Modulation of Host Cell Stress Responses by Human Cytomegalovirus

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Contents

Abstract Human cytomegalovirus (HCMV) induces cellular stress responses during infection due to nutrient depletion, energy depletion, hypoxia and synthetic stress, e.g., endoplasmic reticulum (ER) stress. Cellular stress responses initiate processes that allow the cell to survive the stress; some of these may be beneficial to HCMV replication while others are not. Several studies show that HCMV manipulates stress response signaling in order to maintain beneficial effects while inhibiting detrimental effects. The inhibition of translation is the most common effect of stress responses that would be detrimental to HCMV infection. This chapter will focus on the mechanisms by which cap-dependent translation is maintained during HCMV infection through alterations of the phosphatidylinositol-3′ kinase (PI3K)-Akt-tuberous sclerosis complex (TSC)-mammalian target of rapamycin (mTOR) signaling pathway. The emerging picture is that HCMV affects this pathway in multiple ways, thus

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ensuring that cap-dependent translation is maintained despite the induction of stress responses that would normally inhibit it. Such dramatic alterations of this pathway lead to questions of what other beneficial effects the virus might gain from these changes and how these changes may contribute to HCMV pathogenesis.

Abbreviations 4E-BP: eIF4E binding protein; AICAR: 5-Amino-4-imidazole-carboxamide ribose; Akt: The cellular homolog of the oncoprotein of the AKT8 retrovirus; AMPK: AMP-activated kinase; CaMKKβ: Calcium/calmodulin-dependent protein kinase kinase-β; ER: Endoplasmic reticulum; eIF: Eucaryotic initiation factor; FKBP12: FK506 binding protein; HCMV: Human cytomegalovirus; IR: Insulin receptor; IRS: Insulin receptor substrates; mTOR: Mammalian target of rapamycin; mTORC1: mTOR complex 1; mTORC2: mTOR complex 2; PDK1: Phosphainositide-dependent protein kinase-1; PI3K: Phosphatidylinositol-3′ kinase; PIP2: Phosphatidylinositol-4,5-bisphosphate; PIP3: Phosphatidylinositol-3,4,5-triphosphate; PP2A: Protein phosphatase 2A; PTEN: Phosphatase and tensin homolog; S6K: p70S6 Kinase; TSC: Tuberous sclerosis complex

Introduction

Human cytomegalovirus (HCMV) shares a general life cycle strategy with other mammalian double-stranded DNA viruses that replicate in the nucleus: it must adapt the cellular milieu so the host cell can accommodate the increased demand for nutrients, energy and macromolecular synthesis that accompanies viral infection. For example, successful viral replication requires (1) increased glucose uptake, metabolism and oxygen utilization; (2) abrogation of cellular growth controls; (3) manipulation of the cell cycle to a point that is optimal for virus growth; and (4) inhibition of apoptosis during the productive phase of replication.

These massive changes in the cell's physiology induce cellular stress responses, due to nutrient depletion, energy depletion, hypoxia and synthetic stress, e.g., endoplasmic reticulum (ER) stress. Cellular stress responses are designed to signal the cell when it is in potential trouble and initiate conditions to allow the cell to survive the stress. As a last resort, when the efforts to abate stress fail, apoptosis is induced. Stress responses have many effects on cellular processes; among these some may be beneficial to HCMV replication while others may not. Existing data suggest that HCMV may be able to manipulate stress responses in order to maintain beneficial effects while inhibiting detrimental effects (Isler et al. 2005b; Hakki et al. 2006).

Inhibition of translation is among the most common consequences of cellular stress responses (Kaufman et al. 2002; Arsham et al. 2003; Holcik and Sonenberg 2005; Wouters et al. 2005; Wek et al. 2006). Since translation is an energy-intensive process, its inhibition results in decreased demand for ATP/GTP and decreases the load of proteins entering the ER for processing, consequently relieving ER stress. Translation is well suited to respond to stress, since its inhibition can be accomplished rapidly and reversibly by altering the phosphorylation state of translation regulatory proteins. For example, cap-dependent translation, in which translation initiation depends on

recognition of the mRNA's 5′-cap, is controlled through reversible phosphorylation of the eucaryotic initiation factor (eIF) 4E-binding protein (4E-BP) (reviewed in Mamane et al. 2006). This will be discussed in detail below in Sect. 3.

Although inhibition of translation may permit the cell to recover from stress, it would not benefit HCMV replication. The slow replicative cycle characteristic of HCMV requires the virus to maintain the host cell in a metabolically and translationally active state for an extended period; thus HCMV is obliged to abrogate this type of cellular response. A number of studies have shown that HCMV infection induces several mechanisms to overcome the negative effects of stress responses and maintain translation (Child et al. 2004; Kudchodkar et al. 2004; Hakki and Geballe 2005; Isler et al. 2005a, 2005b; Walsh et al. 2005; Hakki et al. 2006; Kudchodkar et al. 2006; Mohr 2006; Kudchodkar et al. 2007). In this chapter, we will concentrate on the mechanisms by which cap-dependent translation is maintained during HCMV infection by modulation of the phosphatidylinositol-3′ kinase (PI3K)-Akt-tuberous sclerosis complex (TSC)-mammalian target of rapamycin (mTOR) signaling pathway. The emerging picture is that HCMV-mediated regulation of this pathway is multifaceted, thus ensuring that cap-dependent translation is maintained despite the induction of a variety of cellular stress responses. Such dramatic alterations of this pathway lead one to ask what other beneficial effects the virus might gain from these changes and how these changes may contribute to HCMV pathogenesis.

Background: PI3K-Akt-TSC-mTOR Signaling

Akt (PKB) is the cellular homolog of the oncoprotein of the AKT8 retrovirus (Bellacosa et al. 1991). Members of the mammalian Akt family, Akt1, 2 and 3, are activated by PI3K in response to tropic factors (e.g., insulin and other mitogens); other routes of activation are suspected (Datta et al. 1999; Plas and Thompson 2005; Sarbassov et al. 2005b). In Fig. 1, the binding of insulin to the insulin receptor (IR) is used as an example of an Akt activator. IR activation results in tyrosine phosphorylation of insulin receptor substrates (IRSs), this allows binding of the p85 regulatory subunit of PI3K to IRSs. Consequently, the PI3K catalytic subunit (p110) is activated and phosphorylates phosphatidylinositol (PI)-4,5-bisphosphate (PIP2) to PI-3,4,5-triphosphate (PIP3) on the plasma membrane. Both Akt and phosphoinositide-dependent protein kinase-1 (PDK1) bind PIP3, allowing PDK1 to be positioned to phosphorylate (activate) Akt on threonine 308 (T308).

Activated Akt affects multiple cellular targets that increase metabolism, growth and proliferation while suppressing apoptosis (Summers et al. 1998; Ueki et al. 1998; Cass et al. 1999; Datta et al. 1999; Hill et al. 1999; Plas and Thompson 2005). All of these are beneficial to HCMV lytic growth. Thus, it is not surprising that Akt is activated during HCMV infection (Johnson et al. 2001; Yu and Alwine 2002; Kudchodkar et al. 2006). One of the downstream effects of activated Akt is the activation of mTOR kinase (also known as RAFT1 or FRAP) in mTOR complex 1 (mTORC1, Fig. 1, described in detail in Sect. 3 below). Activation of mTORC1 is critical for the maintenance of cap-dependent translation.

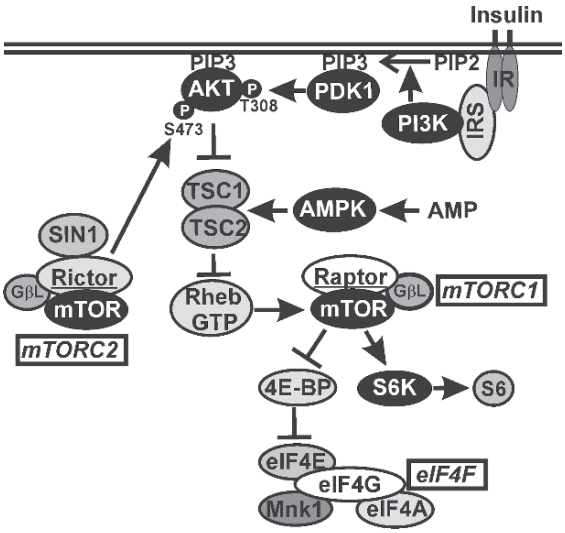

Fig. 1 The PI3K-Akt-mTOR signaling pathway with emphasis on the control of cap-dependant translation via eIF4F. Details are discussed in the text

The link between Akt and mTORC1 is (1) the tuberous sclerosis complex (TSC; reviewed in Luo et al. 2005), made up of TSC1 [hamartin] and TSC2 [tuberin] and (2) Rheb-GTP, a member of the Ras superfamily which binds the N-terminal lobe of the mTOR kinases catalytic domain, allowing mTOR activation (Astrinidis and Henske 2005; Long et al. 2005a, 2005b). Regulation of Rheb-GTP levels is mediated by the GTPase-activating function of the TSC, which stimulates the intrinsic GTPase activity of Rheb, converting it from Rheb-GTP to Rheb-GDP, the inactive form that cannot activate mTORC1. Thus Akt's phosphorylation of the TSC inactivates it, allowing Rheb-GTP levels to remain high in order to activate mTORC1.

Background: The Complexes of mTOR Kinase and Their Activities

mTOR kinase is found in two complexes that differ in their major binding partner (Fig. 1): raptor (*r*egulatory *a*ssociated *p*rotein of *TOR*) in mTORC1 and rictor (*r*apamycin-*i*nsensitive *com*panion of m*TOR*) in mTORC2 (Kim et al. 2002; Sarbassov et al. 2004). Both complexes contain a small protein called GβL that binds to the kinase domain of mTOR kinase and stabilizes the interaction with raptor and rictor (Kim et al. 2003). An additional protein, SIN1, found in mTORC2, maintains the integrity of the complex and regulates activity and substrate specificity (Jacinto et al. 2006; Polak and Hall 2006; Yang et al. 2006a). It is important to note that under normal conditions the two complexes differ in their sensitivity to the drug rapamycin; mTORC1 is sensitive and mTORC2 is insensitive (Sarbassov et al. 2004).

The role of mTORC1 in the control of cap-dependent translation has been extensively studied (Sarbassov et al. 2005a; Reiling and Sabatini 2006). Under conditions of adequate nutrients and oxygen, mTORC1 is activated, permitting phosphorylation of key substrates, p70S6 kinase (S6K) and 4E-BP (Fig. 1). S6K phosphorylation triggers events that promote the formation of translation initiation complexes (reviewed in Mamane et al. 2006); among these is the phosphorylation of ribosomal protein S6, which is often used as a marker for S6K activity. To understand the role of 4E-BP phosphorylation in the control of cap-dependent translation, we must consider its binding partner, eIF4E, and the eIF4F translation initiation complex (Figs. 1 and 2), which binds to the 5′-cap of an mRNA (Fig. 2), the first step in initiation of cap-dependent translation. Functional eIF4F complex consists of the scaffolding protein eIF4G bound to (1) eIF4E, the protein in the complex that directly binds the 5′-cap; (2) Mnk1, a kinase which phosphorylates eIF4E; and (3) eIF4A, an RNA helicase. As indicated in Fig. 2, the functionality of eIF4F depends on eIF4E being bound to eIF4G. However, eIF4E can be removed from eIF4G by binding to 4E-BP, this inhibits cap-dependent translation. It is the role of mTORC1 to control whether or not eIF4E is bound to 4E-BP. Under positive growth conditions, mTORC1 is active and phosphorylates 4E-BP, making 4E-BP unable to bind eIF4E. Thus eIF4E binds eIF4G and completes the eIF4F complex on the 5′-cap. Under these conditions, the polyA binding protein (PABP), bound to the 3′-poly A tail of the mRNA, can also interact with eIF4G (Fig. 2),

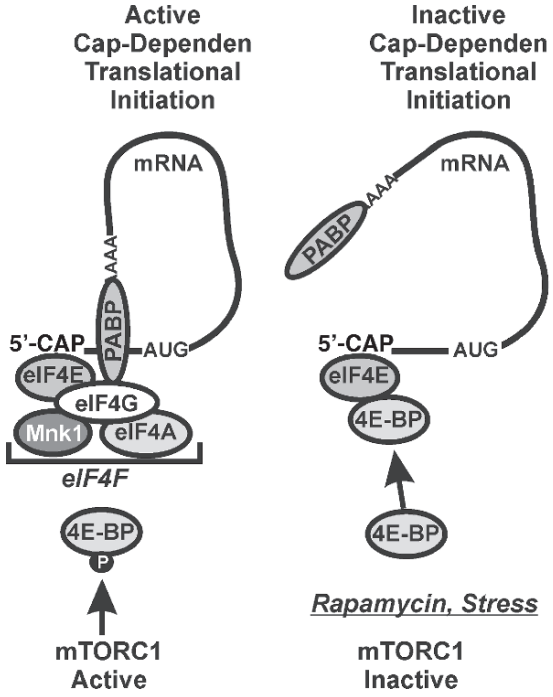

Fig. 2 The control of cap-dependent translation mediated by the activation or inactivation of mTORC1. Details are discusses in the text

setting up conditions in which the 40S ribosome subunit can recognize the mRNA for translation (Mamane et al. 2006). Under negative growth conditions, mTORC1 is inactive and 4E-BP becomes hypophosphorylated. Hypophosphorylated 4E-BP binds to eIF4E, retaining it from eIF4G and the eIF4F complex, thus inhibiting cap-dependent translation.

As discussed below, mTOR kinase activity in mTORC1 is inhibited by many cellular stress responses in order to inhibit phosphorylation of 4E-BP and cap-dependent translation. The drug rapamycin can also do this through direct inhibition of mTOR kinase in mTORC1. It is clear that in order for HCMV to maintain cap-dependent translation, it must find means to maintain hyperphosphorylated 4E-BP. In other words, it needs to circumvent the effects of any cellular stress response that might lead to the inhibition of mTOR kinase activity.

In contrast to the level of understanding of mTORC1, much less is known about the functions of mTORC2. However, RNAi-mediated depletion of rictor in cultured cells demonstrated that mTORC2 plays a role in actin cytoskeleton organization (Jacinto et al. 2004; Sarbassov et al. 2004). At present the only known mTORC2 substrate is serine 473 (S473) of Akt (Sarbassov et al. 2005b; Fig. 1). The role of S473 phosphorylation in Akt activity is controversial, but it has been suggested that it precedes phosphorylation of T308 (the PDK1 site) and may be important for the recognition and phosphorylation of Akt by PDK1 (Sarbassov et al. 2005b) (Fig. 1). This suggests the potential for mTOR and Akt to be involved in an autoregulatory loop. The control of mTORC2 activity is not well understood; however, one study suggests that Rheb-GTP, the activator of mTORC1, does not activate mTORC2 and may inhibit it (Yang et al. 2006b).

HCMV and the Activation of the PI3K-Akt-TSC-mTORC1 Pathway

HCMV infection has dramatic effects on the PI3K-Akt-TSC-mTORC1 pathway. HCMV infection activates Akt through stimulation of T308 phosphorylation via activation of PI3K (Johnson et al. 2001; Yu and Alwine 2002) and stimulation of S473 phosphorylation via activation of mTORC2 (Kudchodkar et al. 2006) (Fig. 1). This occurs by at least two mechanisms. First, transient activation occurs via HCMV attachment to cell receptors which mediate signaling to PI3K (Johnson et al. 2001). However, the identity of this receptor and the means of activating PI3K are under debate (Isaacson et al. 2007; see the chapter by M.K. Isaacson et al., this volume). Second, long-term activation results from the expression of HCMV encoded proteins (Yu and Alwine 2002; Kudchodkar et al. 2006); for example, transfection experiments have shown that expression of the individual major immediate early proteins (either the 72-kDa IE1 or the 86-kDa IE2 proteins) can stimulate phosphorylation of Akt at both sites (Yu and Alwine 2002; Y. Yu and J.C. Alwine, unpublished data).

The HCMV-induced activation of Akt leads to the activation of mTORC1, as indicated by phosphorylation of 4E-BP and S6K, beginning 8-12 h postinfection

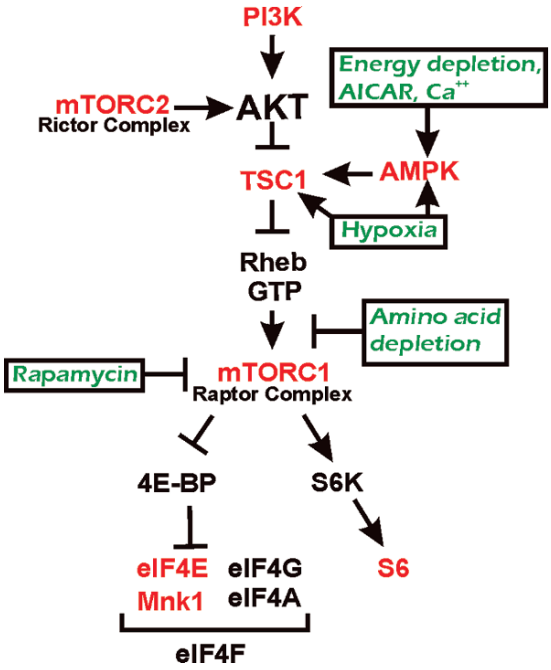

Fig. 3 The effects of stress inducers and HCMV infection on the PI3K-Akt-mTOR pathway, its associated substrates and the components of the eIF4F complex. Stress inducers (*green*) are shown at sites where they affect the PI3K-Akt-mTOR pathway. The points shown in *red* indicate where HCMV infection exerts its effects; Table 1 presents a synopsis of each, see text for details

(Kudchodkar et al. 2004). Although Akt activation is necessary to maintain cap-dependent translation, it is not sufficient. The virus must do more since mTOR kinase activity can be inhibited by many stress responses which exert their effects at points downstream of Akt in the signaling pathway (Fig. 3). Stress responses that do this are induced by the following conditions:

Hypoxia

Hypoxia may occur during the highest rates of metabolic and synthetic activity in HCMV-infected cells. Hypoxia inhibits mTORC1 (Arsham et al. 2002; Brugarolas et al. 2004; Cai et al. 2006) due to the induction of REDD1 (Brugarolas et al. 2004), a protein which activates the TSC (reviewed in van den Beucken et al. 2006). This mechanism is dependent on the induction of hypoxia inducing factor-1 (HIF-1), since the *redd1* gene is a HIF-1 transcriptional target (Schwarzer et al. 2005). A HIF-independent oxygen sensing mechanisms for activation of the TSC involves activation of the AMP-activated kinase (AMPK, Figs. 1 and 3) (Corradetti et al. 2004; Shaw et al. 2004).

Energy Depletion

AMPK activation of the TSC is most often associated with energy depletion (reviewed in Luo et al. 2005; Kimble 2006), which can occur during the highest metabolic and synthetic periods of an HCMV infection. Binding of AMP to AMPK is thought to alter its conformation, permitting subsequent phosphorylation by upstream protein kinases. In contrast, ATP binding prevents phosphorylation and maintains AMPK in its inactive state. Thus, AMPK responds not only to changes in AMP concentration but also to changes in the ratio of AMP to ATP. This means of activating AMPK can be mimicked using the AMP mimetic 5-amino-4-imidazolecarboxamide ribose (AICAR). Under conditions of an increased AMP/ATP ratio or in the presence of AICAR, the tumor suppressor LKB1 phosphorylates AMPK. In addition, the calcium/calmodulin-dependent protein kinase kinase-β (CaMKKβ) also phosphorylates and activates AMPK in response to changes in intracellular calcium concentrations (Hardie 2007).

Both LKB1 and CaMKKβ-mediated mechanisms for activating AMPK could occur during HCMV infection. The increased energy utilization in actively infected cells would increase the AMP:ATP ratio, thus activating AMPK. In addition, it has been shown that the HCMV UL37×1 protein mobilizes calcium from the endoplasmic reticulum into the cytosol (Sharon-Friling et al. 2006); this could potentially activate CaMKKβ.

Amino Acid Depletion

Amino acid depletion inhibits mTORC1 through a mechanism that interferes with the binding of Rheb-GTP to mTOR kinase (reviewed in Avruch et al. 2006). Amino acid depletion, resulting in mTORC1 inhibition, could occur during HCMV infection at times of the greatest viral protein synthesis.

Rapamycin

Direct inhibition of mTOR kinase activity in mTORC1 can be achieved using the drug rapamycin, a macrolide antifungal agent isolated from *Streptomyces hygroscopicus* (Vezina et al. 1975). It is used clinically as an immunosuppressant in organ transplantation, an inhibitor of restenosis of arteries after angioplasty, and an anti-cancer drug (Bjornsti and Houghton 2004). Rapamycin forms a complex with a 12-kDa immunophilin called the FK506 binding protein 12 (FKBP12) and can also bind a 100-amino acid domain (E2015 to Q2114) of mTOR kinase known as the FKBP-rapamycin binding domain (Brown et al. 1994; Sabatini et al. 1994; Chen et al. 1995). The affinity of mTOR's FKBP-rapamycin binding domain for

rapamycin in the absence of FKBP12 is modest, but when rapamycin is bound to FKBP12, the affinity is increased by 2,000-fold (Banaszynski et al. 2005). FKBP12-rapamycin bound to the FKBP-rapamycin binding domain is believed to prevent association between mTOR and raptor (Halford et al. 2001; Hara et al. 2002; Kim et al. 2002). The difference in rapamycin sensitivity between mTORC1 and mTORC2 may be due to structural variations that result from the different binding partners in the complexes. In this model, the mTOR-raptor interaction leaves the FKBP-rapamycin binding domain available while the mTOR-rictor interaction occludes it, thus affording rapamycin insensitivity.

These examples of mTORC1 inhibition by stress responses and drugs highlight the need for HCMV to counteract inhibitory mechanisms at multiple points in the PI3K-Akt-TSC-mTOR pathway. That HCMV can do this has been demonstrated in studies showing that HCMV infection can proceed under conditions of hypoxia (Kudchodkar et al. 2004), under conditions where AMPK is activated (Kudchodkar et al. 2007; N.J. Moorman et al., personal communication) and can overcome the inhibitory effects of rapamycin (Kudchodkar et al. 2006). In addition, preliminary data suggest that HCMV can grow, with reduced kinetics, under conditions of glucose and amino acid depletion (our preliminary data). Indeed, a growing body of evidence shows that HCMV infection may affect many aspects of the pathway downstream of Akt. Known points of interaction between the pathway and HCMV are shown in red in Fig. 3. In the following sections we will highlight HCMV's effects on AMPK, TSC, the mTOR complexes and mTOR's downstream effectors.

The Effects of HCMV Downstream of Akt

Experiments using the AMP mimetic AICAR to activate AMPK have been utilized to analyze the effects of activated AMPK during HCMV infection (Kudchodkar et al. 2007; N.J. Moorman et al., personal communication). Addition of AICAR at 4 h p.i. completely inhibits the viral infection due, at least in part, to the AMPK-mediated inhibition of mTORC1 and translation. Under these conditions, the HCMV immediate early proteins are not expressed and the infection cannot be established. However, if immediate early proteins are expressed before the addition of AICAR (e.g., AICAR addition at 12 h p.i.), there is little to no effect on mTORC1 activity, as indicated by the phosphorylation of 4E-BP and S6K. This observation suggests that the expression and action of the immediate early proteins establishes conditions that are resistant to the effects of activated AMPK. This may involve direct effects on AMPK or downstream at the TSC or Rheb-GTP (Fig. 1). Existing data suggest that HCMV targets AMPK and the TSC (Kudchodkar et al. 2007; N.J. Moorman et al., personal communication); no studies have been reported examining Rheb-GTP.

HCMV infection appears to induce a means to limit the level of phosphorylated AMPK in the infected cell, possibly by dephosphorylation (Kudchodkar et al.

2007). However, this mechanism seems to be of marginal effectiveness since it is overwhelmed by the extensive phosphorylation of AMPK caused by the addition of AICAR. However, under conditions of high levels of phosphorylated AMPK, HCMV inhibits its downstream effects by inactivating the TSC. It has been shown that the HCMV protein pUL38 binds to and inactivates the TSC (N.J. Moorman et al., personal communication), thus protecting mTORC1 from inhibition. This function of pUL38, an early protein, may explain why mTORC1-mediated phosphorylation of 4E-BP and S6K became resistant to the effects of AICAR by 12 h p.i. It is important to note that the inhibition of the TSC by pUL38 may not only block the effects of activated AMPK, but also the effects of other stress responses such as hypoxia that function through activating the TSC.

One question that arises is why would HCMV maintain a limited ability to dephosphorylate AMPK as well as inhibit the TSC with pUL38? It would seem that the inhibition of the TSC would be sufficient to protect mTORC1 from inactivation caused by AMPK activity. A putative answer arises from the observation that HCMV strives to maintain beneficial aspects of cellular stress responses while inhibiting disadvantageous ones. The beneficial aspects of having phosphorylated, activated AMPK are that it activates pathways that increase: (1) ATP production, (2) glucose transport and glycolysis, and (3) fatty acid oxidation (Luo et al. 2005). Thus, HCMV's limited ability to dephosphorylate AMPK may provide a situation where some activated AMPK is maintained during infection. In this regard, data suggest that the different effects of AMPK are activated differentially, requiring different levels of phosphorylated AMPK (Jones et al. 2005). For example, the amount of phosphorylated AMPK needed to increase ATP production, glucose transport, glycolysis and fatty acid oxidation is thought to be much less than the amount needed to activate the TSC and inhibit translation. Hence it would be to the virus's advantage to be able to maintain levels of phosphorylated AMPK capable of inducing beneficial effects but below the level needed to activate the TSC. However, under conditions where this mechanism is overwhelmed, and high levels of phosphorylated AMPK accumulate, the pUL38-mediated inhibition of the TSC would still protect mTOR kinase activity and circumvent translation inhibition.

HCMV Effects on the mTOR Complexes and Their Substrates

One might think that the inhibition of the TSC would be sufficient to assure the maintenance of mTORC1 activity and cap-dependent translation in the infected cell. However, HCMV infection also targets the mTOR complexes and functionally alters them in order to maintain 4E-BP phosphorylation and cap-dependent translation. The first indication of such a mechanism was the observation that in the presence of rapamycin, which directly inhibits mTORC1, translation was maintained in infected human fibroblasts (Kudchodkar et al. 2004). Rapamycin causes a 12- to 24-h delay in the first appearance of progeny virions when compared to a normal

viral growth curve; however, once viral production initiates, it proceeds with normal kinetics to produce near normal levels of progeny (Kudchodkar et al. 2004). Thus the normal rapamycin sensitivity of mTORC1 had been altered; the delay of 12-24 h in viral growth indicated a period in which the viral infection adapted to rapamycin. Indeed, the establishment of rapamycin resistance of viral growth correlated with the development of rapamycin-insensitive phosphorylation of 4E-BP (Kudchodkar et al. 2004, 2006). Interestingly, the phosphorylation of mTORC1's other substrate, S6K, does not become rapamycin-insensitive (Kudchodkar et al. 2004, 2006). Thus HCMV's effect on mTOR kinase activity is substrate-specific, suggesting that HCMV either (1) targets and alters the mTOR complexes directly for the purpose of facilitating 4E-BP phosphorylation or (2) it induces an mTOR-independent mechanism for 4E-BP phosphorylation.

Present data suggest that HCMV alters the mTOR complexes. Experiments using shRNAs to deplete rictor or raptor have shown that HCMV infection alters the substrate specificity of the rictor-containing complex (Kudchodkar et al. 2006). Under normal conditions in uninfected cells, only the raptor-containing complex uses 4E-BP and S6K as substrates; thus depletion of raptor with shRNA results in a severe inhibition of 4E-BP and S6K phosphorylation while depletion of rictor has little effect (Sarbassov et al. 2006b). However, in HCMV-infected cells similar depletion of raptor or rictor caused only about 50% inhibition of 4E-BP and S6K phosphorylation. This suggests that 4E-BP and S6K can be phosphorylated by either the rictor- or the raptor-containing complex; this was confirmed by other experiments (Kudchodkar et al. 2006). Thus the substrate specificity of the rictor-containing complex is expanded, most likely by structure/function modification, during HCMV infection.

An additional indication of HCMV-induced structural modification of the raptor- and rictor-containing complexes is the observation that the rapamycin sensitivity of 4E-BP phosphorylation is altered in infected cells. The raptor-containing complex, normally sensitive to rapamycin, is insensitive in infected cells; and the rictor-containing complex, normally insensitive for the phosphorylation of Akt, is rapamycin-sensitive with respect to 4E-BP phosphorylation (Kudchodkar et al. 2006). How the viral infection mediates these changes remains to be determined; however, virally induced structural modifications of each complex could account for them. Just as structural modifications could alter the substrate specificity of the rictor-containing complexes, they could also change the accessibility of mTOR's FKBP-rapamycin binding domain such that it becomes accessible in rictor-containing complexes and occluded in raptor-containing complexes, thus altering rapamycin sensitivity.

HCMV Effects on eIF4E and Mnk-1

While the HCMV-induced phosphorylation of 4E-BP may seem sufficient to allow eIF4E to be in the eIF4F complex to promote cap-dependent translation, HCMV maintains an additional mechanism. During HCMV infection, the phosphorylation of

eIF4E by Mnk-1 is enhanced to maintain translation; moreover, the levels of eIF4E are increased in infected cells (Walsh et al. 2005). Increasing the amount of eIF4E would be predicted to titrate out hypophosphorylated 4E-BP while still maintaining sufficient free eIF4E to bind eIF4G and preserve the integrity of the eIF4F complex. This is again a situation where HCMV appears to use redundant mechanisms: (1) hyperphosphorylation of 4E-BP to lower its affinity for eIF4E and (2) increased levels of eIF4E to titrate out hypophosphorylated 4E-BP. Such redundancy indicates the importance of maintaining cap-dependent translation during HCMV infection. In addition, the two different mechanisms may be required to maintain cap-dependent translation in different cell types and under different growth conditions.

Conclusions, Questions, Speculations

The points shown in red in Fig. 3 indicate where HCMV infection exerts its effects on the PI3K-Akt-TSC-mTOR pathway, its associated substrates and the components of the eIF4F complex; Table 1 presents a synopsis of each. The striking feature is the number of ways the virus has evolved to manipulate this pathway and thereby manipulate the effects of cellular stress responses. The effects of stress responses that would be inhibitory to viral growth are circumvented, while potentially beneficial effects may be maintained. It is also striking that in more than one case the virus has introduced multiple means to circumvent inhibitory effects of stress responses: for example, the phosphorylation of 4E-BP plus the increased levels of eIF4E and the inhibition of the TSC plus the dephosphorylation of AMPK. The maintenance of redundant mechanisms suggests that they provide the virus with additional capabilities yet to be discovered and understood.

Table 1 Effects of HCMV infection on the PI3K-Akt-TSC-mTOR pathway, its associated substrates and the components of the eIF4F complex

Target	HCMV-mediated mechanism
PI3K	Activated by viral attachment, MIEPs and possible other viral or induced cellular proteins
Akt	Phosphorylated by HCMV activated PI3K-PDK1 and mTORC2
AMPK	Inactivated by dephosphorylation
TSC	Inactivated by HCMV UL38
mTORC1	Activated by HCMV infection Altered producing rapamycin insensitivity
mTORC2	Activated by HCMV infection Altered producing rapamycin sensitivity and gaining 4E-BP and S6K as substrates
eIF4E	Level increased during HCMV infection
Mnk1	Phosphorylation of eIF4E increased by HCMV infection
S6	Phosphorylated by an HCMV-induced mechanism that does not involve S6K (Kudchodkar et al. 2004)

A number of important and exciting questions remain:

1. How does HCMV infection activate PI3K? Initially, viral attachment to receptors may transiently activate PI3K but later in the infection viral protein synthesis is required. The observation that the major immediate early proteins (IE1 and IE2) can mediate PI3K activation suggests a mechanism involving transcriptional activation of viral or cellular genes, the products of which may activate PI3K. However, IE1 and IE2, being complex proteins, may mediate functions, such as the activation of PI3K, which do not depend on transcriptional mechanisms.
2. How are mTORC1 and mTORC2 altered such that their rapamycin sensitivities are reversed and mTORC2 gains 4E-BP and S6K as substrates? Structural modifications of the complexes have been discussed above. It is clearly possible that a cellular protein, induced or misplaced by the viral infection, or a viral protein, becomes part of the complexes and alters their specificities and functions. Indeed, it is conceivable that mTOR is not the only kinase associated with raptor and rictor in infected cells. The shRNA-mediated depletion of mTOR kinase in HCMV infected cells significantly decreases but does not eliminate 4E-BP and S6K phosphorylation (Kudchodkar et al. 2006), thus another kinase could be active with rictor and raptor in infected cells.
3. What mechanism is HCMV using to trigger mTORC2-mediated phosphorylation of Akt S473? As mentioned above in Sect. 3, the control of mTORC2 activity is not well understood. One study suggests that Rheb-GTP, the activator of mTORC1, may inhibit mTORC2 (Yang et al. 2006b). If this is correct, the inhibition of the TSC by pUL38 would be expected to increase Rheb-GTP levels and potentially inhibit mTORC2. However, this does not happen: the rapamycin insensitive phosphorylation of Akt S473 by mTORC2 is activated during an HCMV infection (Kudchodkar et al. 2006). Hence there is still much to learn about HCMV's effects of mTORC2 and the phosphorylation of Akt.

In the above discussion, we have not considered the potential role of phosphatases in the control of the PI3K-Akt-mTOR pathway. PTEN (phosphatase and tensin homolog) is the phosphatase that counteracts PI3K (Baker 2007); its inhibition during infection could account, in part, for the activation of Akt. In human fibroblasts, PTEN levels do not change during infection (Y. Yu and J.C. Alwine, unpublished observations), but it has not been determined whether its activity is altered. One report suggests that PTEN activity may be increased in primary human aortic endothelial cells infected with HCMV (Shen et al. 2006). This could reduce Akt activation and slow the growth of HCMV in these specialized cells, possibly resulting in specific pathogenesis. Protein phosphatase 2A (PP2A) is believed to be the phosphatase that counteracts mTOR kinase by dephosphorylating 4E-BP and S6K. Indeed, PP2A is a central controlling factor in many cellular processes (Mumby 2007). Little is known about the HCMV's interactions with PP2A, but it is likely to be an HCMV target.

Finally, our discussion has largely used the maintenance of cap-dependent translation as the main reason for HCMV targeting the PI3K-Akt-mTOR pathway. However, this is a narrow view. The many interactions that HCMV has with this

pathway (Fig. 3 and Table 1) opens the possibility for vast effects on cellular physiology. The resulting pathogenic effects could implicate HCMV as a subtle, and unexpected, cofactor in many maladies. For example, nearly every one of the targets listed in Table 1 can be an oncoprotein when mutated, inappropriately activated or inappropriately expressed. While not suggesting that HCMV is a frank transforming agent, it is possible that the virus serves as a co-factor with other agents/mutations to promote transformation. The effects of HCMV on oncoproteins such as PI3K, Akt, mTOR, mTOR's effectors and eIF4E could increase the oncogenic potential of a cell, serving as one factor among several which cause transformation, as suggested by the Knudson multi-hit hypothesis (Knudson 1988). The understanding of the means by which HCMV adapts cellular stress response signaling will provide new insight into HCMV pathogenesis.

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