titers were assessed at 48 hpi on a Typhoon fluorescent image scanner. Data shown represent average results of 3 biological replicate experiments, with control results set to 100%.

a significant decrease in levels of extracellular genomes, indicating a defect in assembly or egress that was confirmed by transmission electron microscopy (TEM). In summary, this study showed that KSHV requires multiple central carbon metabolism pathways for distinct stages of lytic replication.

RESULTS

Glycolysis is required for maximal KSHV virion production. To ascertain if glycolysis is necessary for KSHV lytic replication, we measured the production of infectious virus following lytic infection in the presence or absence of the glycolysis inhibitor oxamate, an inhibitor of the enzyme lactate dehydrogenase (LDH). For the Tert-immortalized microvascular endothelial (TIME) cell lytic assay, we infected cells with KSHV and then immediately superinfected the cells with an adenovirus expressing replication and transcription activator (RTA). This approach induces lytic replication in a high percentage of the cells without allowing cells to establish latent infection first. We allowed cells to produce virus for 48 h in the presence or absence of increasing concentrations of oxamate and then harvested the supernatant from each sample. Importantly, the concentrations of oxamate used, 25 and 50 mM, do not affect TIME cell viability, as we have previously shown (8). After pelleting cellular debris from the supernatant, we determined virus titers in the cell-free supernatant on fresh TIME cells. At 48 h post-titer infection, we harvested cells for immunofluorescence assay (IFA) and counted LANA-positive cells to determine the infection rate. There was a clear dose-dependent decrease in levels of LANA-positive cells with oxamate treatment (Fig. 1A), indicating that oxamate inhibits production of infectious KSHV in endothelial cells.

We also performed a lytic replication assay using iSLK cells, an inducible cell line that stably maintains the bacterial artificial chromosome 16 (BAC16)-KSHV viral genome, as well as an integrated, inducible RTA-expressing locus (24). These cells can be induced upon treatment with doxycycline (Dox) and sodium butyrate (NaB). Virus was harvested from the supernatant at 24 h postinduction of iSLK cells in the presence or absence of 50 mM oxamate, and viral titers were quantified on TIME cells. As BAC16-KSHV expresses green fluorescent protein (GFP), the titer from iSLK cells was quantified on a Typhoon fluorescent scanner at 48 h postinfection (hpi) of titer plates and relative

fluorescence levels were compared. Oxamate treatment significantly reduced the relative fluorescence by more than 90% compared to control sample levels, supporting the results from the TIME cell lytic assay (Fig. 1B). Importantly, treatment of iSLK cells with oxamate for 24 h does not significantly increase cell death at 24 h posttreatment (99.3 $\pm$ 0.6% were viable compared to treated control cells as determined by a trypan blue exclusion assay). To confirm that the inhibition of glycolysis led to the decrease in virus titer and that the result was not due to a side effect of oxamate, we performed a similar lytic induction experiment in the presence or absence of glucose in the media. iSLK cells were overlaid with glucose-free media with serum dialyzed to remove small molecules, including glucose. The media had glutamine added back, and both glucose and glutamine were added back to the media for the control as described in Materials and Methods. There was a greater than 95% decrease in the relative fluorescence of cells infected with virus produced from the cells treated with glucose-free media compared to replete media (Fig. 1C). Importantly, there was no significant increase in the percentage of cells undergoing cell death when iSLK cells were overlaid with glucose-free media (99% $\pm$ 1.0% of control treated cells were alive). Together, these data indicate that glycolysis is a required metabolic pathway for maximal KSHV virion production.

Glutaminolysis is required for maximal KSHV virion production. We next examined whether glutamine metabolism is a required metabolic pathway for maximal KSHV virus production. We first performed the KSHV lytic assay in TIME cells, in the presence or absence of exogenous glutamine. There was a 75% decrease in infectious virus present in the supernatants from glutamine-deprived cells compared to cells with replete media (Fig. 2A). We have previously published that TIME cells survive in the absence of glutamine (10). We also performed this assay in the absence of glutamine but with supplementation of alpha-ketoglutarate (αKG). Alpha-ketoglutarate is a downstream intermediate of glutaminolysis and can directly enter the tricarboxylic acid (TCA) cycle (25). Supplementation with αKG is sufficient to rescue a significant percentage of virion production in the absence of glutamine (Fig. 2A). We next removed glutamine from the iSLK lytic replication system and found that in the absence of glutamine, iSLK cells produced significantly less infectious virus as well. The levels of fluorescence of cell titers determined with glutamine-deprived supernatant were reduced by over 90% compared to control sample fluorescence (Fig. 2B). As with the oxamate experiments, the absence of glutamine did not lead to increased cell death of iSLK cells in the time frame used for these experiments (not shown).

To confirm that glutaminolysis is important for KSHV virion production, we performed the TIME cell lytic assay in the presence of BPTES [bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide], a specific inhibitor of glutaminase (GLS), the first enzyme of glutaminolysis. BPTES treatment decreased the titer from cells to about 50% of the level seen with replete samples (Fig. 2C). Additionally, we observed a partial rescue of titer in the presence of BPTES upon supplementation with the TCA cycle intermediate αKG (Fig. 2C) or with pyruvate (Fig. 2D), a metabolite that can also support the TCA cycle at a point distinct from αKG. Together, the data described above indicate that glutamine metabolism via glutaminolysis is a required metabolic pathway for maximal KSHV lytic replication.

Fatty acid synthesis is required for maximal KSHV virion production. We next asked whether fatty acid synthesis is a required metabolic pathway during lytic replication of KSHV. We performed the KSHV lytic replication assay in TIME cells, in the presence or absence of the fatty acid synthesis inhibitor TOFA (5-tetradecyloxy-2-furoic acid). TOFA inhibits acetyl-coenzyme A (CoA) carboxylase (ACC1), the rate-limiting enzyme of fatty acid synthesis (26). TOFA treatment significantly reduced virion production, as the TIME cell titer showed an average 80% reduction in LANA-positive cells compared to control samples (Fig. 3A). Importantly, when we supplemented TOFA-treated samples with the long-chain fatty acid palmitic acid, a fatty acid synthesis intermediate immediately downstream of where TOFA inhibits, we observed a signif-

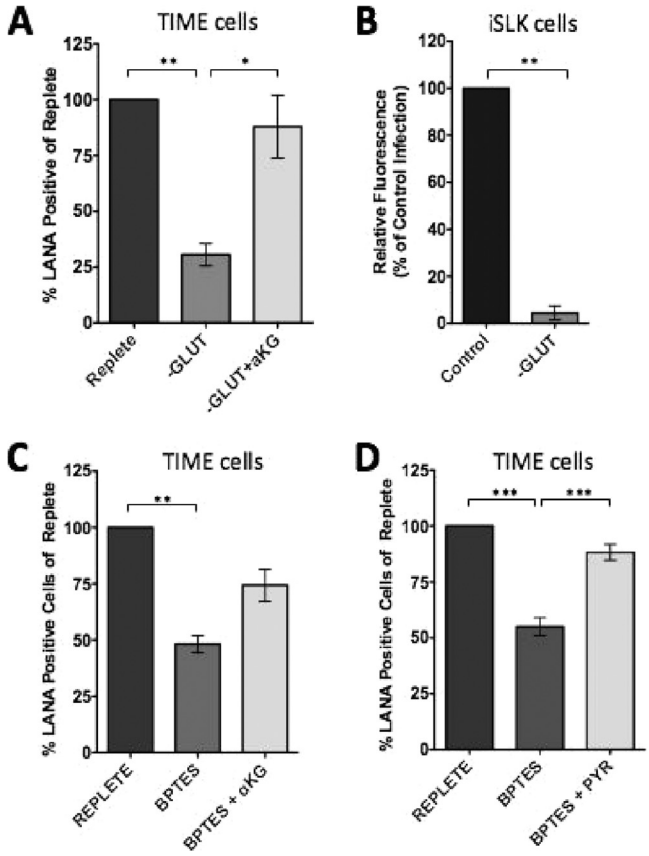

FIG 2 Glutamine metabolism via glutaminolysis is required for maximal KSHV virion production. (A) TIME cells were infected with KSHV and superinfected with an RTA-expressing adenovirus. Cells were overlaid with media with or without glutamine. Replete media contained 4 mM glutamine. One glutamine-free (−GLUT) sample was supplemented with the TCA cycle intermediate alpha-ketoglutarate (αKG). At 48 h, titers of supernatants were determined on TIME cells. Data shown represent the average percentages of LANA-expressing titer cells from three experiments. (B) iSLK cells were chemically induced with doxycycline and sodium butyrate in the presence or absence of glutamine (4 mM). At 24 h, titers of supernatants were determined on TIME cells as described for Fig. 1 by analysis of relative GFP fluorescence levels. Data shown represent the average results of 3 biological replicate experiments. (C and D) TIME cells were infected with KSHV and superinfected with an RTA-expressing adenovirus. Cells were overlaid with replete media in the presence or absence of the glutaminase inhibitor BPTES supplemented with the TCA cycle intermediates 3.5 mM αKG (C) and 8 mM pyruvate (PYR) (D). At 48 h, titers of supernatants were determined on fresh TIME cells. Data shown represent the average percentages of LANA-expressing titer cells of 3 biological replicates.

icant rescue of virus titer to about 60% of control sample levels (Fig. 3A). We previously published that TOFA treatment does not lead to significant cell death in TIME in the time frame used here (7); therefore, the effect was not due simply to death of the host cells.

We confirmed the TOFA inhibition findings, using the inducible iSLK system. When iSLKs were chemically induced, with Dox and NaB, in the presence of TOFA, there was an approximately 90% reduction in the relative level of fluorescence from TIME cells infected with supernatant from the cells at 48 h postinfection (hpi). The reduction of virus production in the presence of TOFA was substantially rescued under conditions of supplementation with 32 μM palmitic acid, increasing the relative fluorescence level to about 50% of the level seen with the control samples (Fig. 3B). Again, TOFA treatment of the iSLK cells did not lead to significant cell death (97.7% $\pm$ 2.3% were alive at 24 h after treatment compared to the control cells). The results of both the TIME cell lytic assay and the iSLK virus production assay support the conclusion that lipogenesis via fatty acid synthesis is a required metabolic pathway for maximal KSHV virion production.

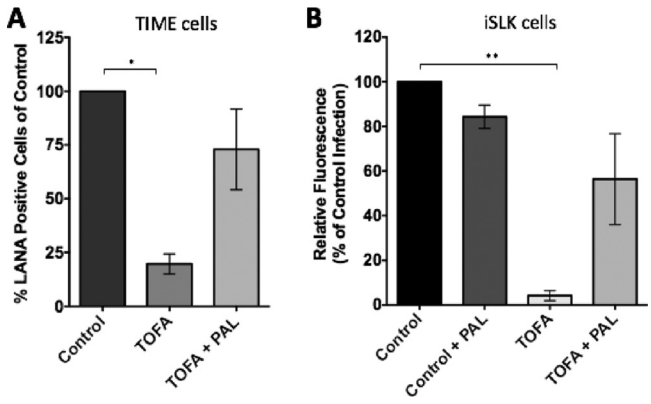

FIG 3 Fatty acid synthesis is required for maximal KSHV virion production. (A) TIME cells were infected with KSHV and then superinfected with an RTA-expressing adenovirus. Cells were overlaid with media with or without the FAS inhibitor TOFA at 2 μg/ml and, where indicated, the long-chain fatty acid palmitic acid (PAL). After 48 h, supernatants were collected and titers were determined on fresh TIME cells. Data shown represent the average percentages of LANA-expressing titer cells of 3 biological replicate experiments. (B) iSLK cells were chemically induced with doxycycline and sodium butyrate in the presence or absence of TOFA (10 μg/ml) and palmitic acid. After 24 h, supernatant was harvested and titers were determined on TIME cells as described for Fig. 1. Data shown represent the average results of 3 biological replicate experiments, with control results set to 100%.

Glycolysis is required for early and late gene expression. To determine at what stage inhibition of glycolysis blocks viral replication, we examined early and late KSHV gene expression upon oxamate treatment of induced iSLK-KSHV cells. Twenty-four hours postinduction of lytic replication in the presence or absence of 50 mM oxamate, supernatant was collected for titer determinations and cells were harvested to extract RNA for quantitative real-time reverse transcription-PCR (qRT-PCR) analysis to determine relative levels of viral transcripts. As before, there was a significant decrease in the viral titer (Fig. 1B and data not shown). qRT-PCR for the immediate early gene ORF45, a tegument protein (27, 28), and the early gene ORF59, a key viral processivity factor (29), revealed a significant reduction in early transcript levels under conditions of normalization to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) host housekeeping gene (Fig. 4A). Furthermore, expression of the late viral genes encoding ORF26, a capsid protein, and K8.1, a KSHV virion glycoprotein (30), was almost completely lost compared to the levels seen with induced control samples (Fig. 4A). Similar results were seen when iSLK cells were induced in glucose-free media and serum (Fig. 4B). Protein levels of the immediate early protein ORF45 were also significantly reduced in oxamate-treated samples compared to induced controls (Fig. 5). Additionally, levels of late virion glycoprotein K8.1 were nearly eliminated by oxamate treatment compared to the levels seen with induced control samples (Fig. 5). The levels of intracellular genomes were also determined by quantitative PCR (qPCR) for genomic DNA. Following induction of KSHV in iSLK-KSHV cells, there was a significant decrease in the levels of intracellular genomes in the presence of oxamate, indicating that inhibition of glycolysis blocks replication of the KSHV genome as expected from the decrease in early gene expression (Fig. 6A). In summary, inhibition of glycolysis blocks replication at a very early step, preventing transcription of KSHV early genes, genome replication, viral late gene expression, and production of infectious virus.

Glutamine metabolism is required for late KSHV gene expression. To determine at what stage depletion of glutamine blocks KSHV replication, we induced iSLK cells with glutamine-deprived media or replete media containing 4 mM glutamine. At 24 h postinduction, cell-free supernatant was harvested for titer determinations, and iSLK cells were harvested for RNA. As expected, there was a significant reduction in viral titer in the glutamine-depleted cells compared to the cells in replete media (Fig. 2B and data not shown). Interestingly, qRT-PCR results for the early genes encoding ORF45 and ORF59 showed no change in gene expression (Fig. 4C). In contrast, the relative

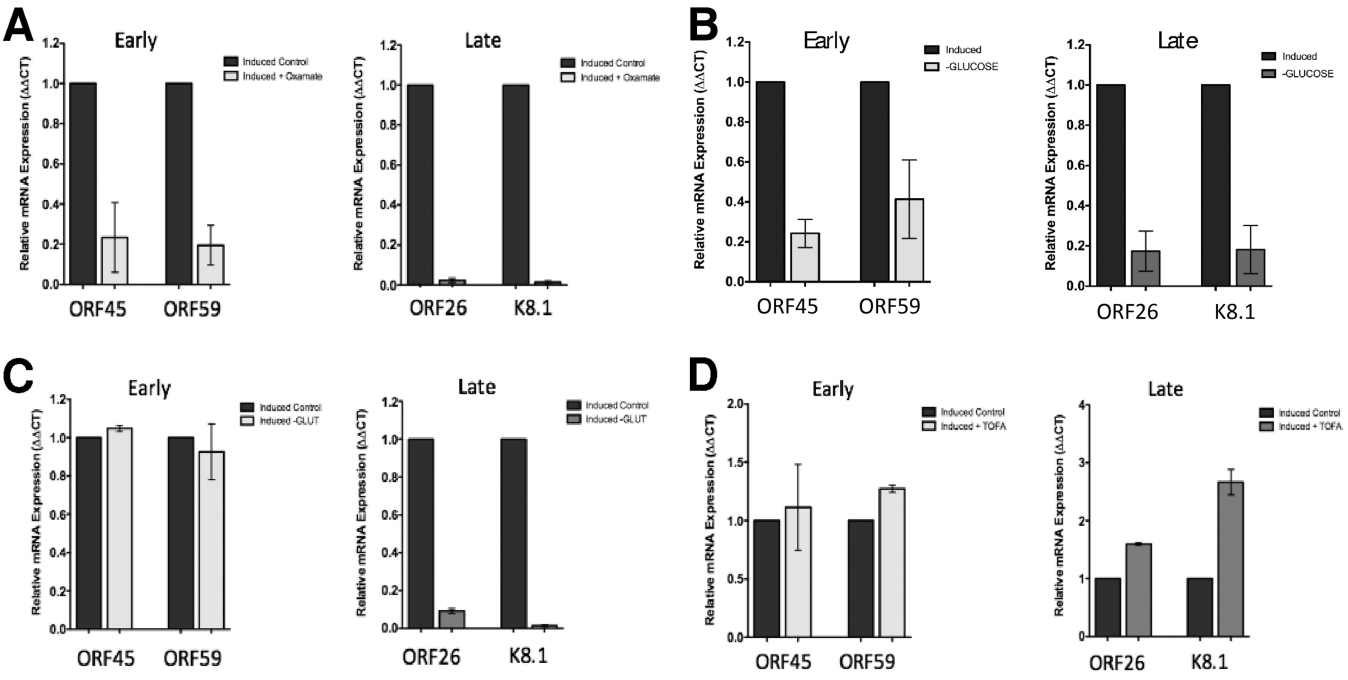

FIG 4 Glycolysis, glutaminolysis, and fatty acid synthesis are required for different steps of KSHV replication. iSLK cells were induced in the presence or absence of (A) oxamate (50 mM), (B) glucose, (C) glutamine, or (D) TOFA (10 μg/ml). Cells were harvested at 24 h postinduction. RNA was extracted, and qRT-PCR was performed on cDNA for early KSHV transcripts (ORF45 and ORF59) and late KSHV transcripts (ORF26 and K8.1). Relative mRNA expression for each viral transcript was normalized to that of the GAPDH housekeeping gene and compared to that of untreated induced samples. Error bars are representative of standard errors from at least 2 biological replicate experiments. ΔΔCT, threshold cycle method.

transcript levels of both late genes, encoding ORF26 and K8.1, were substantially reduced compared to the expression levels of induced control cells (Fig. 4C). In the absence of glutamine, there was a marked loss of both immediate early protein ORF45 and late protein K8.1, similar to what was observed during oxamate treatment (Fig. 5). Induction of lytic replication in the absence of glutamine also significantly decreased KSHV genome replication (Fig. 6A). Taken together, these data suggest that while early gene transcription was occurring as shown in Fig. 4C, early protein translation was blocked. These data suggest that virus production in the absence of glutamine metabolism is blocked after early viral gene transcription but before early viral protein expression, preventing viral genome replication, late gene expression, and production of infectious virus.

Fatty acid synthesis is not required for early or late KSHV gene expression. Finally, we examined whether fatty acid synthesis is required for early and late KSHV gene expression by conducting iSLK induction in the presence or absence of the ACC1 inhibitor TOFA. Supernatant was collected at 24 h postinduction of iSLK-KSHV, and cells

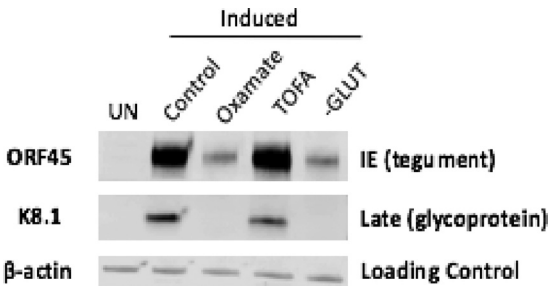

FIG 5 Viral protein expression in the presence of metabolic inhibitors. At 24 h postinduction in the presence or absence of oxamate, glutamine (GLUT), or TOFA, iSLK cell lysates were analyzed by immunoblotting for immediate early protein ORF45 and late protein K8.1. An uninduced (UN) sample was included as a negative control. IE, immediate-early.

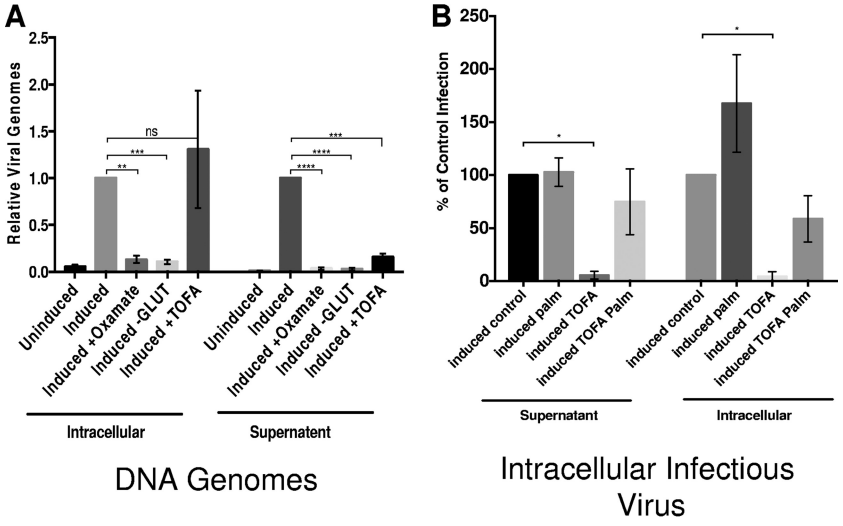

FIG 6 Glycolysis and glutaminolysis are required for viral genome replication, while FAS is required for assembly and egress of infectious KSHV virions. (A) KSHV DNA was quantified by PCR from intracellular total DNA and from virions in the supernatant from iSLK cells induced in the presence or absence of oxamate, glutamine, or TOFA. (B) Relative titers of extracellular or intracellular infectious virions in the presence or absence of TOFA, with or without supplementation of palmitic acid (palm). Intracellular virions were released by three freeze-thaw cycles.

were harvested for qRT-PCR analysis of viral transcript levels. As described above, titer determinations confirmed that virus production was significantly reduced in each TOFA-treated sample (Fig. 3B and data not shown). Transcript levels for both early viral genes, encoding ORF45 and ORF59, were not reduced (Fig. 4D). Surprisingly, transcript levels of both late viral genes, encoding ORF26 and K8.1, were not reduced either (Fig. 4D). If anything, there appears to have been an increase in the synthesis of late genes in the presence of TOFA. This could be due to the fact that virions are produced but fewer virions egress from the cell (see below), leaving the cell fully intact and preventing signaling to shut off the lytic cascade, leading to the accumulation of late gene transcripts. However, TOFA-treated samples showed no significant change in production of either the early or late proteins (Fig. 5). Additionally, there was no decrease in the levels of intracellular KSHV genomes, indicating that in the presence of a fatty acid synthesis inhibitor, KSHV genome replication occurs at the same levels as in untreated cells (Fig. 6A). Overall, these results show that although there was a strong decrease in the production of infectious virus in the presence of the fatty acid inhibitor TOFA, KSHV viral gene transcription and genome replication were unaltered by the loss of cellular lipid production.

Fatty acid synthesis inhibition alters KSHV assembly and egress. Inhibition of fatty acid synthesis leads to a reduction in the levels of infectious particles produced by KSHV, but there is no defect in viral gene expression or replication. While there is a drop in the levels of infectious virus, it is possible that the virions are still produced but are not infectious when fatty acid synthesis is inhibited. Therefore, we determined if there was a defect in assembly and/or egress. To determine if the inhibition of fatty acid synthesis leads to inhibition of assembly, we used three approaches: (i) we quantified extracellular KSHV DNA in virions to determine if the KSHV virions egress from the cell but are not infectious in the presence of TOFA; (ii) we quantified intracellular infectious virus to determine if the KSHV infectious particles are assembled but do not egress in the presence of TOFA; and (iii) we used transmission electron microscopy to determine if virions are assembled in the presence of TOFA.

To quantify extracellular KSHV genomes in virus particles, we pelleted virions from the supernatant of induced iSLK-KSHV cells, released virion DNA with protease K treatment, isolated the nucleic acid, and used qPCR to measure the viral DNA levels.

There was an approximately 80% decrease in the levels of extracellular genomes in supernatants from TOFA-treated cells compared to control treated cells (Fig. 6A). While there was a significant decrease in total production of extracellular virions, the magnitude of decrease was not equivalent to that measured in infectious virus, likely indicating that some of the virions that were produced were noninfectious. To determine if the virions were properly assembled but were unable to egress from the cell, we examined intracellular virus. At 36 h postinduction of iSLK-KSHV cells in the presence or absence of TOFA, we washed the cells and harvested for intracellular virus. The cells were freeze-thawed three times to break open the cells and release the intracellular virus, and then the cellular debris was removed by centrifugation. The resulting supernatant was used to infect TIME cells, and titers were measured by fluorescence. In the vehicle-treated cells, there were significant amounts of intracellular infectious virus such as are seen with many herpesviruses. Treatment of the cells with TOFA led to a nearly complete loss of infectious intracellular virus (Fig. 6B). Treatment of the cells with TOFA and palmitic acid, the fatty acid immediately downstream of the position where TOFA blocks fatty acid synthesis, led to recovery of much of the intracellular infectious virus, indicating that the loss of intracellular infectious virus was not a side effect of the drug treatment. The supernatant taken from these cells prior to lysis revealed, as before, that there was a significant loss of infectious virus produced by the cells. In short, it appears that there was a substantial block in assembly or maturation, as viral replication and late gene synthesis appeared to be at wild-type levels.

To determine if there was a block in the initial steps of virion production or if later steps of assembly or egress were prevented by inhibition of fatty acid synthesis, we used transmission electron microscopy (TEM) of induced iSLK-KSHV cells that had been left untreated or treated with TOFA, the fatty acid synthesis inhibitor. Oxamate, the glycolysis inhibitor, was used as an additional control. Without an inhibitor, there was robust production of virions in this system (Fig. 7). There are large virus factories in the nucleus, and we can identify both B type capsids (formed capsids without DNA, as determined by lack of electron-dense cores) and C type capsids (virions with electron-dense cores indicative of the presence of viral DNA). There were large numbers of cytoplasmic virions, mostly C type, and many cells had virus immediately adjacent to the cells, indicating viral egress outside the cell. In the induced cells treated with oxamate, there was a large decrease in the number of cells with virus factories in the nucleus and the few cells that had any apparent virions had a greatly decreased number compared to the untreated cells (Fig. 7). Interestingly, the numbers of cells with large virus factories in the nucleus in the induced cells treated with TOFA were similar to the numbers seen with induced but untreated cells and there were large numbers of virions in the nucleus and in the cytoplasm. While there were a number of B type and, surprisingly, C type capsids, there was an increase in the number of oddly formed capsids of unknown origin that had small circular spots of staining inside the membrane that were not as electron dense as the cores of C type capsids (see arrows in Fig. 7). While the results were not fully quantitative, the electron microscopy indicated that oxamate clearly decreases the number of cells with virus factories and virus assembly. However, the electron microscopy indicated that TOFA does not have a large effect on the number of virus factories or the production of intracellular virions, though it appears that there are increased numbers of defective capsids made. These data are consistent with the findings showing that induced cells treated with TOFA allow KSHV genome replication and production of early and late viral genes but that there is a block in the production of intracellular infectious virus as well as of released infectious virus.

DISCUSSION

Here we show that central carbon metabolism is required for efficient KSHV virion production. Inhibition of glycolysis, glutaminolysis, or fatty acid synthesis leads to significant loss of infectious KSHV virus production in both endothelial cells and SLK cells. While many viruses induce and require specific metabolic pathways for replication, surprisingly little is known about the exact point in the viral life cycle at which host

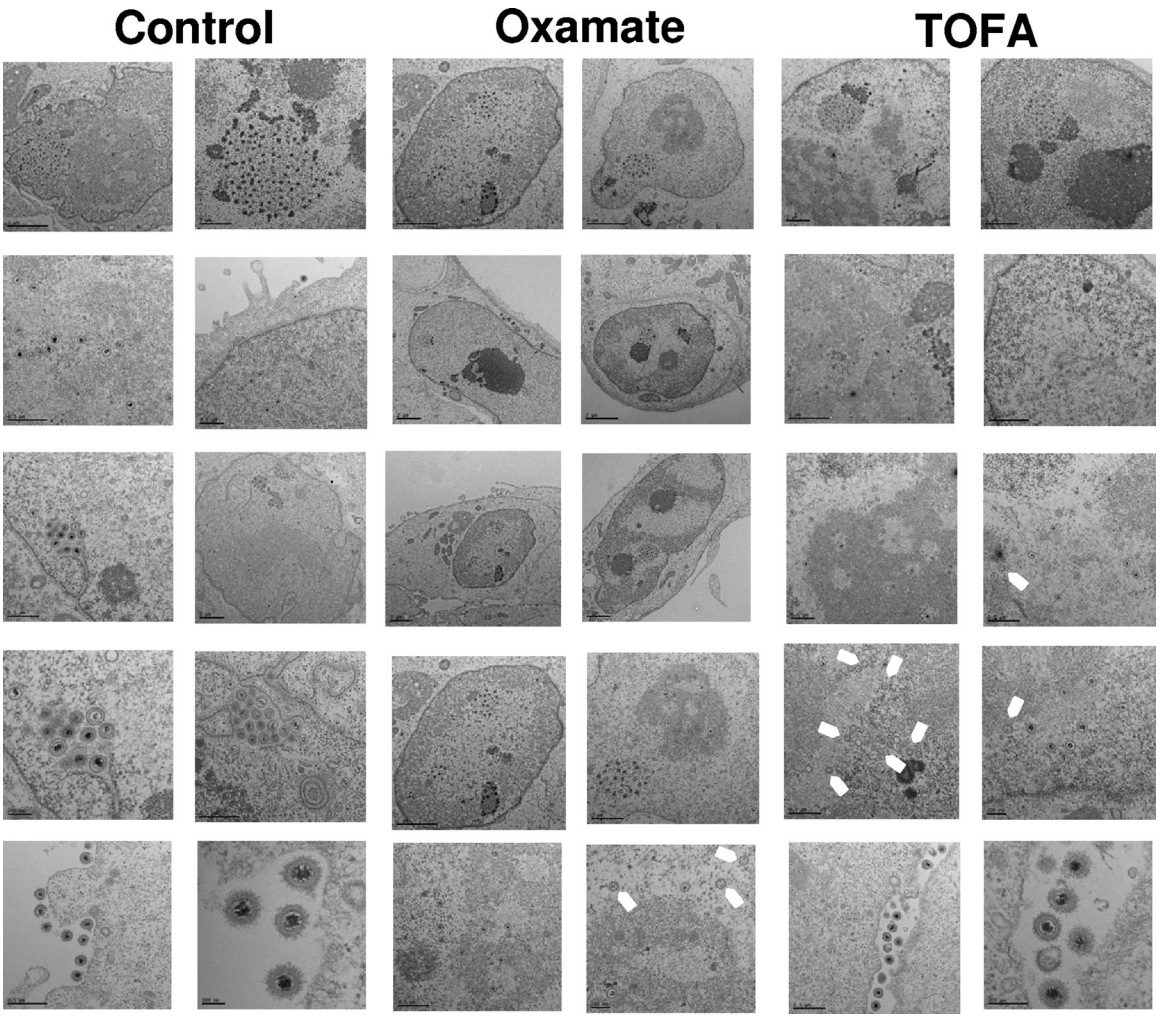

FIG 7 Transmission electron microscopy images of iSLK cells induced in the presence of metabolic inhibitors. iSLK cells were induced to lytic replication in the presence or absence of a vehicle control (DMSO), 50 mM oxamate, or 10 mg/ml TOFA. Cells were fixed and processed for TEM imaging at 36 h postinduction. Representative images are shown for each condition. Arrows indicate immature KSHV particles in the cytoplasm.

cell central carbon metabolism is required (31). Interestingly, inhibition of glycolysis, glutaminolysis, and fatty acid synthesis all blocked KSHV at different points in viral replication (Fig. 8). Inhibition of both glycolysis and glutaminolysis prevented KSHV genome replication and therefore, late gene synthesis and production of virions. Inhibition of glycolysis prevented overall viral gene expression, blocking both early and late gene mRNA production. Whether this block is specific to viral genes is not clear. However, uninfected TIME cells survive for over 48 h with the concentrations of oxamate used to inhibit glycolysis in these studies. Therefore, it is unlikely that there is

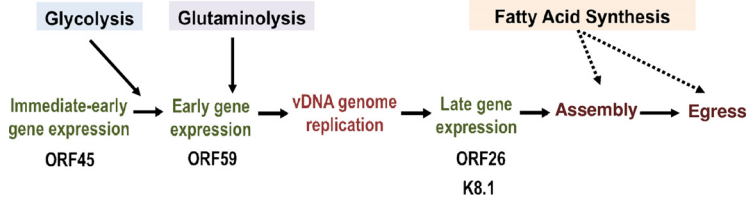

FIG 8 Model of host cell metabolism contribution to KSHV lytic replication. Glycolysis is required for early KSHV gene expression, and glutamine metabolism is required for early gene protein synthesis but not transcription, while fatty acid synthesis contributes to viral assembly and viral egress.

a complete cessation of cellular transcription. If the block is specific to viral gene expression or if there is also inhibition of select classes of cellular genes remains to be determined. In the absence of glutamine, early gene mRNAs were produced but early gene proteins were not present. As with the glycolysis inhibitor, uninfected cells survive for longer than 48 h in the absence of glutamine in the media. Therefore, it is not likely that there is an overall block in cellular protein synthesis but further work will be done to determine if there is a specific block in host protein synthesis as well. Whether loss of viral protein synthesis is due to a lack of energy or requires metabolic intermediates from the TCA cycle remains to be determined. Oxamate deprivation as well as glutamine deprivation does not lead to a significant increase in cell death in either TIME cells or iSLK cells. However, there is limited inhibition of cellular proliferation. Since the different treatments block KSHV replication at different stages, it appears unlikely that this is the cause of the decrease in virus titer. To further examine this, we also treated iSLK cells with 5 mM 5-fluorouracil (5-FU), a drug that inhibits cellular proliferation. There was no increase in cell death, but there was an almost 40% decrease in cell number. This was accompanied by a perfectly matched 40% decrease in cell titer, as expected (not shown). The decrease in cell titer in the presence of oxamate or the absence of glutamine was much stronger ($>$90% in iSLK cells), indicating that the effect was not simply due to the block in cellular proliferation.

In stark contrast to inhibition of glycolysis and glutaminolysis, inhibition of fatty acid synthesis did not appear to inhibit KSHV transcription or translation at any step in viral gene expression. Both early and late genes are expressed at untreated levels in the presence of a fatty acid synthesis inhibitor, and there are equal levels of genome replication as well. A series of experiments indicated that inhibition of fatty acid synthesis prevents proper virion assembly, leading to less viral egress. We first demonstrated that there was an 80% decrease in levels of extracellular KSHV DNA, though the decrease does not appear to have been as strong as the decrease in the levels of infectious particles, indicating that some defective virus particles containing viral DNA are likely produced, indicating partial inhibition of egress. We also quantified intracellular infectious particles to determine if intact virions had been made but failed to egress from cells. Interestingly, there was an almost complete loss of intracellular infectious particles, pointing toward a defect in virion assembly or maturation since the viral genes were produced. We also visualized the intracellular virus particles by transmission electron microscopy. There was no obvious defect in the number or size of the nuclear virus factories in the presence of a fatty acid inhibitor. Importantly, there were very few cells with these factories in cells treated with a glycolysis inhibitor. It is apparent that virions can be produced in the presence of fatty acid inhibitors, including a number of C-type capsids both inside and outside cells, immediately adjacent to the cytoplasmic membrane. However, there were also a larger number of virions that were not C type and that had odd appearances, with membrane-bound, smaller, less-electron-dense round spots present that were not seen in the untreated cells. Taken together, our data indicate that the block in the production of infectious virus caused by inhibition of fatty acid inhibitors is most likely a block in assembly.

The mechanistic details of how inhibition of fatty acid synthesis prevents assembly and exactly where the block occurs will require further studies. It is possible that constant fatty acid synthesis is necessary for assembly by providing specific lipid species required for assembly in the nucleus or in the nuclear membrane. In particular, there could be specific fatty acids or lipids that are necessary to properly envelope viral DNA and tegument in the nucleus or in the intranuclear membrane. Studies to examine the lipid composition of both intracellular and extracellular virions are necessary to address this possibility. Alternatively, fatty acid production is part of many signal transduction pathways in the cell and lipid signaling may be required for providing other specific cellular factors necessary for assembly. Though less likely, it is possible that there is a defect in select viral late gene expression where a viral tegument or glycoprotein is not properly produced when fatty acid synthesis is inhibited and prevents proper assembly.

The surprising finding that glycolysis, glutaminolysis, and fatty acid synthesis block distinct steps in viral replication reveals that each pathway is uniquely required for replication. Increased glycolysis leads to decreases in TCA intermediates as glucose is utilized primarily for lactic acid production rather than for oxidative phosphorylation in the mitochondria, and the induction of fatty acid synthesis can further remove TCA intermediates from the mitochondria. Therefore, it is thought that glutaminolysis is primarily required for aneplerosis, or increasing TCA cycle intermediates drained by glycolysis and fatty acid synthesis. However, our current findings suggest that each pathway is separately required for KSHV lytic replication as inhibition of each pathway blocks a unique step in viral replication.

In these sets of experiments, we have focused on three main pathways involved in central carbon metabolism. It is likely that other metabolic pathways are also required for different steps of replication and production of infectious virions. For example, HCMV requires pyrimidine synthesis to increase production of UDP sugars for glycosylation of viral membrane proteins that are critical for cell entry of the virus (32). This pathway and others could also be required for specific steps of infectious virion production of KSHV. Importantly, the identification of metabolic pathways that are important for the production of infectious KSHV virions could lead to new ways to inhibit KSHV lytic replication. We have previously shown that inhibition of the three metabolic pathways that inhibit lytic replication, i.e., glycolysis, glutaminolysis, and fatty acid synthesis, is also required for the survival of endothelial cells latently infected with KSHV. KS tumors contain spindle cells that maintain latent infection as well as spindle cells undergoing lytic infection. Therefore, identification of drugs that target both latent and lytic infection provides the strongest potential treatment to combat KS tumors and continued spread of the virus. The metabolic pathways identified here provide such targets.

MATERIALS AND METHODS

Cells and media. Tert-immortalized microvascular endothelial (TIME) cells (24, 33) were maintained as monolayer cultures in endothelial basal medium-2 (EBM-2) (Lonza or Cellgro) or EndoGrow media (Millipore) supplemented with a bullet kit containing vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor 3, epidermal growth factor, hydrocortisone, and 5% fetal bovine serum (FBS). Millipore EndoGrow medium, supplemented with dialyzed FBS, depleted of small molecules, including glucose and glutamine, was used for all experiments that compared replete medium (4 mM L-glutamine) and glutamine-free medium. iSLK cells (a kind gift from Jae Jung and Rolf Renne [34]), stably maintaining a selectable GFP-expressing KSHV genome made from BAC16, were maintained in Dulbecco's modified Eagle medium (DMEM) containing 5% FBS, 1% penicillin-streptomycin (Pen-Strep), and 1% L-glutamine selected with puromycin (10 mg/ml), G418 (95 mg/ml), and hygromycin B (50 mg/ml), as previously described (34, 35). iSLK lytic experiments conducted in the absence of glucose or glutamine were performed using DMEM without glucose, L-glutamine, and sodium pyruvate (Corning) supplemented with 10% dialyzed FBS, depleted of small molecules, including glucose and glutamine, with or without 1 g/liter D-glucose (Sigma) or 4 mM L-glutamine added back to the media.

Virus. Titers of KSHV inocula from induced BCBL-1 cells were determined, and the inocula were used to infect TIME cells as previously described (36). Adenoviruses expressing the lytic replication and transcription activator (RTA) were used in lytic replication assays. Adenovirus RTA (AdRTA) was a kind gift from Don Ganem (26). Helper-dependent RTA (HD_RTA), a gutted adenovirus containing the adenovirus packaging signal but no adenovirus viral coding sequences, was produced subsequently following a modified version of previously described published protocols (37, 38). Briefly, the coding region of KSHV RTA driven by a CMV promoter was cloned into the pBluescript II SK plasmid (Stratagene) to create a shuttle vector (pBShuttle) flanked by adenovirus sequences. The RTA/adenovirus expression cassette was excised from this plasmid and electroporated into BJ5183 cells (Stratagene) along with pC4HSu helper adenovirus vector (Microbix Biosystems) to allow homologous recombination. The resulting plasmid (HD_RTA) was transfected into 293Cre4 cells, which stably express a Cre recombinase enzyme, selected with puromycin. Cells were passaged in the presence of helper adenovirus (HD14; Microbix), which contains adenovirus-coding regions and allows the production of HD_RTA adenovirus. The helper adenovirus contains a modified packaging sequence flanked by *loxP* sites; therefore, the helper adenovirus is not packaged along with the HD_RTA. Upon final passage and expansion of adenovirus-producing cells, cells were collected, pelleted, and freeze-thawed three times. Cell debris was then spun out at 2,000 rpm and the cell-free supernatant collected. The cleared supernatant was layered onto a continuous 15%-to-40% CsCl gradient and centrifuged for 2 to 3 h at 25,000 $\times$ *g* using an SW41Ti rotor (Beckman Coulter, Inc., Fullerton, CA). The mature virus band was collected and purified in a second CsCl density gradient. The mature virus band was collected, dialyzed against three changes of A195 buffer, flash frozen in liquid nitrogen, and stored at 80°C.

Chemical reagents. Dimethyl-α-ketoglutarate (alpha-ketoglutarate [αKG]) and pyruvate were purchased from Sigma and used at 3.5 mM and 8 mM, respectively. Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES; Sigma) was solubilized in dimethyl sulfoxide (DMSO), used at a final concentration of 2.5 μM. QVD-OPH (SM Biochemicals) was dissolved in DMSO and used at a final concentration of 20 μM. Oxamate (Sigma) was prepared in EBM-2 or DMEM at a final concentration of 25 or 50 mM. TOFA [5-(tetradecyloxy)-2-furancarboxylic acid] (Sigma or Santa Cruz) was solubilized in DMSO and used at a final concentration of 2 or 10 μg/ml.

Lytic KSHV assay. Immediately following KSHV infection of TIME cells for 4 h, cells were superinfected with an adenovirus expressing RTA (described above) in serum-free EBM-2 containing 0.2 μg poly-L-lysine/ml for 1.5 h. After infection, cells were washed three times with phosphate-buffered saline (PBS). EBM containing metabolic inhibitors or vehicle controls was added to cells. At 48 h post-AdRTA or -HD_RTA infection, cell supernatant was collected and spun down at 2,000 rpm to remove cellular debris. Titers of infectious supernatant were determined on fresh TIME cells by incubating cells with cell-free supernatant supplemented with Polybrene (8 μg/ml) in 6- or 12-well plates for 4 h. At 48 h post-titer infection, cells were harvested and seeded for IFA of LANA and ORF59 expression. For iSLK lytic assays, cells were induced with doxycycline (Dox) and sodium butyrate (NaB) in the presence or absence of metabolic inhibitors. After 24 h of lytic induction, the supernatant from all samples was collected and spun down at 2,000 rpm to remove cellular debris. Titers of the supernatant were determined on fresh TIME cells by incubating cells with cell-free supernatant supplemented with Polybrene (8 μg/ml) in 6- or 12-well plates for 4 h. At 48 h post-titer infection, plates were scanned on a Typhoon fluorescent imager to determine the relative levels of GFP fluorescence of all samples and analyzed using Image-J software.

Immunofluorescence. To determine viral titers for TIME cell lytic assays, aliquots of KSHV-infected TIME cells were seeded on four-well chamber slides and fixed with 4% (vol/vol) paraformaldehyde–phosphate-buffered saline. Infection rates were monitored using antibodies against the latent KSHV protein LANA (a kind gift from A. Polson and D. Ganem) and the lytic protein ORF59 (Advanced Biotechnologies Incorporated) as described previously (24). Cells were incubated with fluorophor-conjugated secondary antibodies goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (Molecular Probes/Invitrogen). Cells were mounted in medium containing DAPI (4′,6′-diamidino-2-phenylindole) before being viewed under a Nikon Eclipse E400 fluorescence microscope (Nikon, Inc.).

Quantitative reverse transcription-PCR. Total RNA was isolated using a Nucleospin RNA II kit (Macherey-Nagel). Two-step quantitative real-time reverse transcription-PCR (Bio-Rad) was used to measure expression levels of ORF45, ORF59, ORF26, and K8.1. The primers used were as follows: ORF45-F (5′-CAACTCTCCGGACGTGAACA-3′), ORF45-R (5′-GGAGATTGGGTTGGGAGGTG-3′), ORF59-F (5′-GACAGC GTCTCGCTGACAGA-3′), ORF59-R (5′-CACACGCGTGAGCTATTCGG-3′), ORF26-F (5′-AGCCGAAAGGATTCC ACCATT-3′), ORF26-R (5′-CCGTGTTGTCTACGTCCAGA-3′, K8.1-F (5′-AAAGCGTCCAGGCCACCACAG-3′, and K8.1-R (3′-GGCAGAAAATGGCACACGGTT-5′). Relative levels of each transcript were normalized by the delta threshold cycle method to the abundance of GAPDH mRNA.

Western blot analysis. All cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and protein was quantified using the bicinchoninic acid (BCA) assay (Pierce). Cells (10 to 20 μg) were subjected to SDS-PAGE on a 4 to 20% gel (Life Technologies or Bio-Rad) and then transferred to an Immobilon F polyvinylidene difluoride membrane (Millipore). The membranes were blocked in Odyssey blocking buffer (Li-COR) for at least an hour and then probed with the indicated primary antibodies diluted in Li-COR buffer for 2 h at room temperature or overnight at 4°C. For viral protein analysis, we used anti-ORF45 lytic antibody (kind gift from Yan Yuan) or anti-K8.1 lytic antibody (Advance Biotechnologies Inc.). Blots were washed 3 times in Tris-buffered saline with Tween 20 (TBST) and then probed with IRDye secondary antibody (Li-COR) diluted in Li-COR buffer for 1 h at room temperature. Blots were washed 3 times in TBST and then scanned and quantified with an Odyssey CLx infrared imaging system (Li-COR) for fluorescent blots.

Viral genome quantification. Samples were harvested at 36 h postinduction with or without metabolic inhibition (Oxamate, glutamine starvation, TOFA). The supernatant and the cells were harvested separately. Genomic DNA was harvested from cell pellets using a DNA extraction kit (Invitrogen). Genomic DNA in the supernatant was pelleted using a Beckman Avanti Ultracentrifuge at 25,000 rpm. The medium was removed, and genomic DNA was extracted (Macherey-Nagel). Viral genomes were quantified by qPCR for the LANA genomic locus normalized to GAPDH from any cellular genomes in the supernatant.

Intracellular virus titer. Cells were induced in the presence or absence of TOFA with or without palmitic acid supplementation. At 36 h, supernatant was collected and saved, cells were washed with PBS, and 5 ml of fresh medium was added to flasks before the flasks were placed at −80°C for three freeze-thaw cycles. During thaw cycles, flasks were vigorously shaken to disrupt cellular but not viral membranes. Debris was pelleted at 2,000 rpm at 4°C, and cell-free supernatant was collected. Titers of samples were then determined on fresh TIME cells, and the cells were scanned on a Typhoon fluorescent scanner at 36 to 48 h after titer infection to compare levels of infectivity of intracellular virions. Titers of the matched supernatant samples were also determined on fresh TIME cells.

Transmission electron microscopy. At 36 h postinduction and posttreatment, iSLK cells were harvested and fixed before being sent to the Fred Hutchison Cancer Research Center EM Core Facility and prepared for TEM. Briefly, iSLK cells were induced with doxycycline and NaB in the presence or absence of metabolic inhibition (Oxamate or TOFA). At 36 h postinduction, medium was aspirated, and cells were gently washed with PBS, which was then replaced with 50% 1/2 Karnovsky's fixative and 50% media. Cells were incubated at 37°C for 10 min. Half the medium was removed and replaced with full 1/2 Karnovsky's fixative. Cells were then incubated at 37°C for an additional 30 min

to 1 h. After a fixing step, cells were gently scraped and collected. Samples were pelleted gently at 2,000 rpm at 4°C for 5 to 10 min, most of the supernatant was removed, and 1 ml fresh fixative was added. Cells were resuspended gently and stored at 4°C until they were imaged at the core facility.

Statistical analysis. Standard errors of the means are shown, and statistical differences between groups were analyzed with Student's (two-tailed) *t* test or one-way analysis of variance (ANOVA). A *P* value of <0.05 was considered significant and is indicated by an asterisk in the figures. A *P* value of <0.01 is indicated by a double asterisk. A *P* value of <0.0001 is indicated by a triple asterisk.

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