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AMPK-Mediated Inhibition of mTOR Kinase Is Circumvented during Immediate-Early Times of Human Cytomegalovirus Infection▽

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Human cytomegalovirus (HCMV) infection increases synthetic rates in infected cells. The resulting increase in energy utilization could potentially increase the AMP:ATP ratio, causing activation of 5′-AMP-activated protein kinase (AMPK). Activated AMPK promotes inhibition of mammalian target of rapamycin (mTOR) kinase, which could be deleterious to the viral infection. Using the AMPK-activating drug 5-amino-4-imidazolecarboxamide ribose (AICAR), we showed that, by 12 h post-HCMV infection, inhibition of mTOR by AMPK is circumvented. However, growth curves showed that progeny virion production is inhibited when AICAR is added, suggesting other inhibitory effects of AICAR or activated AMPK.

5′-AMP-activated protein kinase (AMPK) is a heterotrimer that contains α, β, and γ subunits, each of which has at least two isoforms (5). Decreases in the cellular energy state, as reflected by an increase in the AMP:ATP ratio, cause conformational changes in AMPK that make it susceptible to phosphorylation and activation by an AMPK kinase. Once activated, AMPK stimulates multiple events that either enhance ATP generation or inhibit processes that consume ATP (5). One of the targets inhibited by activated AMPK is mammalian target of rapamycin (mTOR) kinase (Fig. 1), which is a major controller of cap-dependent translation; inhibition of mTOR saves energy by reducing translation. Activated AMPK phosphorylates and activates one of the subunits of the tuberous sclerosis complex, TSC2 (Fig. 1), a GTPase-activating protein that forms a complex with TSC1 and stimulates the intrinsic GTPase activity of Rheb. This results in the conversion of the active form, Rheb-GTP, to the inactive form, Rheb-GDP. Reduction in Rheb-GTP results in loss of activation of mTOR and decreased cap-dependent translation (5).

Human cytomegalovirus (HCMV) is a slow-growing betaherpesvirus which must maintain beneficial host cell functions for an extended period. However, as the infection proceeds, increased metabolism and synthesis of viral proteins induce cellular stress responses. The intense energy utilization during a viral lytic infection could potentially increase the AMP:ATP ratio such that AMPK becomes activated; the resulting inhibition of mTOR would be deleterious to the viral infection. We have previously shown that HCMV infection activates mTOR signaling and maintains cap-dependent translation despite cellular stress signaling that should inhibit it (3, 4). Thus, we predicted that HCMV infection should be able to circumvent the inhibition of mTOR by AMPK.

For these experiments, we have simulated an elevated AMP:ATP ratio, using the AMPK-activating drug 5-amino-4-imidazolecarboxamide ribose (AICAR). Confluent monolayers of life-extended human foreskin fibroblasts (1) were serum starved for 48 h, then infected with purified (serum-free) HCMV (Towne strain) (multiplicity of infection [MOI] = 2) under serum-free conditions. AICAR (1 mM) was added to one set of plates at 4 h postinfection (hpi) and to another set at 12 hpi. Plates from each set, as well as drug-free control plates, were harvested at 24 and 36 hpi, using previously described methods (3). Western blot analysis was used to determine the phosphorylation levels of two mTOR substrates, the eIF4E binding protein (p4E-BP), phosphorylated on T37 and T46, and p70 S6 kinase (pS6K), phosphorylated on T389. We also determined the phosphorylation status of ribosomal protein S6 on S235/S236 and AMPK on T172. Total levels of 4E-BP, S6, AMPK, and the HCMV major immediate-early proteins (MIEPs) were also determined.

The data in Fig. 2 show that the addition of AICAR at 4 h eliminated HCMV-induced phosphorylation of S6K and S6 and hyperphosphorylation of 4E-BP, measured at 24 and 36 hpi, compared to those for HCMV infection with no drug added. Total S6 and 4E-BP levels were relatively constant, and significant amounts of each protein remained at 24 and 36 hpi in the AICAR-treated samples. Thus, the loss of phosphorylated forms of these proteins was due to inhibition of mTOR kinase, not to diminished amounts of the proteins. Examination of HCMV MIEP synthesis showed that the addition of AICAR at 4 hpi eliminated MIEP accumulation, indicating that the viral infection could not get established under these conditions.

In contrast, the addition of AICAR at 12 hpi showed that the infected cells were more resistant; phosphorylation of all three substrates was detected at 24 and 36 hpi. In addition, high levels of the MIEPs were detected at all time points, suggesting that the infection was established prior to the addition of the drug and that the drug could no longer inhibit MIEP accumulation. Thus, between 4 and 12 hpi, HCMV infection can circumvent the inhibitory effects of AMPK activation on mTOR kinase activity.

For Fig. 3, we examined AMPK activation in infected cells

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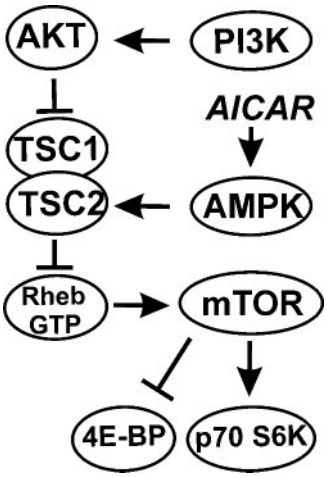

FIG. 1. Control of mTOR activity by the PI3K/Akt pathway and AMPK.

under various conditions of AICAR addition. The antibody used recognized AMPK phosphorylated on T172, which indicated activated AMPK (5). The first five lanes show phosphorylation of AMPK in cells infected for 0, 2, 4, 12, and 24 h in the absence of AICAR. At 0 hpi, the uninfected serum-starved cells contained some activated AMPK; however, this disappeared within 2 h of infection and did not reappear in the untreated cells except for a very low amount detected at the 24-h time point. A second sample without AICAR was also

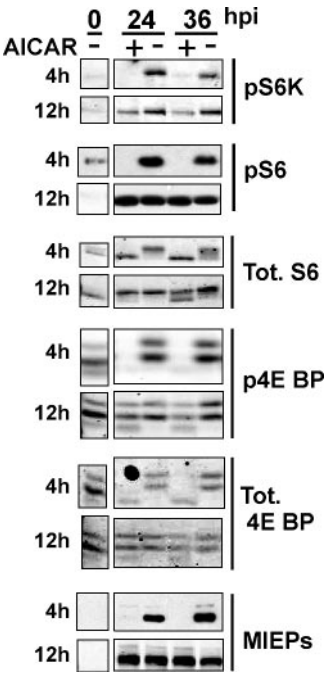

FIG. 2. Western blots showing that by 12 hpi HCMV circumvents the inhibition of mTOR by AICAR-activated AMPK. See the text for details. The separated sections of each panel are from the same Western blot and the same exposure. pS6K, phosphorylated S6 kinase; pS6, phosphorylated S6 protein; Tot. S6, total level of S6; p4E BP, phosphorylated eIF4E binding protein; Tot. 4E BP, total level of 4E BP.

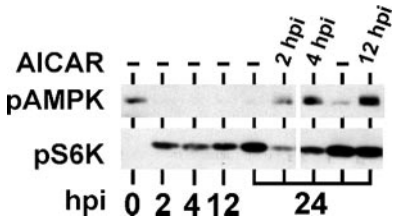

FIG. 3. Western blots showing phosphorylation of AMPK induced by AICAR during HCMV infection. See the text for details. The separated sections of each panel are from the same Western blot and the same exposure. Total AMPK levels did not change significantly during HCMV infection in the absence or presence of AICAR (data not shown). pAMPK, phosphorylated AMPK; pS6K, phosphorylated S6 kinase.

tested at the 24-h time point as the control for the sample in which AICAR was added at 12 hpi (see below). Results for this sample also indicated that a modest activation of AMPK occurred by 24 hpi or later in a normal infection.

The disappearance of phospho-AMPK by 2 hpi is not due to virally induced degradation of AMPK, since total AMPK levels do not change significantly during the infection time course in either the presence or absence of AICAR (data not shown). This suggests that at very early times in infection, prior to 2 hpi, HCMV may promote the dephosphorylation of AMPK. Coordinate with the loss of activated AMPK, the levels of phosphorylated S6K increased in infected cells. We have previously observed this early phosphorylation of S6K (3).

In parallel samples, AICAR was added to the infected cells at 2, 4, or 12 hpi; in each case, the phosphorylation of AMPK was examined at 24 hpi (Fig. 3). In all cases, AMPK was significantly phosphorylated, indicating that HCMV cannot block the robust phosphorylation of AMPK by AICAR. The addition of AICAR at 2 hpi caused inhibition of S6K phosphorylation, reiterating the conclusions from Fig. 2. In contrast, inhibition was not seen if the drug was added at 12 hpi. In this experiment, we detected increased S6K phosphorylation at 4 hpi; we noted that the point in infection when the sensitivity to AICAR changes can vary due to the MOI and the length of time the viruses are allowed to adsorb to cells. Variations in cell passage number may also contribute. However, these data suggest that phosphorylation of AMPK induced by AICAR is not blocked by an established HCMV infection, i.e., after the expression of immediate-early genes. This suggests that AICAR-resistant mTOR kinase activity results from virus-induced effects that act either directly on phosphorylated AMPK or downstream of it.

We next examined the effect of AICAR on HCMV growth over a 120-h time course. Cells treated with AICAR from the beginning of the infection produced no virions (data not shown), apparently because the viral infection never got established. Additionally, exposure to the drug over this extended period was deleterious to the cells. Therefore, human foreskin fibroblast cells were infected with HCMV at an MOI of 2.5 in the absence of the drug. Twenty-four hours prior to the harvest time points of 60, 72, 84, 96, and 120 hpi, AICAR (1 mM) was added to the medium of one set of plates, while the control set was left untreated. In this manner, we allowed the infection to become established for different amounts of time and limited

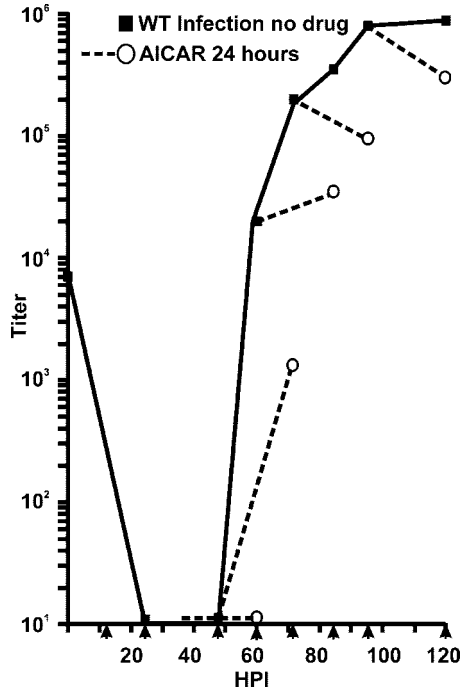

FIG. 4. HCMV growth curves showing that the addition of AICAR for a 24-h interval at any point in the infection time course causes inhibition of progeny virion formation. See the text for details. WT, wild type.

drug exposure to only 24 h. Thus, we could determine whether activation of AMPK at different points in the infection resulted in different effects on virus production. The growth curves (Fig. 4) show that addition of the drug for 24 h at any time in infection significantly inhibited further production of infectious progeny virions.

The data in Fig. 2 and 3 suggest that HCMV infection may induce two mechanisms to block the cellular stress response caused by low energy levels, specifically, the inhibition of mTOR kinase caused by activated AMPK. One mechanism is suggested in Fig. 3, where prior to 2 hpi in the absence of AICAR, the endogenous phospho-AMPK in serum-starved cells is dephosphorylated. Given the very early time of this effect, viral gene expression may not be needed, suggesting that signaling activated by attachment, or tegument, proteins may be involved. However, this mechanism cannot overcome the robust phosphorylation and activation of AMPK caused by AICAR. That the viral infection is able to maintain 4E-BP and S6K phosphorylation under these conditions suggests a virally induced mechanism for circumventing activated AMPK. The time of appearance of this mechanism, between 4 and 12 hpi, suggests that the expression of an immediate-early gene product is required and that this product acts either directly on phosphorylated AMPK or at a point downstream from it (Fig. 1).

mTOR exists in two complexes, which differ in the binding partner, which is either raptor or rictor. In normal cells, the raptor complex phosphorylates 4E-BP and S6K and the rictor complex does not (2, 6–8). However, we have recently shown that both the raptor and rictor complexes can phosphorylate 4E-BP and S6K in HCMV-infected cells (4). Data suggest that the rictor complex is not activated by Rheb-GTP (9), suggesting that it is not under the control of AMPK (Fig. 1). Therefore, the AICAR-resistant phosphorylation of 4E-BP and S6K detected in HCMV-infected cells may be due, at least in part, to the altered substrate specificity of the rictor complex.

Despite HCMV's ability to overcome AICAR inhibition of mTOR kinase, we found that AICAR stops production of progeny virions when added at any time during the time course of an infection (Fig. 4). This suggests that activated AMPK or AICAR may affect another cellular pathway which is critical for virion maturation.

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