TRANSLATION REGULATION DURING HCMV INFECTION

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology in the School of Medicine.

Chapel Hill
2015

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ABSTRACT

Benjamin Ziehr: Translation Regulation During HCMV Infection
(Under the direction of Nathaniel Moorman)

During viral infection competition for resources is inevitable. One of the fiercest areas of competition is mRNA translation. Host ribosomes are utilized by both host and viral mRNAs, since no virus encodes its own ribosome. Human Cytomegalovirus (HCMV) is a ubiquitous human pathogen that causes significant disease in newborns and immunocompromised individuals. While most viruses suppress host mRNA translation to limit competition for ribosomes, HCMV stimulates host mRNA translation. The goal of this dissertation is to investigate the mechanisms that allow HCMV mRNAs to efficiently compete for access to host ribosomes. mRNA translation begins with translation initiation, which is the rate limiting step of mRNA translation. To determine how viral mRNAs undergo translation initiation we investigated whether the host eIF4F translation initiation complex controlled HCMV mRNA translation. We found that despite eIF4F disruption that suppressed host mRNA translation, HCMV mRNAs continue to translated in the absence of eIF4F. To identify factors that may promote HCMV mRNA translation independent of eIF4F we performed a mass spectrometry screen for mRNA m^7G cap binding proteins in HCMV infected cells. Two viral proteins were identified in our screen, pTRS1 and pIRS1. In addition to binding mRNA during infection, pTRS1 associates with the host translation machinery. We also demonstrated that pTRS1 is sufficient to preferentially stimulate translation of luciferase reporters containing viral sequences outside the context of infection. pTRS1 is a known inhibitor of the host antiviral kinase PKR. However,

pTRS1 was able to stimulate translation in cells lacking PKR, indicating that pTRS1 has functions other than antagonizing PKR. Finally, we evaluated HCMV replication in the absence of pTRS1 or its redundant homolog pIRS1. Using a novel infection model we confirmed that either pTRS1 or pIRS1 is necessary to antagonize PKR for efficient HCMV replication. We also show that pTRS1 is necessary and sufficient to prevent stress granule formation. In fact pTRS1 can also prevent stress granule formation as a result of HRI activation suggesting that pTRS1 may antagonize HRI in addition to PKR. Using our novel infection system we also demonstrate the necessity of the PKR binding domain of pTRS1 for efficient HCMV replication.

ACKNOWLEDGEMENTS

I gratefully thank my beautiful wife Natalie for putting up will all the late nights and weekends in lab, more importantly for being ever supportive and loving.

I owe a debt to Nathaniel Moorman. His never flagging energy and insightful mind were always available to discuss ideas, experiments and projects. He taught me to be a scientist.

To the rest of the Moorman lab thanks for all the support. Having great friends with you makes science so much more fun.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

4EBP1	eukaryotic translation initiation factor 4E binding protein 1
eIF2	eukaryotic translation initiation factor 2
eIF2α	eukaryotic translation initiation factor 2 subunit α
eIF4A	eukaryotic translation initiation factor 4A
eIF4E	eukaryotic translation initiation factor 4E
eIF4F	eukaryotic translation initiation factor 4F
eIF4G	eukaryotic translation initiation factor 4G
G3BP1	GTPase activating protein (SH3 domain) binding protein 1
HCMV	human cytomegalovirus
mTOR	mechanistic target of rapamycin
m7G	7-methylguanosine
PABP1	poly(A) binding protein, cytoplasmic 1
PERK	RNA-dependent protein kinase (PKR)-like ER kinase
PKR	double stranded RNA-dependent protein kinase

Chapter 1: Introduction

Both host and viral mRNAs use host ribosomes for their translation as no virus encodes a ribosome. Therefore, competition for ribosomes is inevitable during viral infection. Viruses have evolved multiple mechanisms to allow viral mRNAs to efficiently compete with host transcripts for access to ribosomes. The reliance of viral mRNAs on host ribosomes has been exploited by host defenses that inactivate the translation machinery upon sensing viral infection. As a result most viruses have evolved countermeasures to ensure the continued translation of viral mRNAs. Therefore, the interface of viral mRNAs with the host translation machinery serves as a critical determinant of viral infection.

Many viruses avoid competition for ribosomes with the host by limiting host mRNA translation. HCMV, however, does not limit host mRNA translation. (1,2) Instead HCMV activates host signaling pathways controlling mRNA translation to ensure the continued synthesis of host and viral proteins, while simultaneously antagonizing host defenses that limit viral protein synthesis. The overarching goal of this dissertation is to better understand how HCMV manipulates the host translation machinery during infection to synthesize host and viral proteins needed for virus replication. Therefore an overview of mRNA translation is provided below.

mRNA Translation Initiation

The Scanning Model of Translation Initiation The rate-limiting step of mRNA translation is

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[1]This introduction has been prepared as part of a review by Ziehr, B., Vincent H.A., and Moorman, N.J.

translation initiation. (3,4,5, reviewed in 6) Translation initiates with the assembly of the eIF4F translation initiation complex on the 7-methyl guanosine cap (m^7G cap) found on the 5' end of mRNAs. (7,8,9, reviewed in 10) The eIF4F complex consists of three proteins: eIF4E, eIF4G and eIF4A. eIF4F assembly begins with the binding of eukaryotic initiation factor 4E (eIF4E) to the m^7G cap. (7,11) After binding to the m^7G cap, eIF4E recruits the eIF4G scaffold protein, which in turn recruits the eIF4A RNA helicase. (12,13,14, reviewed in 15) eIF4G serves as a scaffold that coordinates the assembly of the eIF4F complex and subsequent interactions with additional translation factors. (16) eIF4G also interacts with poly A binding protein (PABP). (17) PABP binds the poly A tail of an mRNA and through its interaction with eIF4G circularizes the mRNA. This circularization event is hypothesized to promote mRNA translation. (18) eIF4A is an RNA helicase that unwinds RNA secondary structure ahead of the scanning ribosome. (19) Additional translation initiation factors associate with eIF4F to modify its activity. For example, the eIF4B protein also associates with the eIF4F complex to promote eIF4A helicase activity, but is not considered part of the eIF4F complex. (20) Following eIF4F assembly the complex recruits the 43S preinitiation complex (see below) through an interaction between eIF4G and eIF3. (21)

Prior to binding to the eIF4F complex, multiple initiation factors associate with the 40S ribosomal subunit to prepare it for translation initiation. The 40S subunit and its associated factors are referred to as the 43S preinitiation complex. (22, reviewed in 23) This complex contains the 40S ribosomal subunit, multiple initiation factors (eIF1, eIF1A, eIF3, and eIF5), and a ternary complex composed of eIF2, GTP and a charged methyionyl-tRNA ($tRNA^{Met}$). eIF3 plays a critical role during 43S assembly, acting as a scaffold for the recruitment of the additional initiation factors. (24) eIF3 exists as a multiprotein complex containing up to 13 subunits, five of which are essential for translation. (25) These subunits directly bind the 40S

ribosomal subunit and help recruit the ternary complex to the 43S complex. eIF3 subunits also nucleate the recruitment of eIF1, eIF1A and eIF5. (26)

Interactions between the eIF3 complex and the eIF4G subunit of the eIF4F complex recruit the 43S ribosomal subunit to the 5' end of the mRNA. (21) Together the 43S preinitiation complex, the eIF4F complex and the bound mRNA constitute the 48S initiation complex. (27) Once assembled, the 48S complex scans the 5' end of the mRNA in search of the translation start site. Scanning of the 48S complex can be impeded by RNA structures present in the mRNA 5' untranslated region (5'UTR). (28,29) In the standard model of scanning the eIF4A helicase unwinds RNA structures in an ATP-dependent manner. However additional RNA helicases including DDX3, DHX9 and DHX29 can also facilitate scanning through areas of high RNA secondary structure. (30,31,32)

The 48S complex ceases scanning upon recognition of a translation start site, which almost always consists of the AUG methionine codon. (33) Start site recognition is influenced by the nucleotides surrounding the AUG codon; AUG codons in the context of a Kozak consensus sequence are recognized most efficiently. (34) The combined actions of eIF1, eIF1A and eIF5 position the $tRNA^{Met}$ over the AUG codon triggering hydrolysis of eIF2-bound GTP, release of some initiation factors and binding of the 60S ribosomal subunit. (35) eIF5 directs subsequent joining of the 40S and 60S subunits to form a functional 80S ribosome, after which peptide elongation commences. (36) Elongation continues until a translation termination or "stop" codon is encountered, at which point eukaryotic release factor 1 (eRF-1) together with eRF-3 terminates elongation by displacing the nascent peptide from the ribosome. (37,38)

Alternative Translation Initiation Mechanisms Translation of some viral RNAs do not require ribosome scanning, but rather ribosomes are recruited directly to the site of translation initiation by specific RNA sequences and/or structures, called Internal Ribosome Entry Sites

(IRESs). (39,40, reviewed in 41) Ribosome recruitment to IRESs often requires only a subset of translation initiation factors. For example translation initiation driven by the poliovirus IRES is independent of the eIF4E cap binding protein, but requires eIF4A, eIF4G and the 43S preinitaition complex. (42) On the other end of the spectrum, the cricket paralysis virus (CrPV) IRES requires only the 40S and 60S ribosomal subunits to initiate translation. (43) Most IRESs contain significant RNA structure that mimic portions of the translation initiation machinery and therefore allow IRES mediated translation independent of canonical translation initiation factors. (44)

However, some IRESs require additional factors to efficiently recruit ribosomes termed IRES trans activating factors, or ITAFs. Numerous studies have implicated cellular factors as ITAFs with limited supporting evidence. Despite the uncertainty surrounding ITAFs, two factors have consistently been demonstrated to be ITAFs, the polyprimidine tract binding protein (PTB) and the lupus autoantigen (La). Both of these proteins have been found to be critical for the activity of multiple IRESs. (45,46, reviewed in 47) PTB and La promote IRES driven translation during HCV, poliovirus and rhinovirus infection. (48,49) PTB is an RNA binding protein associated with RNAs in both the nucleus and cytoplasm. (50) PTB directly binds to RNA secondary structures and promotes RNA folding to maintain IRES structures. (51) In the case of Poliovirus PTB promotes the recruitment of eIF4G to stimulate IRES activity. (52) La stimulates recruitment of 40S ribosomal subunits to IRES containing mRNAs and may promote correct start codon usage. (53)

While IRESs are rare in mammalian mRNAs, sequences in the 5'untranslated regions (5'UTRs) of some mRNAs can promote translation initiation under conditions that generally limit mRNA translation. For example upstream open reading frames (uORFs) are translation competent ORFs consisting of less than 30 codons situated individually or in clusters upstream of a protein coding region on the same mRNA. (54,55, reviewed in 56) Under normal growth

conditions scanning ribosomes initiate translation at the first AUG codon they encounter, and therefore translate uORFs rather than the downstream protein coding region. Thus uORFs act as translational repressors in unstressed cells. However, during period of stress that limit ternary complex availability (reviewed below), uORFs counterintuitively stimulate translation of the downstream protein coding region. (57) The mechanism governing uORF-dependent translation enhancement of downstream reading frame is currently unclear, however ribosome pausing at the uORF termination codon is thought to stimulate the reinitiation of translation on adjacent 5' start codons. (58)

Signaling Pathways Regulating Translation Initiation

mTOR Signaling The target of rapamycin (TOR) kinase is conserved throughout eukaryotes, where it plays a critical role in modulating protein synthesis in response to the intracellular and extracellular environment. (59,60, reviewed 61) In mammalian cells mTOR (mammalian Target Of Rapamycin) is found in two complexes, the mTOCR1 and mTORC2 complexes. (62) The two complexes share several subunits, including mLST8, DEPTOR and the mTOR kinase itself. Each complex also has unique components that define the complex; mTORC1 contains the protein RAPTOR, while the presence of RICTOR defines the mTORC2 complex. (63,64)

mTORC1 plays a central role in regulating translation initiation by controlling the assembly of the eIF4F complex. (65,66, reviewed in 67) In the hypo-phosphorylated state, the 4EBP family of translational repressors bind eIF4E and prevent formation of the eIF4F complex. (68) Specifically, hypo-phosphorylated 4EBP1 competitively competes with eIF4G for binding to eIF4E, and therefore limits eIF4F formation and recruitment of the 43S preinitiation complex. Phosphorylation of 4EBP1 by mTOR dramatically reduces 4EBP1 affinity for eIF4E, significantly

increasing the abundance of the eIF4F complex and stimulating the overall level of protein synthesis. (69)

mTORC1 activity also promotes protein synthesis by phosphorylating and activating the 70 kDa ribosomal protein S6 kinase (p70S6K). (70) There are two p70S6K isoforms, p70S6K1 and p70S6K2, and each is activated by mTOR phosphorylation. Active p70S6K phosphorylates several factors involved in translation such as the eIF4B protein. (71) When phosphorylated by p70S6K, eIF4B binds the eIF4A subunit of the eIF4F complex and increases its activity, allowing for more efficient 48S scanning through highly structured 5'UTRs. Like mTOR, p70S6K also phosphorylates and deactivates a translation repressor, the eukaryotic elongation factor kinase (eEFK2). (72,73) eEFK2 inhibits translation by phosphorylating and thereby inhibiting the activity of eukaryotic elongation factor 2(eEF2). (74) eEF2 stimulates the incorporation of additional amino acids to the growing peptide chain during translation elongation. (75) When eEF2 is phosphorylated eEF2 fails to bind the ribosome and the rate of translation elongation is reduced. Phosphorylation of eEFK2 by p70S6K decreases eEFK2 activity, promoting the association of eEF2 with the ribosome and thereby stimulating translation elongation.

Protein synthesis is one of the most energy intensive cellular processes. Given mTOR's central role in the control of protein synthesis, it is perhaps not surprising that mTOR itself is subject to both positive and negative regulation in response to environmental cues. Many of these signals converge on the tuberous sclerosis (TSC) complex, which negatively regulates mTOR activity. (76,77 reviewed in 78) The heterodimeric tuberous sclerosis complex consists of the tuberous sclerosis 1 (TSC1) and tuberous sclerosis 2 (TSC2) proteins. The TSC complex acts as a GTPase activating protein (GAP) that hydrolyzes GTP bound to the mTOR cofactor Rheb. (79) Energy or amino acid depletion activates signaling pathways that stimulate TSC complex GAP activity, resulting in mTOR inhibition. For example elevated AMP to ATP ratios resulting from nutrient deprivation activates the AMP-regulated protein kinase (AMPK). (80)

Active AMPK phosphorylates TSC2, stimulating TSC GAP activity and thereby decreasing mTOR activity and protein synthesis. (81) Conversely, growth factor signaling stimulates the PI3K and Akt kinases, which phosphorylate and inhibit the TSC complex. (82) Thus the TSC complex integrates upstream signaling pathways to tune mTOR activity and protein synthesis to match the cell's environment.

Mnk-1 Mnk-1 is a MAP kinase-dependent kinase that can be stimulated by extracellular growth factors through ERK. (83) Mnk-1 binds eIF4G and when active phosphorylates eIF4E. In some cases phosphorylated eIF4E stimulates the rate of translation, however this is not always the case. (84,85) It has also been proposed that phosphorylated eIF4E binds the mRNA m^7G cap with a higher affinity than unphosphorylated eIF4E. However, the effect of eIF4E phosphorylation on mRNA translation is an active area of investigation.

eIF2α Kinases The eIF2α translation initiation factor plays a critical role in translation initiation as part of the trimeric eIF2 complex, which recruits a ternary complex composed of eIF2, GTP, and $tRNA^{Met}$ to the ribosome. (86, reviewed in 87) Recognition of the AUG initiation codon by $tRNA^{Met}$ stimulates the hydrolysis of eIF2-associated GTP, and triggers release of eIF2-GDP from the ribosome. (88) Eukaryotic initiation factor 2B (eIF2B) exchanges GDP for GTP in the eIF2 complex, allowing eIF2 to participate in subsequent rounds of initiation. (89) A critical regulatory step in this process is the phosphorylation of eIF2α. Phosphorylation of eIF2α by one of four eIF2α kinases (see below) increases the affinity of eIF2B for eIF2, preventing eIF2B release. (90) As eIF2α is present at 10 fold greater concentration than eIF2B, even small increases in eIF2α phosphorylation can essentially sequester all of the available eIF2B, leading to a dramatic decrease in protein synthesis. (91)

Similar to the mTOR signaling pathway, eIF2α kinases serve to match protein synthesis rates to current conditions in the cell. Four eIF2α kinases have been identified, each of which

phosphorylates eIF2α in response to a different cell stress. (reviewed in 92) The heme-regulated kinase (HRI) phosphorylates eIF2α when reactive oxygen species accumulate, potentially linking the rate of protein synthesis to the respiratory capacity of the cell. (93) Protein synthesis rates that exceed the cell's folding capacity activate the endoplasmic reticulum-associated PERK. (94) Phosphorylation of eIF2α by PERK temporarily halts protein synthesis to allow the cell to clear the backlog of unfolded proteins. The eIF2α kinase GCN2 is activated by binding to uncharged tRNAs that accumulate during amino acid deprivation, directly linking amino acid availability to the rate of protein synthesis. (95) Most relevant for viral infections, protein kinase R (PKR) is activated by binding to double stranded RNAs (dsRNAs) generated during virus replication. (96) Subsequently eIF2α phosphorylation inhibits the synthesis of viral proteins, and thus limits viral replication.

HCMV Manipulation of Translation

Unlike many viruses HCMV does not limit host translation. Instead, overall levels of protein synthesis are maintained or perhaps slightly elevated in HCMV-infected cells. As both host and viral mRNAs rely on the same pool of ribosomes for their translation, this raises the question of how HCMV mRNAs efficiently compete with host transcripts for access to the translation machinery. In addition HCMV replication activates antiviral defenses and stress response pathways, yet viral protein synthesis and virus replication remains unaffected. (97,98, reviewed in 99) While the full spectrum of strategies HCMV uses to deregulate host responses is beyond the scope of this introduction, below I highlight ways in which HCMV counteracts host defenses and stress responses to maintain viral protein synthesis and facilitate virus replication.

HCMV Increases eIF4F Abundance As discussed above the eIF4F complex plays a critical role in translation initiation by recruiting the 43S pre-initiation complex to the 5' end of mRNAs. Recruitment of the eIF4F complex to mRNAs is thought to be the rate-limiting step of

translation initiation, in particular the binding of the eIF4E subunit to the mRNA m^7G cap. In fact overall levels of translation correlate with eIF4F abundance. (100) During HCMV infection, both host and viral mRNAs must recruit ribosomes to their respective mRNAs. This should lead to competition for binding to the eIF4F complex. How might viral RNAs efficiently compete with host transcripts for eIF4F? One explanation might be found in the increased abundance of the eIF4F translation initiation complex found in infected cells. Walsh et al. observed an increase in the abundance of the eIF4F subunits and intact eIF4F complex following HCMV infection. (101) These findings suggest that one mechanism HCMV uses to limit competition for the cellular translation machinery is to increase the abundance of translation factors allowing for efficient translation of both host and viral mRNAs.

Consistent with the idea that eIF4F is a critical determinant of HCMV replication, several studies have shown that disrupting or inhibiting the eIF4F complex prior to infection profoundly limits viral replication. (102,103) Depletion of individual eIF4F components or the associated PABP greatly reduced the synthesis of HCMV early proteins, limited viral DNA accumulation and reduced virus replication. (104,105) Similarly, a small molecule inhibitor of the eIF4A helicase suppressed HCMV replication when given at the time of infection. (104) While eIF4F is certainly necessary for efficient replication it is unclear whether eIF4F is required for the translation of viral mRNAs, host mRNAs or both during the course of infection.

HCMV Stimulates eIF4F Formation Through mTOR In addition to increasing the abundance of eIF4F subunits, HCMV activates signaling pathways such as mTOR that increase eIF4F complex formation. (106) As reviewed above active mTOR promotes eIF4F formation by phosphorylating and inactivating the 4EBP family of translational repressors, which competitively inhibit eIF4F formation when hypophosphorylated. Early studies found that mTOR remains active in infected cells under conditions such as hypoxia that normally limit mTOR activity and disrupt eIF4F formation. (106) Subsequent work demonstrated that mTOR activity is

similarly maintained despite activation of AMPK during HCMV infection, which alters glucose metabolism to facilitate virus replication. (107) In addition, mTOR activity was refractory to further chemical induction of AMPK activity in infected cells, suggesting that HCMV actively limits AMPK antagonism of mTOR. (107)

The dichotomy of continued mTOR activity despite AMPK activation was subsequently shown to be the result of the HCMV UL38 protein. (108) HCMV replicates poorly in the absence of pUL38, which correlates with diminished mTOR activity. pUL38 binds the host TSC2 protein and suppresses the GAP activity of the TSC complex. As a result pUL38 alone is sufficient to prevent mTOR inhibition by nutrient deprivation, starvation or AMPK activation. pUL38 was also found to induce mTOR activity in a TSC2-independent manner, although the mechanism remains unclear. Thus HCMV pUL38 severs the connection between AMPK and mTOR signaling, allowing HCMV-induced AMPK to remodel metabolism while maintaining eIF4F formation through continued mTOR activity.

HCMV also stimulates the PI3K/AKT pathway to increase mTOR activity, and thus eIF4F complex formation. (109) HCMV increases PI3K/Akt activity during the very earliest phase of infection. In fact, UV-inactivated virus is sufficient to increase PI3K and AKT activity through its interaction with cellular growth factor receptors. (109) In addition, the immediate early proteins IE1 and IE2 also stimulate Akt activity through an unknown mechanism, potentially decreasing TSC GAP activity and stimulating mTOR. (110)

Given the multiple mechanisms employed by HCMV to induce and maintain mTOR activity, it comes as no surprise that decreasing mTOR activity inhibits virus replication. (102,103,104) Depletion of mTOR, rictor or raptor decreases HCMV replication, as do ATP-competitive inhibitors of mTOR kinase activity. When added at the time of infection, mTOR inhibitors inhibit viral DNA accumulation and thus the transcription of HCMV late genes.

However mTOR inhibitors have little if any effect on viral protein synthesis or HCMV replication when added later in the replicative cycle.

HCMV Regulation of eIF2α Kinases HCMV infection generates cellular stresses that in uninfected cells would trigger eIF2α phosphorylation. For example, HCMV generates dsRNAs that should activate PKR. (98) In addition, HCMV induces an unfolded protein response (UPR) that includes PERK activation which is necessary for efficient viral replication. (97) HCMV also increases protein synthesis, which should activate GCN2. (1,2) However, minimal eIF2α phosphorylation occurs in infected cells during the immediate early to early stages of infection. (97) During the later stages of infection when virus-induced stress peaks a moderate, but consistently observed, increase in eIF2α phosphorylation occurs. While eIF2α phosphorylation is described as a potent inhibitor of protein synthesis even at low levels host and viral mRNAs continue to translate despite the mild eIF2α phosphorylation late in infection. It is unclear whether eIF2α phosphorylation can no longer restrict translation initiation late in infection or if the level of eIF2α phosphorylation late in infection is insufficient to suppress protein synthesis. Regardless, the significant cellular stress during HCMV infection and continued protein synthesis suggests that HCMV encodes viral proteins that prevent activation of eIF2α kinase activity, or actively limits eIF2α phosphorylation in response to virus-induced stress.

PERK is a sensor of ER stress, namely an accumulation of unfolded proteins and similar to PKR, phosphorylates eIF2α to suppress translation (see above). HCMV infection induces ER stress at early times post infection. (97) Many of the outcomes of ER stress could be beneficial to HCMV replication, but certainly not the shut off of mRNA translation. In addition to ER stress, HCMV infection stimulates the accumulation of PERK and activates PERK autophosphorylation. (97) However eIF2α remains minimally phosphorylated. The mechanism to prevent PERK and

eIF2α phosphorylation during HCMV infection is unknown, but likely important for HCMV replication.

The role of the HRI and GCN2 kinases during HCMV infection has not been established, however it seems likely that HCMV regulates both kinases during infection. The increase in nucleotide and fatty acid synthesis in infected cells should imbalance cellular redox potential, potentially activating HRI. (111) Similarly the metabolic demands of the viral anabolic processes results in at least mild nutrient deprivation as evidenced by AMPK activation. (107) Decreased nutrient availability could increase GCN2 activity during HCMV infection.

Ambisense transcription of the HCMV genome gives rise to antisense transcripts capable of forming dsRNA structures. dsRNA accumulates in infected cells, yet PKR is minimally activated and eIF2α is minimally phosphorylated during HCMV infection. (97) These results suggest that like other herpesviruses, HCMV actively suppresses PKR activation to maintain viral protein synthesis. Using a screen to identify regions of the HCMV gene capable of rescuing growth of a vaccinia virus mutant lacking its PKR antagonist E3L (VVΔE3L), the laboratory of Dr. Adam Geballe discovered two viral proteins that prevent PKR activation, the HCMV TRS1 and IRS1 proteins (pTRS1 and pIRS1, respectively). (112)

pTRS1 and pIRS1 are partially encoded by the terminal repeats flanking the unique short region of the HCMV genome. The first two thirds of the pTRS1 and pIRS1 coding region lie in the terminal repeat, and thus the first 550 amino acids are identical. (113) The carboxyl terminal third of each coding region lies is in the unique short region, and therefore pTRS1 and pIRS1 have distinct carboxyl terminal domains. However the C-terminal domains of pTRS1 and pIRS1 are similar, sharing 35% amino acid identity.

Several pTRS1 and pIRS1 domains important for PKR antagonism have been identified using the heterologous vaccinia virus system. Two domains of pTRS1 were necessary to rescue

replication of VVΔE3L, the RNA binding and PKR binding domains. (114) pTRS1 contains a non-canonical RNA binding domain in the conserved amino terminus. Deletion of the pTRS1 RNA binding domain ablated its ability to rescue VVΔE3L replication, suggesting that pTRS1 in part prevents PKR activation by sequestering dsRNA ligands. Further support for this idea came from studies showing that pTRS1 competes with PKR for binding to dsRNA in vitro. (114) An additional domain in the unique C-terminus of pTRS1 was also necessary for VVΔE3L replication. This domain mediates pTRS1 binding to PKR in vitro, suggesting that a physical interaction between pTRS1 and PKR is required for PKR antagonism. (114) However the role for these pTRS1 functional domains during HCMV infection has not been investigated.

In addition to binding PKR and its ligands, pTRS1 might also limit PKR activation by changing is location in the cell. HCMV infection relocalizes a portion of PKR to the nucleus and outside the context of infection pTRS1 is sufficient to relocalize PKR to the nucleus. (115) Trafficking PKR to the nucleus could limit PKR activation by spatially separating PKR from its cytoplasmic dsRNA ligands and its substrate eIF2α.

HCMV might also counteract the effects of eIF2α kinase activation by recruiting cellular phosphatases to limit phosphorylation of eIF2α. In general, host phosphatases lack substrate specificity. (116) Rather scaffold proteins target phosphatases to their substrates. For example the related herpes simplex virus-1 (HSV-1) hijacks cellular phosphatases to limit eIF2α phosphorylation. The HSV-1 protein ICP34.5 binds both the host phosphatase PP1 and eIF2α, resulting in dephosphorylation of eIF2α and continued viral protein synthesis. (117) HCMV infection increases the abundance and activity of the host PP1 and PP2A phosphatases, suggesting that HCMV might similarly encode a protein that redirects PP1 or PP2A to eIF2α allow for efficient viral mRNA translation. (118)

Alternative Translation Initiation Mechanisms During HCMV Infection

IRES Activity During HCMV Infection Many viruses use IRES elements to ensure translation of viral mRNAs under stress conditions that limit host protein synthesis. As reviewed above, IRESs direct recruitment of ribosomes to mRNAs independent of canonical translation initiation pathways. Only one IRES has been identified in HCMV and is located within UL138. (119) HCMV UL138 is encoded on a polycistronic mRNA that contains four distinct ORFs, pUL138 is the last of the four ORFs on the transcript, and the UL138 initiating methionine is located over 2.4 kb from the mRNA cap. This suggested that cap-mediated translation initiation of UL138 would be exceedingly inefficient. Grainger et al. identified an IRES-like element adjacent to the UL138 ORF that directs translation of pUL138. In addition the authors showed that the IRES allows for increased UL138 protein expression under stress conditions. While the biological significance of the UL138 IRES is unknown, it may play a critical role in regulating HCMV latency. pUL138 is expressed during latency, and serves as a molecular switch that regulates virus reactivation. (120) The UL138 IRES may therefore allow the UL138 ORF to be efficiently translated under stress conditions that trigger HCMV reactivation.

HCMV infection also stimulates the translation of cellular mRNAs containing IRESs. For example the endoplasmic reticulum chaperone BiP (immunoglobulin binding protein) is stimulated by the UPR and necessary for efficient viral replication. (121) During HCMV infection IRES mediated translation of BiP is specifically stimulated. (122) HCMV infection appears to stimulate BiP translation by increasing the abundance of the Bip ITAF La. BiP is one characterized example of a cellular IRES stimulated by HCMV infection. Given the increase in La abundance stimulated by HCMV infection there are likely additional IRES dependent mRNAs that are specifically translated during HCMV infection.

Through a distinctly different approach HSV also stimulates cellular IRESs. The HSV protein vhs has recently been shown to stimulate IRES activity. (123) Many studies have detailed the ability of vhs to limit host protein synthesis by vhs RNase activity. In contrast vhs appears to stimulate a specific subset of IRESs. The mechanism of vhs induced IRES activity is unclear, but has been proposed to be the result of vhs induced stress pathways that limits m^7G cap dependent translation. Alternatively, vhs may recruit translation initiation machinery to stimulate IRES mediated translation.

uORF Dependent Translation During HCMV Infection Cellular mRNAs utilize uORFs to translate under conditions that induce eIF2α phosphorylation, reviewed above. Briefly, when eIF2α is not phosphorylated uORFs direct translation of short peptide coding regions upstream of the main ORF of an mRNA. Following eIF2α phosphorylation mRNAs containing uORFs preferentially reinitiate translation of the main ORF on an mRNA, this stimulates translation of uORF containing mRNAs during cellular stress. The HCMV transcriptome contains many mRNAs with uORFs, might these mRNAs direct eIF2α phosphorylation dependent translation?

Ribosome profiling experiments have provided very detailed information on the location of ribosomes that are actively translating viral mRNAs. (124) Not only do HCMV mRNAs contain uORFs, but profiling experiments have detected 80S ribosomes within the 5'UTR of canonical viral mRNAs. (125) This suggests that HCMV mRNAs have ORFs within the 5'UTR of annotated ORFs capable of recruiting a ribosome, a model consistent with uORF suppression of translation. Similar experiments have not been done under conditions that phosphorylate eIF2α to determine if uORF containing mRNAs preferentially remain associated with ribosomes. While the significance of uORFs in viral mRNAs has not been directly tested, it remains an attractive hypothesis to explain continued translation of viral mRNAs despite eIF2α phosphorylation late in infection.

Rationale for Dissertation

The goal of this dissertation is to better understand the mechanism controlling HCMV mRNA translation. Knowledge of the necessary components in viral mRNA translation will allow identification of host and viral factors that may be excellent candidates for the development of antivirals. To investigate the control of viral mRNA translation I first evaluated the role of eIF4F in the translation of HCMV mRNAs. As reviewed above mTOR signaling can promote eIF4F accumulation and activity. While mTOR activity is clearly necessary for HCMV replication, the relevance of eIF4F activity to viral mRNA translation has not been established. mTOR activity may be necessary for a wide range of host functions during HCMV infection as mTOR stimulates many cellular processes. mTOR may be necessary for stimulating glycolytic flux, promoting nucleotide biosynthesis and driving fatty acid synthesis; in addition to its pro-translation functions of promoting eIF4F dependent translation and regulating S6K activity. Therefore a direct measurement of viral mRNA translation in the absence of eIF4F is needed. Specifically, direct inhibition of eIF4F formation and the activity of its members are necessary to determine whether eIF4F is necessary for HCMV mRNA translation. In this dissertation I sought to directly measure the efficiency with which viral mRNAs associated with ribosomes during HCMV infection in the absence of the eIF4F complex or the activity of its members.

My work in the first chapter demonstrates that viral mRNAs are translated independent of mTOR activity. In the second chapter of this dissertation ,I therefore, sought to identify the factors that allow viral mRNAs to translate independent of eIF4F. I identified a pair of viral proteins bound to mRNA m^7G caps during HCMV infection, pTRS1 and pIRS1. To investigate whether pTRS1 contributes to eIF4F independent translation during HCMV infection I characterized the biochemical interaction of pTRS1 with mRNA. I also determined that pTRS1 outside the context of infection can stimulate protein synthesis. pTRS1 is known to inhibit PKR activation in a heterologous viral infection. To determine if the stimulation of translation I

observed was entirely dependent on PKR antagonism I tested if pTRS1 could stimulate translation in the absence of PKR. Interestingly pTRS1 stimulated translation independent of PKR, although to a lesser extent than in PKR replete cells.

In chapter three I evaluate the role of pTRS1 during HCMV infection. To do so I needed to construct a novel system to separate the roles of pTRS1 and pIRS1 during HCMV infection as pTRS1 and pIRS1 are functionally redundant. Using shRNAs specific for IRS1 I confirmed for the first time that pTRS1 or pIRS1 is sufficient for efficient HCMV replication. I also confirmed that the cellular restriction factor that inhibits HCMV replication in the absence of either pTRS1 or pIRS1 is in fact PKR. During our characterization of HCMV infection lacking both pTRS1 and pIRS1 I observed the formation of stress granules. Previous work demonstrated that stress granules do not form in HCMV infected cells. (126) Our work demonstrates that pTRS1 restricts stress granule formation in HCMV infected cells. Interestingly I also observed that pTRS1 is sufficient to prevent stress granule formation through antagonism of eIF2α phosphorylation, independent of PKR. Following eIF2α phosphorylation the resulting inhibition of mRNA translation leads to stress granule formation. These data suggest pTRS1 may antagonize eIF2α kinases generally or directly antagonize eIF2α. Finally I generated a mutant HCMV strain expressing a truncated pTRS1 lacking the PKR binding domain, which is necessary for pTRS1 to antagonize PKR in a heterologous system. pTRS1 lacking the previously described PKR binding domain failed to antagonize PKR activation or replicate confirming its role in PKR antagonism during HCMV infection.

This dissertation expands our understanding of HCMV biology. My work provides insight into the mechanisms by which viral mRNAs translate, demonstrates that pTRS1 can both antagonize host antiviral pathways and stimulate translation of viral mRNAs, and provides the first characterization of HCMV replication in the absence of pTRS1. I have identified novel viral

protein interactions that may promote HCMV replication, as well as confirmed the importance of antagonizing PKR antiviral sensing to HCMV replication. This dissertation highlights pTRS1 as an ideal candidate for antiviral drug development. Compounds that would prevent pTRS1 from stimulating mRNA translation, either by blocking binding to the mRNA m^7G cap or blocking antagonism of PKR, should be effective at limiting HCMV replication.

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Chapter 2: Differential role for host translation factors in host and viral protein synthesis during HCMV infection[1]

Introduction

As obligate intracellular parasites viruses are reliant on cellular processes for their replication. At a minimum, viruses require host ribosomes to synthesize viral proteins. While host cells have evolved to limit mRNA translation during infection, viruses have evolved to limit host control of protein synthesis in infected cells to maximize viral protein expression. Thus the interaction of viral mRNAs with the host translation machinery represents a fundamental aspect of the host:pathogen interface.

The recruitment of a ribosome to host mRNAs occurs through an ordered assembly of translation factors on the 5' terminus of the message. In particular the eIF4F complex is a critical host translation initiation complex required for the efficient recruitment of ribosomes to mRNAs containing a 7-methylguanosine cap (m7G cap) on their 5' terminal nucleotide (5, 12, 16). Most cellular messages are capped (31), and therefore the eIF4F complex is thought to be required for the translation of the majority of cellular mRNAs. The three components of eIF4F each play specialized roles in translation initiation (19). The eIF4E protein nucleates the assembly of the eIF4F complex by binding to the m7G mRNA cap (37). eIF4E

[1]This chapter previously appeared as an article in the Journal of Virology. The original citation is as follows: Lenarcic EM, Ziehr B, De Leon G, Mitchell D, Moorman NJ. 2014. Differential role for host translation factors in host and viral protein synthesis during human cytomegalovirus infection. J Virol. Feb;88(3):1473-83. doi: 10.1128/JVI.02321-13. Supplemental data available online through link above.

recruits the eIF4G scaffold protein, which in turn recruits the eIF4A RNA helicase to complete the eIF4F complex. The eIF4A helicase stimulates translation initiation by resolving secondary structures in the 5' UTR of mRNAs, thereby facilitating ribosomal scanning to the initiating codon (15, 36). Recruitment of the eIF4F complex to the m7G cap is a rate-limiting step in the initiation of mRNA translation, and reducing the amount of eIF4F complex results in a global decrease in protein synthesis.

Herpesviruses do not encode obvious homologs of eIF4F subunits, and herpesvirus mRNAs are thought to be translated in a cap-dependent manner, though cap-independent translation has been described for a limited number of herpesvirus mRNAs (3, 14, 17, 32). This is based in part on studies showing that HCMV infection induces the accumulation of eIF4F subunits (45). In addition HCMV infection activates signaling pathways that stimulate eIF4F complex formation. For example HCMV activates the mTOR kinase (21, 27, 28, 45). Active mTOR facilitates eIF4F complex formation by phosphorylating and antagonizing the translation repressor 4EBP-1, which prevents the formation of eIF4F on the mRNA cap (11, 13). HCMV also stimulates the ERK and MEK kinase cascades, resulting in phosphorylation of eIF4E by the Mnk1/2 kinases (45). In sum these events ensure that the eIF4F complex remains active during infection despite the induction of a cellular stress response.

The above findings suggest that HCMV infection increases eIF4F activity to stimulate the translation of viral mRNAs. However several recent studies have suggested a more complicated role for eIF4F during herpesvirus infection (8). For example, while inhibiting the mTOR kinase from the start of HCMV infection disrupts the eIF4F complex and limits virus replication, some HCMV mRNAs continue to be efficiently translated (28). As infection progresses both total protein synthesis and virus replication become increasingly resistant to the effects of mTOR inhibitors, despite significant disruption of the eIF4F complex (8). This suggests that mTOR has additional eIF4F-independent roles in virus replication, perhaps in the metabolic remodeling of

HCMV-infected cells (38). These data suggest that the eIF4F complex may not be required for viral mRNA translation during the later stages of infection. However the requirement for eIF4F activity for HCMV protein synthesis has not been directly assessed.

In this study we measured the impact of eIF4F inhibition on host and viral mRNA translation during HCMV infection. Inhibiting eIF4F at the time of infection limits progression through the viral lytic cycle despite the efficient expression of HCMV immediate early proteins. Following the onset of viral DNA replication both viral protein synthesis and replication became increasingly resistant to eIF4F inhibition. We found that disrupting the eIF4F complex inhibited the association of host mRNAs with polysomes and consequently limited host protein synthesis during HCMV infection. In contrast, eIF4F inhibition had a minimal effect on the synthesis of representative viral proteins from each kinetic class, and did not affect the association of the corresponding viral mRNAs with polysomes. Global analysis of the translation efficiency of viral transcripts revealed robust translation of HCMV mRNAs despite a significant reduction in eIF4F abundance. Our data suggest a differential requirement for eIF4F activity for the translation of host and viral mRNAs during the late stage of infection. While host mRNAs continue to require eIF4F for their translation during infection, the association of viral mRNAs with ribosomes is insensitive to changes in eIF4F abundance or activity.

Materials and Methods

Cells, Viruses and Inhibitors Primary human foreskin fibroblasts (HFFs) were passaged in DMEM (Sigma) containing 10% newborn calf serum and used between passages 7 and 14. Unless otherwise indicated cells were seeded at confluence and then serum starved for 48 hours prior to infection. HCMV infections were performed with the BADinGFP strain (ADGFP) (46), which contains the green fluorescent protein (GFP) open reading frame under the control of the SV40 promoter inserted in the non-essential UL21.5 locus. HCMV infections

were performed at a multiplicity of infection (MOI) of three in serum free media unless otherwise noted. Cell free HCMV was titered by the tissue culture infectious dose 50 (TCID50) method on primary human fibroblasts. The eIF4AI/II inhibitor hippuristanol (4) was generously provided by Dr. Jerry Pelletier (McGill University). Cycloheximide, actinomycin D and phosphonoacetic acid (PAA) (all from Sigma) were used at a final concentration of 100 µg/ml, 5 µg/ml or 200 µg/ml, respectively. Unless otherwise noted Torin1 (Tocris) was used at a final concentration of 250 nM.

Western blot analysis of host and viral proteins Cell pellets were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EDTA) containing protease inhibitors (complete EDTA-free; Roche). Cells were incubated on ice in RIPA buffer for 15 minutes and then cleared of debris by centrifugation for ten minutes at 14,000 x g. The protein concentration of each sample was determined using the Bradford reagent. Equal amounts of protein from each sample were resolved on 10% polyacrylamide gels, transferred to nitrocellulose membranes (Whatmann) and then blocked for at least one hour in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% non-fat milk. Primary mouse monoclonal antibodies were diluted in TBS-T containing 1%BSA and incubated with the membrane for one hour at room temperature. Rabbit polyclonal antibodies were incubated overnight at 4oC in TBS-T containing 5% BSA. Following washing with TBS-T, blots were incubated with HRP-conjugated secondary antibodies for one hour at room temperature. Blots were again washed in TBS-T and proteins visualized by treatment with ECL reagent (Amersham or Advansta)) and exposure to film. The following antibodies were used in this study: IE1 (1:100), UL44 (1:100), pp28 (1:100), tubulin (Sigma, 1:5000), rpS6 (1:1000), phospho-rpS6 (1:2000), eIF4A (1:1000), eIF4E (1:1000), eIF4G (1:1000), 4EBP1 (1:1000) from Cell Signaling, hsp90 (Enzo,1:1000), RACK1 (Santa Cruz, 1:1000)

Metabolic labeling of HFFs At the indicated time post infection, the media was removed and replaced with serum free DMEM lacking methionine and cysteine (Sigma). Following a fifteen minute incubation, 125 µCi 35S-labeled methionine/cysteine (EasyTag Express Protein Labeling Mix; Perkin Elmer) was added directly to each well. In experiments measuring total protein synthesis the cells were labeled for 30 minutes. Cells were labeled for one hour where the synthesis of specific proteins was measured. The media was then removed and the cells washed three times with ice cold PBS. Cells were pelleted and stored at -80oC until analyzed. Where the effect of inhibitors was tested, the inhibitors were included in both the methionine/cysteine free incubation and labeling periods.

Analysis of total protein synthesis 35S-labeled cells were lysed in 100 µl RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate) containing protease inhibitors (complete EDTA-free protease inhibitor, Roche). The protein concentration of the lysate was then determined by the Bradford assay. Fifteen microliters of cell extract was mixed with 0.1 ml of 1 mg/ml BSA containing 0.02% (w/v) sodium azide (NaN3). One milliliter of 20% trichloroacetic acid (TCA) was added to the sample, and the mixture was vortexed and incubated on ice for 30 minutes. The precipitate was vacuum filtered onto 2.5 cm glass microfiber filters (Whatmann). The filters were washed twice with 20% TCA, once with 100% ethanol, and allowed to air dry for 30 minutes. The amount of radioactivity retained on the filters was measured using a scintillation counter. The amount of precipitated radioactivity in each sample was normalized to the total amount of protein in the sample. In some cases metabolically labeled proteins were visualized by resolving 10 µg of protein on 10% SDS-PAGE gels. Gels were dried for one hour and then exposed to film to visualize radiolabeled proteins.

Quantification of viral DNA HCMV DNA accumulation was measured essentially as described in ref ((26)). Briefly, HFFs were seeded at confluence in six well plates, and then serum starved for forty-eight hours prior to infection. The cells were infected at a multiplicity of

0.05, and inhibitors were added following the removal of the inoculum. Control infected cells were treated with DMSO vehicle. Ninety six hours post infection the cells were scraped from the dishes, pelleted, and frozen at -80ºC. Frozen cell pellets were resuspended in 100 µl lysis buffer (RIPA) and digested overnight with Proteinase K (10 mg/ml). The lysates were extracted with phenol/chloroform, and the DNA was precipitated with isopropanol. The DNA pellet was resuspended in distilled water and quantified using a NanoDrop spectrometer. Five hundred nanograms of DNA were analyzed by quantitative real-time PCR (qPCR) exactly as described previously (28) using primers specific for the HCMV major immediate early promoter (MIEP) (see Supplemental Table 1). The number of HCMV genomes in each sample was determined by comparing the results to a series of DNA standards containing from 108 to 101 HCMV genomes. To control for variations in pipetting, the results were normalized to the total amount of DNA in each sample by qPCR using primers specific for GAPDH.

Polysome isolation Polysomes were isolated by centrifugation through 10-50% linear sucrose gradients. Linear sucrose gradients were prepared by pouring sucrose step gradients and allowing them to equilibrate overnight at 4oC. Step gradients consisted of 10, 20, 30, 40, and 50% sucrose steps, each prepared in polysome gradient buffer (20 mM Tris-HCl, pH 7.4, 140 mM KCl, 5 mM MgCl2) containing 100 µg/ml cycloheximide. Confluent serum starved fibroblasts were infected at a multiplicity of three in serum free media. At seventy two hours post infection cells were treated with Torin1 or left untreated. Sixteen hours later the cells were incubated in serum free media containing cycloheximide for ten minutes at 37oC. The cells were washed three times in PBS containing cycloheximide and scraped from the dishes and pelleted (5 minutes at 3000 x g). The cell pellets were resuspended in polysome gradient buffer containing cycloheximide, 1 mM DTT and 1% Triton X-100. The cells were swollen on ice for ten minutes, and then disrupted by five passes through a 27 gauge needle. Nuclei were removed by centrifugation for 5 minutes at 2500 x g. The resulting supernatant was cleared of insoluble

debris by centrifugation for ten minutes at 13,000 x g. The resulting supernatant was layered onto the sucrose gradients and spun in an ultracentrifuge (Beckman-Dickinson) for two hours at 32,500 rpm in an SW41 swinging bucket rotor. The centrifuge brake was disabled to avoid disrupting the gradients during deceleration. Following centrifugation the gradients were manually fractionated from the top into fourteen 750 µl fractions. The bottom fraction of the gradient (fraction 15) containing any pelleted debris, was discarded. RNA was extracted from one third of each gradient fraction and resolved on 2% non-denaturing agarose gels to visualize ribosomal RNA. We routinely monitored the efficiency of fractionation by performing Western blots for the nuclear protein lamin A/C and cytoplasmic protein tubulin (data not shown). In each experiment the efficiency of mTOR inhibition was measured by performing Western blots on whole cell extracts using antibodies specific for either phosphorylated or total ribosomal protein S6 (rpS6).

Quantification of viral mRNA abundance The abundance of specific mRNAs in total cellular RNA was quantified by reverse-transcriptase real time PCR (qRT-PCR) as previously described (9). Briefly, frozen cell pellets were resuspended in Trizol (Invitrogen). The mixture was extracted with chloroform, and the RNA was precipitated with isopropanol. RNA pellets were resuspended in RNAse-free water and RNA was quantified using a NanoDrop spectrometer. Two micrograms of RNA was reverse transcribed with High Capacity Reverse Transcription Kit (ABI) using random hexamers as primers. Two microliters of the resulting cDNA was mixed with gene specific primers and SYBR Green Master Mix and amplified in a Roche LightCycler 4800 using the following cycling parameters: 95°C for 5 minutes, then 40 cycles consisting of 95°C for 30 seconds and 60°C for 30 seconds. The amount of viral transcript in each sample was determined using the □□Ct method, with GAPDH as the reference sample.

To quantify the abundance of mRNAs in sucrose gradient fractions, RNA was extracted from one third of each gradient fraction. An equal volume of RNA from each fraction was reverse transcribed, and an equal volume of the cDNA reaction was analyzed by real-time PCR analysis as above. The abundance of each transcript was determined by comparing the threshold values for each sample to a standard curve specific for each primer set. For viral transcripts, the standard curve consisted of a range of HCMV BAC DNA concentrations ranging from 108 to 101 copies. For host transcripts, the PCR product of each primer pair was cloned into the pGEM-T vector. The threshold values obtained from a series of DNA standards containing from 108 to 101 copies was used to determine the abundance of each transcript in each fraction. The total amount of each transcript in the gradient was determined by summing the copy number of the transcript across all fractions. The percent of the mRNA present in each fraction of the gradient was then graphed to demonstrate the relative distribution of a transcript across the gradient. Primer sequences for both host and viral genes are listed in Supplemental Table 1.

shRNA-mediated depletion of host mRNAs Lentivirus shRNA expression constructs targeting eIF4A1 were obtained from the University of North Carolina Lentivirus Core Facility. Lentivirus stocks were prepared by transfecting 293T cells with lentivirus shRNA construct together with packaging vectors. Cell free supernatants were harvested three days post transfection and filtered through a 0.45 µm filter. One day prior to transduction HFFs were seeded at confluence into 6 well plates. The cells were transduced with lentivirus overnight in the presence of 5 µg/ml polybrene. The next morning the media was removed and the cells were washed with PBS. Serum free media was then added to the wells, and the cells were incubated for an additional 48 hours prior to infection with HCMV as described above.

Analysis of cap structures on viral mRNAs Polysome-associated mRNAs were isolated from untreated of Torin1 treated, infected cells. The RNAs were treated with calf-

intestinal phosphatase to remove 5' phosphates from uncapped RNAs, and then treated with TAP to remove the m7G cap. An RNA oligonucleotide was then ligated to the decapped mRNA. All reagents were from the RLM-RACE kit (Ambion) essentially as described in the product manual. The RNA was then reverse transcribed and PCR amplified using primers specific for the viral sequence and the RNA oligonucleotide. The omission of TAP should prevent ligation of the RNA oligo if the mRNA contains a 5' m7G cap, and therefore amplification of the cDNA would be prevented. In some samples the TAP digestion step was omitted to test for the presence of the mRNA cap structure.

Microarray Analysis of Translation Efficiency Polysome-associated and total RNA were isolated as described above. Sample quantity and quality was verified via the Agilent 2100 Bioanalyzer. Total RNA and polysome RNA was labeled with Cy3 or Cy5 respectively, using the Agilent LIQA two color labeling protocol per manufacturer's instructions. Samples were hybridized to a custom microarray developed on the Agilent Human GE 4x44K v2 Microarray platform. In addition to probes specific for the whole human genome the microarray contained oligonucleotide probes specific for all of the annotated genes of the WT Merlin HCMV genome (NCBI accession: NC_006273). Microarray hybridization, washing, and signal intensity analysis were performed according to the manufacturer's direction. Data was collected using Feature Extraction software. Data analysis was carried out using Partek Genomics Suite software. Only probes showing positive signal in all samples were subjected to further analysis. To determine the translation efficiency of a given gene the signal intensity from the polysomes RNA was divided by that in the total RNA. To determine the relative change in translation efficiency in the presence of Torin1, the translation efficiency of an mRNA in Torin1 treated cells was divided by that in untreated cells. Statistically significant changes in translation efficiency in the presence of Torin1 were calculated using a students paired two-tailed t-test. The experiment was performed three times using RNAs derived from three independent experiments.

Results

To test the requirement for eIF4F activity for the translation of viral mRNAs we measured the effect of a specific inhibitor of the eIF4A RNA helicase on viral replication and protein synthesis. Hippuristanol is an ATP-competitive inhibitor that binds directly to the active site of eIF4AI and eIF4AII and inhibits their RNA helicase activity (4, 22). Importantly, hippuristanol is a specific inhibitor of eIF4AI and eIF4AII, and it does not affect the activity of other RNA helicases (22). Therefore unlike other eIF4F inhibitors that target signaling pathways that control eIF4F integrity, hippuristanol directly targets the enzymatic activity of the eIF4F complex. Consistent with the requirement of eIF4A activity for the translation of most cellular mRNAs, hippuristanol inhibits protein synthesis in mammalian cells (4, 22). We found that 100 nM hippuristanol potently inhibited the total rate of protein synthesis in uninfected cells (figure 1A) at a concentration that did not affect cell viability (figure 1B) or mTOR signaling (figure 1C). We therefore used hippuristanol treatment to determine the requirement for eIF4A activity for the translation of viral mRNAs during HCMV infection.

We first determined the effect of hippuristanol on HCMV immediate early (IE) protein expression. Cells were serum starved for 48 hours and then infected with HCMV and treated with hippuristanol at the time the inoculum was removed. Serum starvation was included to better assess the stimulatory effects of HCMV infection on eIF4F activity and to allow us to compare our results to previously published studies. The expression of HCMV immediate early protein IE1 was then measured by Western blot at six hours post infection. As expected cycloheximide, an inhibitor of peptide elongation, prevented IE1 protein expression (figure 1D). Consistent with our previous studies (28), the mTOR inhibitor Torin1 did not limit IE1 protein expression. Surprisingly hippuristanol treatment also had no effect on IE1 protein levels despite limiting total protein synthesis to a similar degree as cycloheximide. A potential explanation for this result could be that inhibiting eIF4A helicase activity resulted in a compensatory increase in

IE1 mRNA transcription. However quantitative reverse transcriptase real-time PCR (qRT-PCR) analysis of IE1 mRNA levels showed that similar amounts of the IE1 mRNA were present in untreated and hippuristanol treated cells (figure 1E). As similar amounts of IE1 mRNA and protein were made when eIF4A was inhibited, this result demonstrates that inhibiting eIF4A helicase activity does not decrease the efficiency of IE1 mRNA translation despite a global reduction in the rate of protein synthesis.

We next determined the effect of inhibiting eIF4A at the time of infection on the progression of the HCMV lytic cycle. As shown in figure 2A, the IE1 protein was expressed throughout the HCMV lytic cycle in the presence of hippuristanol. The expression of the HCMV early protein UL44 (pUL44) was delayed and reduced by hippuristanol treatment, however pUL44 continued to increase in abundance as infection

Figure 1

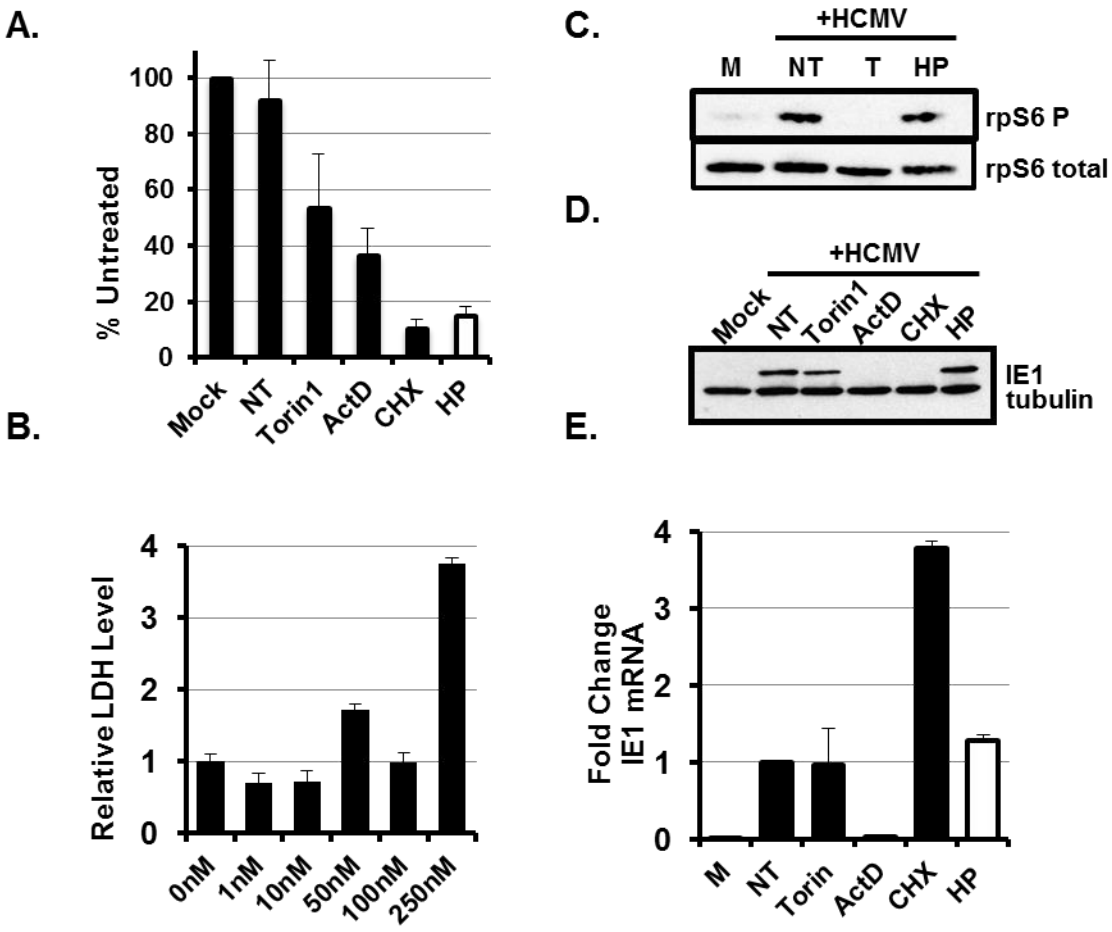


Figure 1 An HCMV immediate early protein is efficiently translated in the presence of an eIF4A inhibitor. (A) Human foreskin fibroblasts (HFFs) were infected with HCMV (MOI of 3) and treated with vehicle (NT), Torin1 (T, 250 nM), actinomycin D (ActD, 10 µg/ml), cycloheximide (CHX, 100 µg/ml) or hippuristanol (HP, 100 nM) when the inoculum was removed. The rate of incorporation of radiolabeled amino acids into acid-insoluble protein during the final thirty minutes of the infection was measured (n=3). Values are expressed as a percentage of the untreated, uninfected control. (B) Uninfected HFFs were treated with the indicated concentrations of hippuristanol for five days. Cell viability was determined by the LDH assay (n= 2) (C) Cells were mock infected (M) or infected and treated as in B. Cells were harvested at 6 hpi and the levels of phosphorylated rpS6 and total rpS6 were determined by

Western blot (n=2) (D) As in C, except IE1 and tubulin levels were measured by Western blot (n=3). (E) Same as D, except that RNA was harvested at 6 hpi and IE1 mRNA abundance was measured by qRT-PCR. The abundance of IE1 mRNA in untreated cells was set to one (n=3).

progressed. In contrast the expression of pp28 was significantly diminished in hippuristanol treated cells at all times post infection. We investigated the defect in pp28 expression in more detail by determining if the decrease in pp28 protein levels was due to decreased transcription or translation of the pp28 mRNA. Quantitative real-time PCR analysis found that pp28 mRNA levels were reduced when hippuristanol was added to the cultures at the time of infection (figure 2B). As transcription of HCMV late mRNAs is dependent on viral DNA replication, we hypothesized that the defect in pp28 transcription might be due to a requirement for eIF4A activity for efficient viral DNA accumulation. Infected cells were treated with hippuristanol at the time of infection, and viral DNA was isolated and quantified by real-time PCR at ninety six hours post infection. As a control, we also measured the level of viral DNA in cells treated with phosphonoacetic acid (PAA), a well-described inhibitor of HCMV DNA replication. Hippuristanol and PAA similarly reduced viral DNA accumulation by approximately two orders of magnitude (figure 2C). Therefore the reduction in pp28 expression when hippuristanol is added at infection is due to a requirement for eIF4A activity for viral DNA replication.

Figure 2

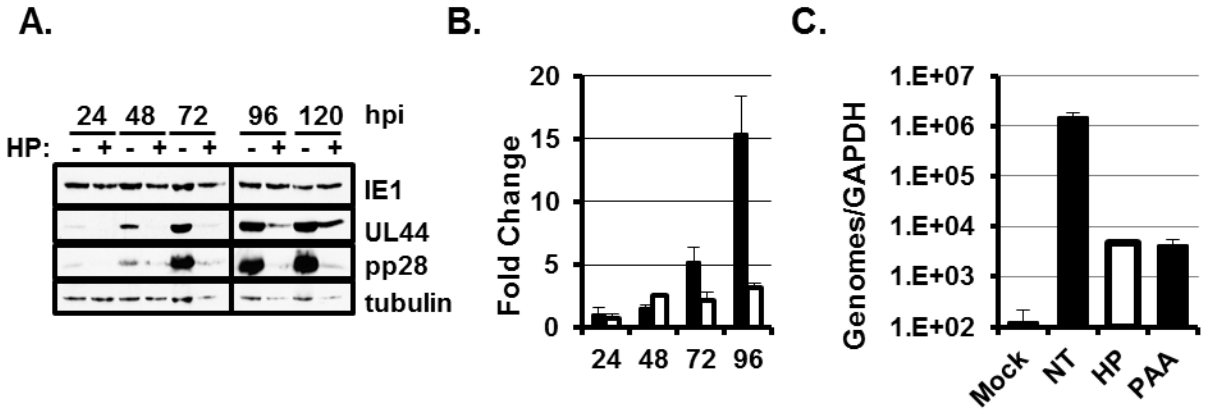


Figure 2 Hippuristanol inhibits progression through the HCMV lytic cycle. (A) Serum starved confluent HFFs were infected with HCMV at a multiplicity of three. Cells were left untreated or treated with hippuristanol (HP; 100 nM) when the inoculum was removed. Cells were harvested at twenty four intervals and viral protein expression was measured by Western blot (n=3). (B) Cells were infected and treated as in A and pp28 mRNA abundance was determined by qRT-PCR (n=3). The fold change in pp28 mRNA levels relative to the 24 hour time point is shown. Closed bars = untreated samples, open bars = HP treated samples) (C) Serum starved confluent HFFs were infected at an MOI of 0.05. Hippuristanol (HP; 100 nM) or phosphonoacetic acid (PAA; 200 µg/ml) was added at the time of infection. At 96 phi viral DNA was quantified by qPCR. The results were normalized to the abundance of GAPDH DNA in the sample to control for variations in loading (n=3).

Consistent with the effect of hippuristanol on viral DNA accumulation and late gene transcription, hippuristanol potently inhibited the production of cell free progeny dose dependent (figure 3B) consistent with the specific inhibition of eIF4A in a non-toxic manner. We used shRNA-mediated depletion of eIF4A1 as an additional approach to confirm the phenotypes observed following hippuristanol treatment. Similar to hippuristanol treatment, the HCMV IE protein IE1 and the early protein pUL44 were expressed in eIF4A1-depleted cells, although pUL44 expression was delayed and reduced (figure 3C). Limiting eIF4A1 expression prior to infection also decreased the yield of cell free virus by approximately 150 fold (figure 3D). Similar results were obtained using a separate eIF4A-specific shRNA (data not shown). These data confirm the results obtained in hippuristanol treated cells, and support the conclusion that eIF4A activity is required at the onset of infection for progression through the HCMV lytic cycle.

Figure 3

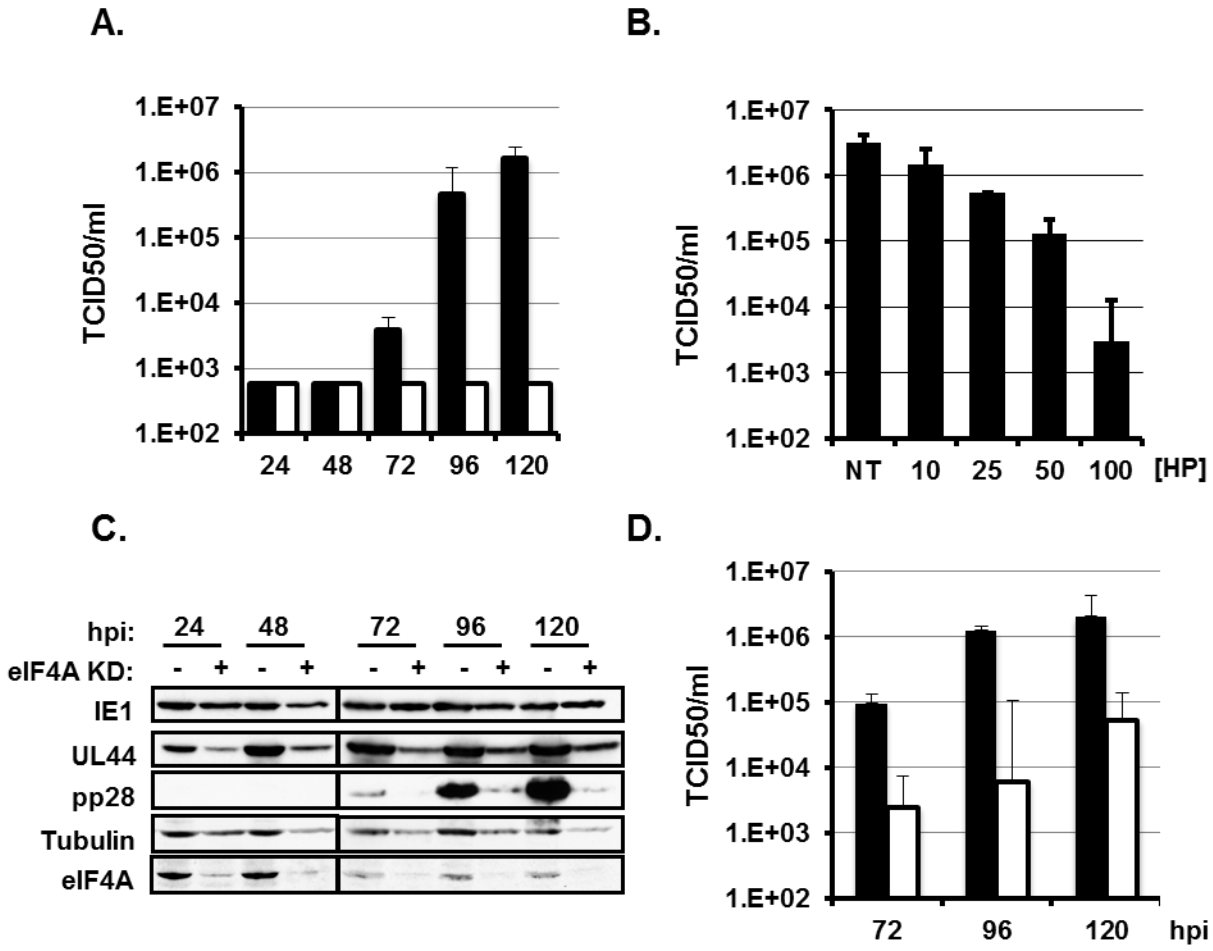


Figure 3 Inhibiting eIF4AI at the time of infection limits HCMV replication. (A) Serum starved confluent HFFs were infected with HCMV at an MOI of three. Cells were left untreated (closed bars) or treated with hippuristanol (100 nM, open bars) at the time of infection. Cell free virus was quantified by the TCID50 method (n=3). (B) Cells were infected as in A. The indicated concentrations of hippuristanol were added at the time of infection. The amount of cell free virus supernatants at 120 hours post infection was determined by the TCID50 method (n=2). (C) Cells expressing control (closed bars) or eIF4AI (open bars) specific shRNA were infected with HCMV at an MOI of 3. Viral protein expression was measured as in figure 2A (lanes marked with (-) = scrambled shRNA, eIF4AI KD = eIF4AI shRNA; n=3) (D) As in C, except the amount

of cell free virus in the supernatant was quantified by the TCID50 assay (n=3). Closed bars = scrambled shRNA; open bars = eIF4AI shRNA)

We next performed experiments to address the temporal requirement for eIF4A activity in HCMV mRNA translation and virus replication. Cells were infected with HCMV, and then hippuristanol was added to the infected cells for a twenty four period beginning at different times post infection (e.g. from 24 to 48 hours, 48 to 72 hours, etc.). For comparison, vehicle treated cells were harvested at the time of hippuristanol addition, and at the end of the 24 hour treatment period. We first measured the rate of incorporation of radiolabeled amino acids into protein in the presence of hippuristanol of different times post infection. Hippuristanol inhibited total protein synthesis in both uninfected and infected cells by greater than 70% (figure 4A). We also visualized proteins made during the final thirty minutes of the hippuristanol treatment on acrylamide gels by autoradiography. Interestingly, in HCMV-infected cells a subset of proteins continued to be efficiently synthesized in the presence of hippuristanol during the later stages of infection (figure 4A). These proteins were only observed in infected cells, suggesting that they were viral proteins.

Using the same treatment protocol we next evaluated the effect of hippuristanol on viral protein levels. Adding hippuristanol at any time following infection did not affect levels of the IE1 protein (figure 4B). Levels of the early UL44 protein increased between 24 and 48 hours in the presence of hippuristanol, although to a lower level than in untreated cells. The addition of hippuristanol at later times following infection did not affect the levels of pUL44. The late protein pp28 was first detected at 48 hours in untreated cells, however pp28 was undetectable in cells treated with hippuristanol between 24 and 48 hours. Hippuristanol also limited the accumulation of pp28 between 48 and 72 hours post infection. Beyond 72 hours hippuristanol had minimal effect on the steady state levels of pp28 protein. As a more direct measure of the rate of viral protein synthesis we measured the incorporation of radiolabeled amino acids into specific viral

proteins during the final hour of hippuristanol treatment. Representative immediate early, early, and late proteins were immunoprecipitated from the labeled cell lysates and visualized by autoradiography. The results show that the HCMV IE1 and UL44 proteins were efficiently synthesized in the presence of hippuristanol (figure 4C). Hippuristanol had a modest effect on the synthesis of the pp28 protein, however nascent pp28 protein was clearly detectable. In contrast cycloheximide completely inhibited nascent protein synthesis (figure 4C). These results suggest that viral protein synthesis is largely independent of eIF4A activity during the late stage of HCMV infection.

We also determined the temporal requirement for eIF4A activity for the production of HCMV progeny virus. In marked contrast to the results obtained when eIF4A was inhibited at the start of infection, hippuristanol had minimal impact on the accumulation of cell-free virus (figure 4D). In control cells the amount of cell-free virus increased by approximately two orders of magnitude between 48 and 72 hours post infection. Hippuristanol treatment had less than a fivefold effect on virus replication during the same time frame. Virus titers increased an additional tenfold between 72 and 96 hours post infection in untreated cells. Hippuristanol treatment had no negative effect on virus replication during this time and in fact slightly increased the yield of cell-free virus. These data show that HCMV replication becomes increasingly resistant to eIF4A inhibition as infection progresses.

Figure 4

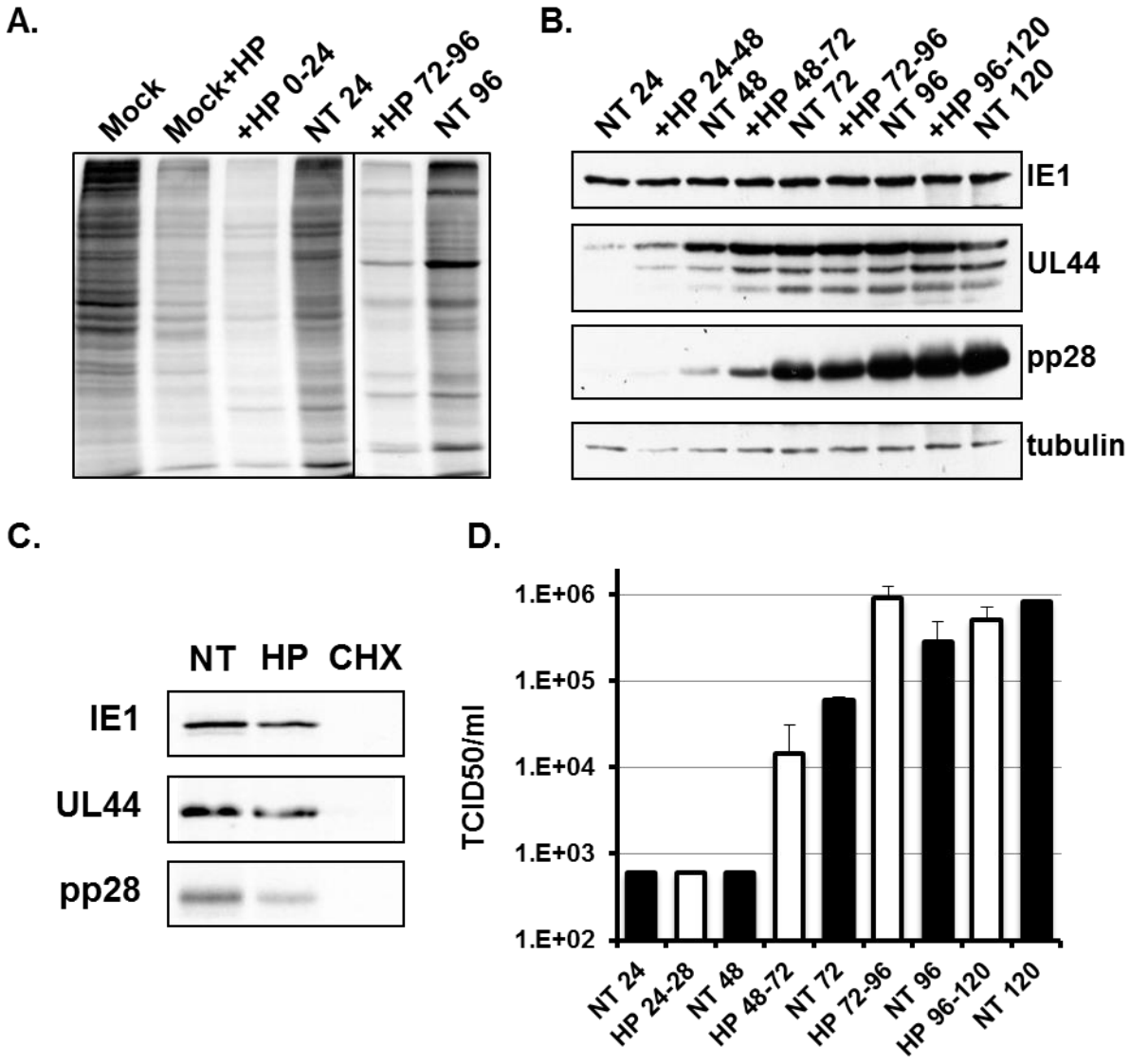


Figure 4 HCMV replication and protein synthesis becomes increasingly resistant to inhibition by hippuristanol as infection progresses. (A) HFFs were left uninfected (Mock) or infected with HCMV at a multiplicity of three. Cultures were treated with 100 nM hippuristanol for the indicated 24 hour period. Nascent proteins were metabolically labeled during the final thirty minutes of drug treatment and visualized by autoradiography (n=3). (B) Cells were infected and treated as in A. Cells were harvested at the indicated times and viral protein expression was measured by Western blot (n=3). (C) Cells were infected as in A. Eighty hours post infection

cells were treated with either hippuristanol or cycloheximide. Nascent proteins were metabolically labeled during the final hour minutes of the drug treatments, and the indicated viral proteins were immunoprecipitated from the lysates and visualized by autoradiography (n=2). (D) HFFs were infected as in A. At the indicated times hippuristanol (HP, 100 nM) was added to the cultures. Cell free virus present at the end of the 24 hippuristanol treatment (open bars) was quantified by the TCID50 assay. Supernatants from untreated cultures (NT, black bars) were harvested and titered at the indicated times (n=3).

As part of the eIF4F complex the eIF4A helicase resolves secondary structures in the 5' UTR of mRNAs that might otherwise impede ribosome scanning (15, 36). A possible explanation for the translation of viral mRNAs when eIF4A is inhibited could be that viral mRNAs lack extensive secondary structure in their 5'UTRs and therefore do not require the eIF4A helicase activity for their mRNA translation. However assembly of the eIF4F complex might still be required to recruit 40S ribosomal subunits to viral mRNAs, as the eIF4G subunit bridges interactions between eIF4F and the 43S pre-initiation complex. We therefore determined the impact of eIF4F disruption on HCMV mRNA translation. We used the mTOR inhibitor Torin1, a specific ATP-competitive inhibitor of mTOR kinase activity, to decrease eIF4F abundance in infected cells (41). We focused on the late stage of infection as our data from hippuristanol-treated cells suggested a minimal requirement for eIF4F activity for the synthesis of viral proteins during this stage of infection. Torin1 treatment decreased the amount of eIF4G that co-purified with m7G-sepharose while conversely increasing the binding of the translational repressor 4EBP1 (figure 5A) demonstrating that Torin1 disrupted the eIF4F complex during the late stage of infection. Torin1 also limited nascent protein synthesis in both infected and uninfected cells. Similar to the results in hippuristanol-treated cells, the synthesis of a subset of infected cell specific proteins appeared to be less affected by Torin1 (figure 5B). We also found that polysome formation in infected cells was resistant to the effects of Torin1, while Torin1

treatment diminished polysomes abundance in uninfected cells (figure 5C). Our results show that a subset of mRNAs efficiently recruits ribosomes in infected cells despite significant disruption of the eIF4F complex.

Figure 5

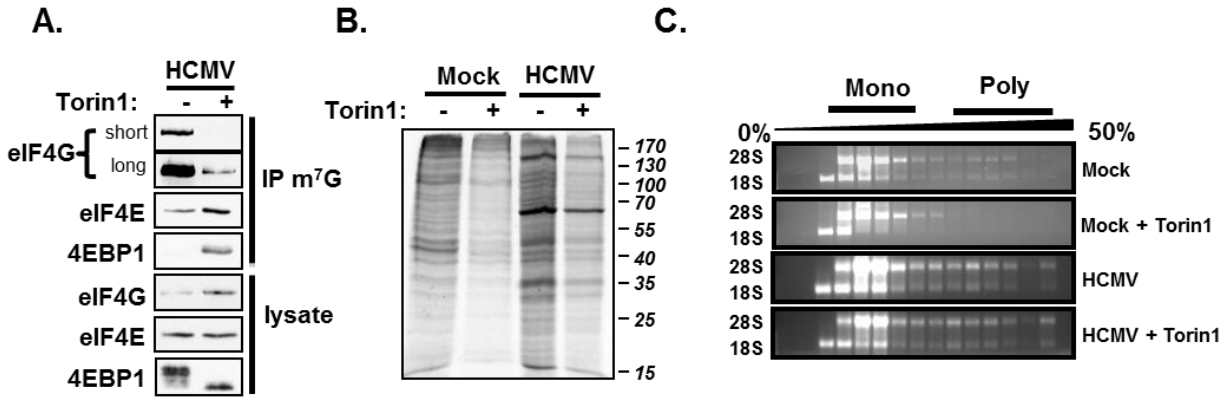


Figure 5 Effect of eIF4F disruption on protein synthesis in HCMV infected cells during the late stage of infection. (A) Eighty hours post infection (MOI of 3) cells were treated with Torin1 (250 nM) for sixteen hours or left untreated. The integrity of the eIF4F complex was measured using m7G sepharose affinity purification. (B) Same as in A, except nascent proteins were metabolically labeled during the final thirty minutes of Torin1 treatment and visualized by autoradiography (C) Same as A, except cytoplasmic lysates were resolved through 10-50% sucrose gradients to separate ribosomal subunits, monosomes, and polysomes. The location of ribosomes in the gradient was monitored by visualizing rRNA isolated from each fraction on agarose gels.

We next determined the effect of eIF4F disruption on the translation of host mRNAs during the late stage of infection. Cytoplasmic extracts from control or Torin1-treated infected cells were resolved through sucrose density gradients to separate ribosomal subunits, monosomes, and polysomes. The relative abundance of specific host mRNAs in each gradient fraction was measured by qRT-PCR. If the recruitment of ribosomes to the mRNA is dependent

on the abundance of eIF4F complex, then Torin1 treatment should result in a shift of mRNAs from the polysomes-containing fractions to the lighter, monosome-containing fractions of the gradient. Conversely if ribosomes bind the mRNA equally as well when eIF4F is disrupted, the distribution of the mRNA in the gradient should be unaffected by Torin1. Importantly this assay only measures the distribution of cytoplasmic mRNAs across the gradient, allowing us to normalize for any potential effects of the mTOR inhibitor on mRNA transcription, processing, or nuclear export.

Using this approach we determined the extent of polysome association for three host mRNAs previously shown to require the eIF4F complex for their efficient translation (18, 40). Figure 6A shows that the host HSP90 and GNB2L1 transcripts are efficiently translated in infected cells as determined by their relative abundance in polysome-containing fractions (fractions 11-14). While the rps20 mRNA is less efficiently translated than either of the above, rps20 transcripts were clearly present in polysomes. Treating infected cells with Torin1 decreased the association of each of the host mRNAs with polysomes, with a corresponding increase in the amount of each mRNA in the monosome-containing fractions (figure 6A). Torin1 decreased the steady state levels of the HSP90 and GNB2L1 proteins (figure 6B) demonstrating that changes in polysomes association are reflective of changes in protein abundance. We also measured the rate of incorporation of radiolabeled amino acids into nascent HSP90 and GNB2L1 protein during the final hour of Torin1 treatment. Fewer radiolabeled amino acids were incorporated into nascent HSP90 or GNB2L1 proteins in the presence of Torin1, confirming that their translation was inhibited (figure 6C). These results show that the degree of eIF4F disruption achieved with Torin1 is sufficient to inhibit the translation of eIF4F-dependent mRNAs. We conclude that host eIF4F-dependent mRNAs continue to require eIF4F for their translation during HCMV infection.

Figure 6

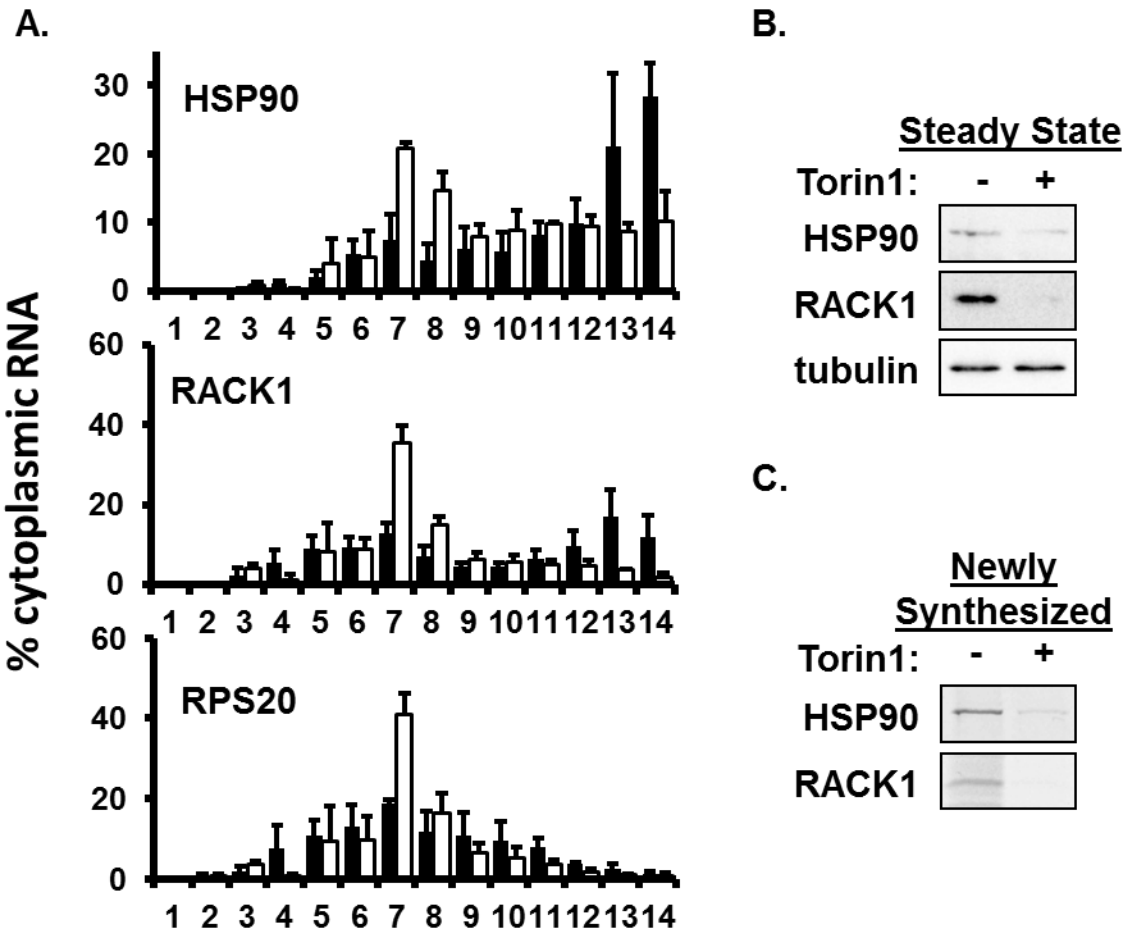
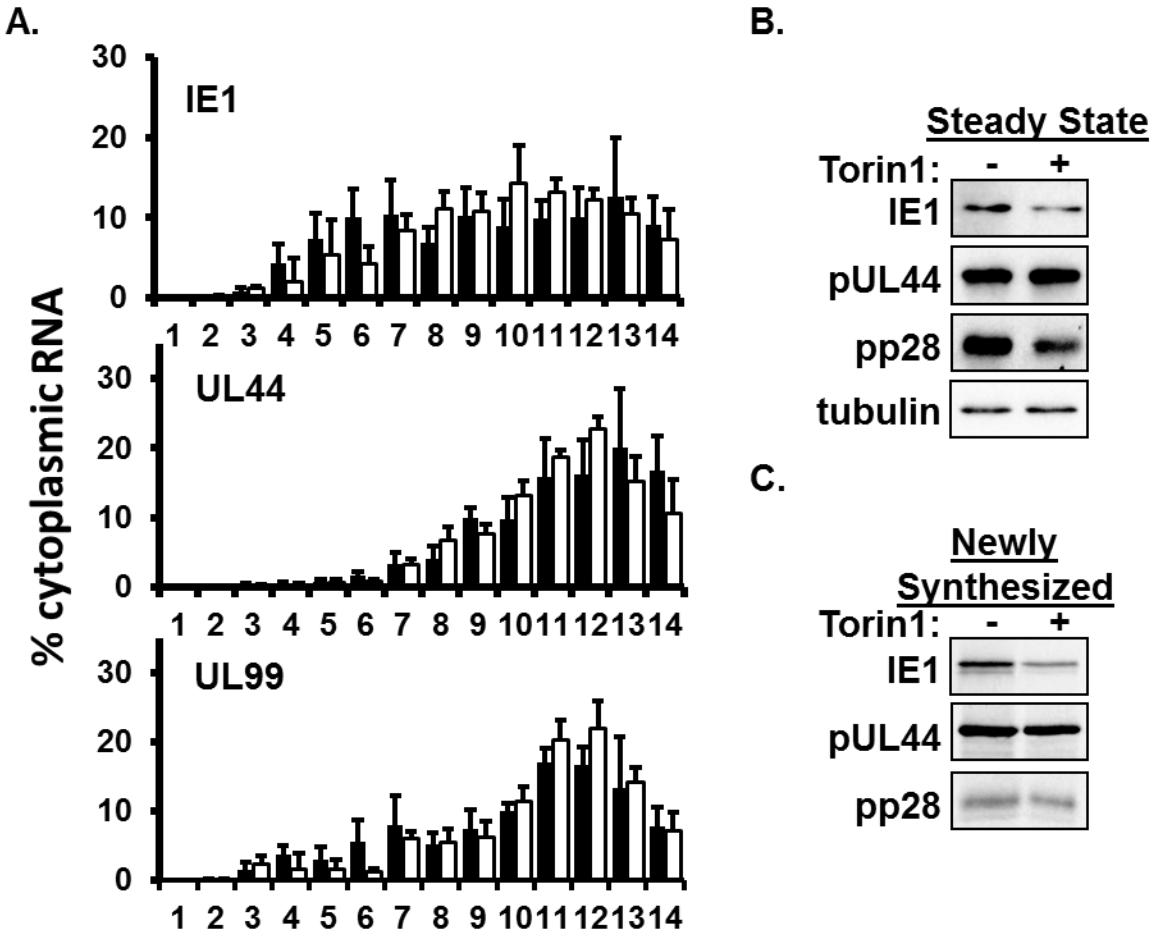


Figure 6 eIF4F disruption limits the association of host mRNAs with polysomes. (A) HFFs were infected and treated as in figure 5. At 96 hpi cytoplasmic extracts were resolved through sucrose density gradients. The abundance of the indicated mRNAs in each gradient fraction was determined by qRT-PCR. The percent of the total RNA in the gradient in each fraction is shown. (closed bars = untreated, open bars = Torin1 treated; n=3) (B) Cells were treated as in A. Steady state protein levels were measured by Western blot. (n=3) (C) Cells were treated as in A. Nascent proteins were metabolically labeled during the final thirty minutes of the assay. Immune complexes specific for the indicated proteins were visualized by autoradiography. The results of a representative experiment (n=3) are shown in panels B and C.

We next determined the effect of eIF4F disruption on the translation of viral mRNAs. We found representative viral mRNAs from each kinetic class associated with polysomes as efficiently in Torin1 treated cells as in untreated cells (figure 7A). The steady state levels of each viral protein were minimally affected by Torin1 treatment as determined by Western blot (figure 7B). Similarly, the rate of nascent protein synthesis for each viral protein was unaffected by Torin1 treatment (figure 7C). A potential caveat of this approach stems from the fact that HCMV mRNAs are packaged into the tegument of virions. Therefore the migration of viral mRNAs in the gradient could reflect their association with virions rather than polysomes. However we found that the inclusion of EDTA in lysis buffer, which disrupts polysomes (ref (2) and figure S1), shifted both the IE1 and UL99 mRNAs to lighter fractions of the gradient. Together these data show that substantial disruption of the eIF4F complex does not inhibit the recruitment of ribosomes to HCMV mRNAs.

Figure 7 eIF4F disruption does not affect the association of viral mRNAs with polysomes late in infection. (A) HFFs were infected and treated as in figure 5. The distribution of viral mRNAs across a sucrose gradient was determined by qRT-PCR as in figure 6A (n=3) (B) Cells were treated as in figure 6B. Steady state protein levels were measured by Western blot. (n=3) (C) Cells were treated as in figure 6C. Nascent proteins were metabolically labeled during the final thirty minutes of the assay. Immune complexes specific for the indicated proteins were

Figure 7



visualized by autoradiography. (n=3) The results of a representative experiment (n=3) are shown in panels B and C.

Several potential explanations existed for the differential effect of eIF4F disruption on host and viral mRNA translation. We considered the hypothesis that the viral mRNAs examined lacked sufficient 5'UTR structure to require eIF4F activity for their translation. In vitro studies have shown that mRNAs lacking stable secondary structure have a minimal requirement for eIF4F for their translation (39). The 5'UTR for each of the examined viral mRNAs has previously been mapped. In each case the free energy constraints of the viral 5'UTRs would predict that eIF4F was needed for their efficient translation (figure S2A). Another possibility was that HCMV

mRNAs are not capped and therefore would not require the eIF4F complex for their translation. Surprisingly we were unable to find any information concerning the presence of an m7G mRNA cap on either the UL44 or UL99 mRNAs. We therefore used tobacco acid pyrophosphatase (TAP)-mediated RNA ligation to test for the presence of an m7G mRNA cap on the viral mRNAs (see Materials and Methods). We found that polysome-associated mRNAs for both viral genes were capped and therefore have the potential to interact with eIF4F (figure S2A).

Based on these results we performed a global analysis of the effect of eIF4F disruption on the translation of host and viral mRNAs. For each mRNA we determined the translation efficiency, which is the ratio of mRNA abundance in the polysome compared to the total RNA sample. We then compared the translation efficiency for each mRNA in untreated cells and Torin1 treated cells. In these experiments we used a custom microarray containing oligonucleotide probes specific for all annotated HCMV ORFs as well as 44,000 human genes. Table S1 lists host mRNAs with statistically significant changes in their translation efficiency in the presence of Torin1. We identified over 340 host mRNAs whose translation efficiency was decreased by greater than 40% in Torin1 treated cells. The results of our analysis of host mRNAs were similar to those described previously (18, 40). For example we found that Torin1 inhibited the translation of mRNAs encoding ribosomal proteins (figure 8A), which are translated in an eIF4F-dependent manner. We found that several host mRNAs previously shown to require eIF4F for their translation during HCMV infection were translated less efficiently in the presence of Torin1 (35).

In contrast the relative translation efficiency of HCMV mRNAs was for the most part similar in untreated and Torin1 treated cells (figure 8B and Table S2). Torin1 treatment resulted in a statistically significant decrease in the translation of only one viral mRNA (UL17). In addition the translation of four viral mRNAs (UL34, UL25, US1, and UL99) showed a statistically significant increase in translation efficiency in Torin1 treated cells. Torin1 treatment did not have

a significant impact on the translation of the remaining 98 viral genes measured. The array data were confirmed by qRT-PCR for several viral mRNAs (figure S3) using monosome and polysome fractions isolated from two additional experiments. No signal was obtained from either step when reverse transcriptase step was omitted, indicating the absence of DNA contamination (data not shown). These results further demonstrate that the Torin1 treatment used in these experiments is sufficient to inhibit the translation of host mRNAs that utilize the eIF4F complex. However the translation of HCMV mRNAs as a group does not correlate with the abundance or activity of the eIF4F complex.

Figure 8

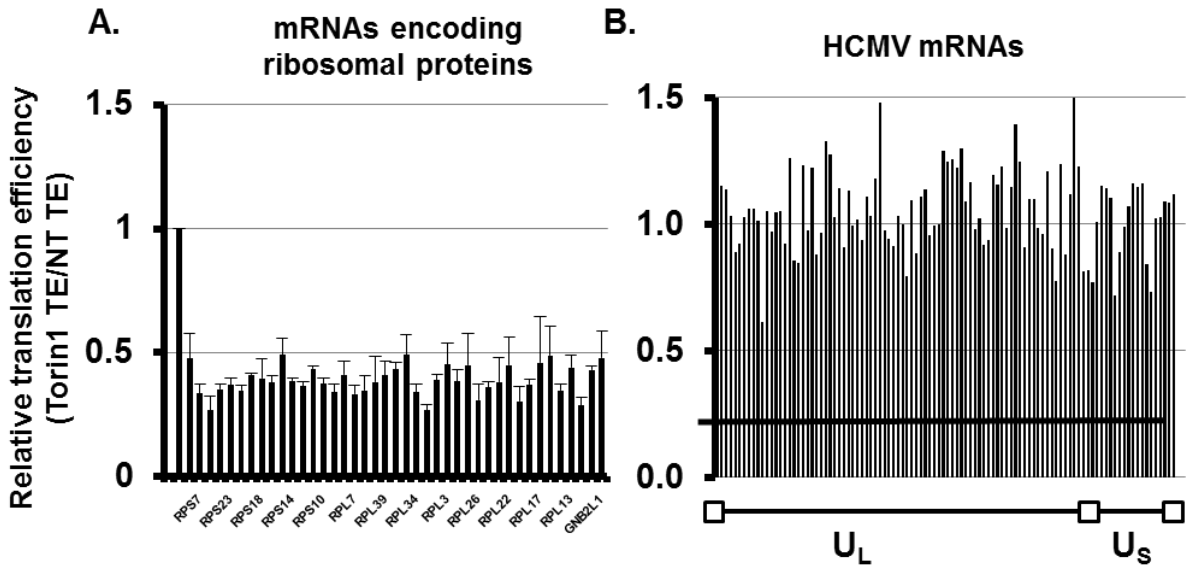


Figure 8 Global analysis of the effect of eIF4F disruption on the translation efficiency of host and viral mRNAs. (A) HFFs were infected and treated as in figure 5. Polysome-associated and total RNA were isolated from infected cells treated with Torin1 or left untreated. The translation efficiency of each mRNA was calculated by determining the ratio of the mRNA in the polysome and total fractions by microarray analysis. The graph shows the ratio of the translation efficiency (TE) in Torin1 treated cells to that in untreated cells. (n=3) mRNAs translated as efficiently in both conditions have a value of 1. Values <1 indicate that translation was inhibited in the

presence of Torin1. Panel A shows the relative translation efficiency of representative mRNAs encoding ribosomal proteins in Torin1 treated cells. (B) The relative translation efficiency of HCMV mRNAs in Torin1-treated cells is shown. (n=3) The translation efficiency of both host and viral mRNAs in untreated and Torin1 treated cells is given in Supplemental Tables S1 and S2.

Discussion

In this study we measured the requirement for the host eIF4F translation initiation complex for the translation of host and viral mRNAs during HCMV infection. Our results demonstrate a differential requirement for eIF4F in the translation of host and viral mRNAs. We find that eIF4F is required at the start of infection for efficient progression through the viral lytic cycle. However as infection progresses both viral protein synthesis

and replication become increasingly insensitive to inhibition or disruption of the eIF4F complex. In contrast host eIF4F-dependent mRNAs continue to require eIF4F for their translation in infected cells. Our results therefore reveal a fundamental difference between host and HCMV mRNAs in their requirement for host translation factors.

We found that inhibiting or depleting the eIF4A subunit of the eIF4F complex from the start of infection inhibited progression through the HCMV lytic cycle. Specifically inhibition of eIF4A from the start of infection prevented viral DNA replication. These data are consistent with previous studies showing that inhibition of the mTOR kinase with Torin1 from the start of infection limits HCMV DNA accumulation and replication. Precisely how eIF4F contributes to viral DNA replication is currently unclear. Seven HCMV proteins are required for viral DNA replication in vitro (34). Perhaps one or more of these viral factors is dependent on eIF4F activity for their synthesis prior to viral DNA replication. This is consistent with our finding that pUL44 expression is delayed and reduced when eIF4A is depleted or inhibited at the start of infection despite the efficient expression on an IE protein. Alternatively eIF4F activity might be

required for the expression of a host protein needed for viral DNA replication or for the expression of viral DNA replication proteins. Recent studies have shown that eIF4F is required for the translation of several host metabolic enzymes (18). As HCMV infection remodels host metabolism (6, 24, 29, 30, 38), perhaps the increased eIF4F abundance and activity in infected cells promotes the expression of metabolic enzymes needed for virus replication.

Of particular interest is our finding that eIF4A activity is not required for the efficient translation of viral mRNAs during the later stages of infection. One explanation could be that the viral mRNAs examined have minimal structure in their 5' UTRs. In this case 40S ribosomal subunits would be capable of scanning viral 5'UTRs without a requirement for helicase activity. However the 5'UTRs of the representative viral mRNAs examined in this study have been defined and are predicted to have sufficient structure to impede ribosome scanning (figure S2B), suggesting that a helicase is required for their translation. While our data demonstrate a minimal requirement for eIF4A activity, perhaps another RNA helicase is required for the resolution of secondary structures in viral mRNAs. Human cells encode sixty four potential RNA helicases and HCMV itself encodes a putative RNA helicase (42). If indeed an alternative helicase contributes to viral mRNA translation, our data suggests that this helicase is directed specifically to viral mRNAs as total protein synthesis remains significantly dependent on eIF4A activity in infected cells.

Perhaps the most surprising finding was that ribosomes efficiently associate with HCMV mRNAs despite significant disruption of the eIF4F complex. Early in infection expression of the HCMV immediate early protein was resistant to eIF4A inhibition, while later in infection most mRNAs efficiently associated with ribosomes despite eIF4F disruption. In a stochastic model in which eIF4F binds to mRNA in a sequence-independent manner, one would expect that the translation of all mRNAs would be equally affected by disruption of the eIF4F complex. Our results demonstrating the differential effects of eIF4F inhibition on host and viral mRNAs argue

against this model. However if eIF4F preferentially associated with a subset of mRNAs in a sequence-specific manner, disruption of the eIF4F complex should preferentially affect the translation of that subset of mRNAs. In fact two recent studies found that the eIF4F complex is most important for the translation of host mRNAs containing a tract of pyrimidines (TOP) or pyrimidine rich motif (PRTE) immediately adjacent to the 5' mRNA cap (18, 40). Our microarray analysis of host mRNA translation closely matches the results of these previous studies. The translation of host mRNAs containing TOP motifs (e.g. ribosomal mRNAs) was preferentially inhibited by eIF4F disruption. Analysis of the 5'UTRs of the representative viral mRNAs studied herein did not reveal TOP or PRTE-like motifs, consistent with a minimal role for the eIF4F complex in their translation. Furthermore, examination of additional HCMV mRNAs with defined transcript structure did not reveal the presence of these motifs (unpublished observations). However the 5'UTRs for the majority of HCMV mRNAs have not been defined. A more comprehensive understanding of HCMV message structure is needed to globally assess a role for cryptic TOP motifs in HCMV mRNA translation.

Our results raise the question of how ribosomes are recruited to HCMV mRNAs when eIF4F activity is inhibited. While Torin1 treatment resulted in sufficient eIF4F disruption to limit host mRNA translation, some residual eIF4F complex was still present. Therefore one explanation could be that a host or viral factor preferentially recruits the residual eIF4F complex to HCMV mRNAs. An example might be found in the herpes simplex virus (HSV) ICP6 protein, which stimulates eIF4F formation (43). Perhaps a viral protein specifically recruits any remaining eIF4F to viral mRNAs. Alternatively a protein or protein complex expressed during infection might limit the association of the 4EBP1 translation repressor with viral mRNAs. To this end the HCMV UL69 protein (pUL69) binds to m7G mRNA cap and associates with viral mRNAs. While infection with wild type HCMV limits 4EBP1 binding to the mRNA cap, 4EBP1 robustly binds m7G sepharose in cells infected with a pUL69 mutant virus (1). Perhaps pUL69 specifically

inhibits 4EBP1 binding to eIF4E-associated viral mRNAs resulting in the preferential accumulation of eIF4F on viral messages. The interaction of pUL69 with poly A binding protein (PABP) further suggests a role for pUL69 in viral mRNA translation. In uninfected cells PABP promotes translation initiation via its interaction with eIF4F, thereby promoting the formation of the “closed loop” (20). In addition PABP is required for efficient HCMV replication (25). pUL69 could bridge the association of PABP with the mRNA cap on viral transcripts when eIF4F is limiting. However in both of the above scenarios some mechanism must exist to discriminate between host and viral messages, as the degree of eIF4F disruption obtained in our experiments was sufficient to limit the translation of host eIF4F-dependent mRNAs.

It is also possible that the recruitment of ribosomes to viral mRNAs does not require the eIF4F complex. Many viruses limit the abundance of the eIF4F complex while efficiently synthesizing viral proteins. For example many RNA viruses encode proteases that cleave eIF4G and inactivate the eIF4F complex (44). Viruses that inactivate eIF4F often rely on an alternative suite of translation initiation factors (10). Host cells also utilize alternative translation initiation complexes, most notably the cap binding complex (CBC) (23). Like eIF4F, the CBC binds to the m7G cap and can recruit ribosomes to facilitate mRNA translation. Unlike eIF4F, the CBC does not require the eIF4E protein and is therefore insensitive to inhibition by 4EBP1 (7). Perhaps viral mRNAs utilize the CBC to recruit ribosomes to viral mRNAs when eIF4F activity is limiting. Equally plausible is the existence of an HCMV-encoded protein or protein complex that facilitates the recruitment of ribosomes to viral transcripts. For example the N protein of bunyavirus is capable of replacing the entire eIF4F complex in the translation of viral mRNAs (33). If viral proteins contribute to HCMV mRNA translation, these factors would be excellent candidates for novel antiviral therapeutics, as they would specifically limit viral protein synthesis while leaving host protein synthesis intact. Clearly additional studies are needed to further define the complement of viral and/or host factors that govern HCMV protein synthesis.

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Chapter 3: Human cytomegalovirus TRS1 protein associates with the 7-methylguanosine mRNA cap and facilitates translation[1]

Introduction

The regulation of mRNA translation is a critical interface between viruses and the infected host cell. Viral mRNAs are completely reliant on host machinery for the synthesis of viral proteins, as viruses require host ribosomes to translate their mRNAs. The infected cell senses the presence of viral RNAs and activates signaling pathways that attempt to suppress viral protein expression. At the same time the cell must translate antiviral proteins to limit virus replication. The interface of viral mRNAs with the host translation machinery is therefore a fundamental aspect of the host: pathogen relationship.

Most eukaryotic mRNAs initiate translation through the ordered assembly of translation initiation factors on their 5' terminus [1, 2]. The eIF4F complex binds to the 7-methylguanosine (m^7G) mRNA cap and recruits 40S ribosomal subunits to the 5' end of the transcript [3]. Bound 40S subunits then scan the 5' untranslated region (5'UTR) of the mRNA until reaching the translation initiation codon, where the eIF2 complex pairs a charged methionine tRNA to the AUG initiation codon triggering ribosome assembly and translation elongation. Multiple regulatory cues govern the initiation phase, which is the

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[1]This Chapter previously appeared as an article in Proteomics. The original citation is as follows: Ziehr, B., Lenarcic, E., Vincent, H. A., Cecil, C., Garcia, B., Shenk, T. and Moorman, N. J. 2015. Human cytomegalovirus TRS1 protein associates with the 7-methylguanosine mRNA cap and facilitates translation. Proteomics, 15: 1983–1994. doi: 10.1002/pmic.201400616. Supplemental data available online through link above.

rate-limiting step in protein synthesis. The mTOR kinase promotes translation initiation by

antagonizing the translation repressor 4EBP1, which blocks assembly of the host eIF4F translation initiation complex [4]. The phosphorylation of the eIF2α subunit of the eIF2 complex is an additional regulatory step in translation initiation. Cell stressors including ER stress, nutrient deprivation or viral infection activate kinases that phosphorylate and inactivate eIF2α, which inhibits its ability to recycle GDP to GTP and thus blocks its ability to participate in further rounds of translation initiation [5]. Together these regulatory steps ensure tight control of mRNA translation that is matched to the physiological state of the cell.

Viral infection induces cell stress responses that would limit mRNA translation if left unchecked. Not surprisingly, viruses have evolved mechanisms to counteract these stress responses to ensure efficient viral mRNA translation. Human cytomegalovirus (HCMV) encodes proteins that ensure eIF4F and eIF2α remain active throughout infection. HCMV pUL38 antagonizes the tuberous sclerosis complex (TSC), preventing it from suppressing mTOR activity and limiting eIF4F abundance [6]. HCMV pTRS1 prevents activation of the antiviral eIF2α kinase PKR during infection with a heterologous virus [7-10]. As a result, host translation initiation factor activity and host protein synthesis are maintained during HCMV infection.

It is also likely that HCMV encodes additional proteins that regulate translation initiation. While HCMV stimulates the accumulation of the eIF4F complex in infected cells [11, 12], the role of eIF4F in viral protein synthesis is unclear. mTOR activity is required for ongoing host protein synthesis during HCMV infection. However during the late stage of infection, HCMV protein synthesis and viral replication are minimally affected by disruption or inhibition of the eIF4F complex [13, 14]. HCMV protein synthesis similarly becomes resistant to inhibition by stressors that inactivate eIF2α late in infection [15, 16]. Infection strongly activates the eIF2α kinase PERK, yet eIF2α is minimally phosphorylated and translation is maintained. These results suggest that, at least late in infection, viral mRNAs can preferentially recruit residual

active eIF4F or eIF2α, or that an alternative set of host or viral factors acts to promote viral protein synthesis.

In this study we focused on identifying HCMV proteins that regulate mRNA translation. Using a proteomics based approach we found that the HCMV pTRS1 and pIRS1 proteins associate with the mRNA cap in infected cells. pTRS1 association with the mRNA cap did not require additional viral proteins or the host eIF4F translation initiation complex. We find that pTRS1 associated with actively initiating mRNAs during infection, and increased the translation of reporter genes outside of infection. While pTRS1 generally enhanced translation, it preferentially stimulated the translation of mRNAs containing specific sequences or features. Our results suggest that the preferential stimulation of translation was dependent on the ability of pTRS1 to inhibit PKR. However, pTRS1 was sufficient to increase translation in cells lacking PKR, and thus pTRS1 also enhances translation in a PKR-independent manner. Our data suggests that pTRS1 expression may provide a mechanism to ensure that viral transcripts are efficiently translated under conditions that limit the activity of the host translation machinery while also limiting the host antiviral response.

Materials and Methods

Cells, Viruses and Plasmids Primary human foreskin fibroblasts (HFFs), passages 5-14, were cultured in DMEM containing 10% newborn calf serum. HeLa cells were cultured in DMEM containing 10% fetal bovine serum. The BAC-derived BAD*in*GFP strain of HCMV was used as the wild-type virus in these studies [17]. The BAD*in*TRS1GFP and BADinIRS1GFP variants were made using lambda/red-mediated recombineering using the BAD*wt* BAC as the parental strain using methods described previously [6]. The mutants express either pTRS1 or pIRS1, respectively, with a C-terminal GFP fusion from their endogenous locations in the HCMV genome under the control of their native promoters, and both replicate with the same kinetics as its parental strain. Viruses were propagated and titered by the TCID50 method in HFFs.

pTRS1 expression plasmids were generously provided by A. Geballe (Univ. of Washington), and have been previously described [8, 9]. The full length or truncated UL99 5'UTR (nucleotides 144,872 to 145,348 or 145,115 to 145,348, respectively) was amplified by PCR from the HCMV AD169 genome (GenBank FJ527563.1) using primers containing HindIII or NotI sites flanking the viral sequence (UL99FL-F 5' GATCATCAAGCTTGACGCCGCTGGCGGCGGCGCTGATC 3'. UL99trunc-F 5' GATCATCAAGCTTATTTCCGCGACCTGCCTACCGTC 3', UL99FL-R 5' GATGATCCCATGGATCGGTAGGTTCGTCTTGCG 3'). The resulting PCR products were cloned into the HindIII and NotI sites of the pGL3 Control vector (Promega), and sequence verified. The UL44 5'UTR was PCR amplified and cloned using the following primers: UL44F 5' GATCATCAAGCTTGGCTCGGCGCGGCTGTATTATTAG 3', UL44R 5' GATGATCCCATGGCCCGGACAGCGTGCAAGTCTC 3'. The IE1 5'UTR was amplified from infected cell cDNA using the following primers: IE1F 5' GGAGGCCTAGGCTTTTGCAAAACAGATCGCCTGGAGACGCCATC 3', IE1R 5' TTATGTTTTTGGCGTCTTCCATCGTGTCAAGGACGGTGAGTCAC 3'.The PCR products was cloned by Gibson assembly into the Hind III and NotI sites of the pGL3-Control vector. The GAPDH 5' UTR (accession# NM_002046.4) was synthesized (IDT) and cloned into the HindIII and NcoI sites of the pGL3-Control vector using Gibson assembly. Two oligonucleotides each containing half of the actin UTR (accession # NM_001101.3) were annealed and extended with Klenow to generate double stranded DNA, and then cloned into the HindIII and NcoI sites of pGL3-Control by Gibson cloning. The tubulin 5'UTR (accession # NM_178014.2) construct was generated in the same manner. All constructs were sequenced verified.

Analysis of m^{7}G-Sepharose-Bound Proteins HFFs were infected with BAD*in*GFP virus at a multiplicity of 3 IU/cell. The affinity purification of proteins associated with the m^{7}G sepharose was performed similarly to that described in ref [18] with the following modifications. Cell lysates were prepared at 72 hours after infection and incubated with m^{7}G-sepharose for 1

hour in cap binding buffer (40mM HEPES, pH 7.6; 120mM NaCl; 1mM EDTA; 0.3% CHAPS). The beads were washed at room temperature three times in cap binding buffer, once in cap binding buffer containing 500 mM KCl and then incubated in elution buffer (100 □M m^7GTP, 100 mM KCl, 1 mM DTT, 50 mM HEPES, pH 7.6) for 1 hour to release proteins from the beads. Eluted proteins were reduced with DTT (10mM) and alkylated with iodoacetamide. The samples were then digested with trypsin (Promega), and desalted and purified on Zip-Tip C18 columns (Millipore). The peptides were then separated by reverse phase nanospray liquid chromatography on C18 resin with an Agilent 1200 series HPLC. Mass spectrometry was performed on a LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific). Protein identification was performed using Protein Discoverer software version 1.4 with the MASCOT or SEQUEST search algorithms against the most current version of the Uniprot Human database. A custom HCMV reference proteome was derived from the HCMV AD169 genome (Genbank # FJ527563). We allowed for 2 missed cleavages. Precursor mass tolerance was set to 10 ppm, and fragment mass tolerance to 0.6 Da. Dynamic modifications included oxidized methionine and carbamidomethylated cysteine. Target FDR was set to 0.05. To confirm the specificity we performed our capture with sepharose only (no m^7GTP) beads and identified co-purifying proteins by mass spectrometry as above. Proteins recovered from sepharose only beads that were also purified with m^7G-sepharose (Fig. S5) were considered likely contaminants, and are denoted as such in Table S1.

A similar approach was used to measure the association of specific host and viral proteins with the m^7G mRNA cap. Transfected or infected cells lysates or partially purified pTRS1 was incubated with m^7G agarose (Jena Bioscience #AC-155S) as above, and the bound proteins were analyzed by Western blot.

Partial Purification of pTRS1 293T cells were transfected with his epitope-tagged pTRS1 expression vector and harvested 48 hours after transfection. Cells were lysed in cap binding buffer (above) containing 5 mM imidazole and treated with micrococcal nuclease.

pTRS1 was captured from the lysates using His-Select Affinity Gel (Sigma #114K70151) and eluted with 250mM imidazole in cap binding buffer. Protein concentration was determined using a BCA assay (Pierce). pTRS1 (5 pmol) in cap binding buffer was used in the m^7GTP agarose-binding assay.

Luciferase Assays HeLa cells in 12 well dishes were transfected with 0.5 µg of the luciferase reporter together with the indicated concentrations of the TRS1 expression plasmid. Luciferase activity was measured in a luminometer (Molecular Devices) at 24 hours after transfection. Luciferase activity was normalized to the protein content of the lysate as determined by Bradford assay. In each case the results are compiled from at least three independent experiments.

RNA Quantification Total RNA was extracted using Trizol reagent. DNase-treated RNA was reverse transcribed as described previously [19]. Quantitative real-time PCR (qRT-PCR) was performed to assess changes in luciferase mRNA abundance using the following primers: luciferase 5'-ACAAAGGCTATCAGGTGGCT-3', 5'-CGTGCTCCAAAACAACAACG-3'; GAPDH 5'-CTGTTGCTGTAGCCAAATTCGT-3', 5'-ACCCACTCCTCCACCTTTGAC-3'. The abundance of luciferase RNA was determined by the ΔΔCt method using GAPDH as the reference transcript as previously described [20].

Protein Analyses For metabolic labeling of nascent proteins, cells were incubated with 125 µCi ^{35}S-labeled amino acids (EasyTag Express; GE Health Sciences) for 30 minutes in methionine- and cysteine-free media at 24 h post transfection. The amount of radioactivity incorporated into TCA-insoluble protein was quantified using a scintillation counter and normalized to the protein concentration in the sample as determined by the Bradford assay (Sigma-Aldrich).

For immunofluorescence analysis, HFFs were infected with the indicated virus at a multiplicity of 0.5 IU/cell, and 72 hours later cells were fixed and stained as described previously [6]. In some cases cells were treated with thapsigargin (1 µM) or sodium arsenite (0.5 mM) for 1

hour prior to fixation to induce the formation of stress granules [21]. Images were captured on a Zeiss confocal microscope. Antibodies specific for G3BP1 (BD Biosciences), eIF4E, eF4G, or eIF4A (Cell Signaling; 1:100 dilution) and DAPI were used to visualize stress granules, translation initiation factors and nuclei, respectively.

For Western blot assays, equal amounts of protein were analyzed as described previously [22] using antibodies specific to the following antigens: his epitope tag, eIF4E, eIF4G, 4EBP1, and PABP1 (Cell Signaling) at 1:1000 dilution; PKR (Santa Cruz), PKR phospho-T446 (Epitomics), and GFP (Roche) at 1:1000 dilution; pTRS1 [23] at 1:100 dilution.

Velocity Sedimentation Analysis of Ribosomal Subunits Cytoplasmic extracts from pTRS1-GFP infected cells (multiplicity of 3 IU/cell; 72 hpi) were treated with puromycin for 1 hour prior to harvest. The cells were lysed in polysome lysis buffer [14] and the extracts resolved on 5-20% linear sucrose gradients. The proteins in a portion of each fraction were precipitated with TCA and analyzed by Western blot. The gradient was manually fractionated from the top, and total RNA was extracted from a portion of each fraction. RNA from each fraction was resolved by electrophoresis on non-denaturing 2% agarose gels to visualize the distribution of ribosomal RNA.

Depletion or Disruption of PKR HeLa cells were transduced with lentivirus expressing PKR shRNAs (TRCN0000001380 and TRCN0000001382, PKR #1 and #2 respectively). Lentivirus stocks were generated by co-transfecting 293T cells with the TRCN expression vector (obtained from the UNC Lentivirus Core Facility) and packaging plasmids as previously described [14]. HeLa cells were transduced in the presence of polybrene (5μg/ml). shRNA expressing cells were selected with puromycin (1μg/mL) and PKR knockdown was evaluated by western blot analysis.

pLX-sgRNA and pCW-Cas9 [24] vectors were obtained from Addgene. HeLa cells were transduced with pCW-Cas9 lentivirus and then clonally selected in the presence of puromycin (1μg/mL) to create a stable HeLa line expressing Cas9 under the inducible control of the Tet ON

promoter (HeLa-Cas9). Oligonucleotides containing PKR-specific guide RNAs were (ref[24], 5' TTCAGCAGGTTTCTTCATGGAGG 3'; PAM motif underlined) cloned into pLX-sgRNA in the place of the AAVS1 target sequence to create pLX-sgPKR. pLX-sgPKR was transfected into HeLa-Cas9 and the cells were grown and passaged in the presence of doxycycline (1µg/ml) and blasticidin (1µg/ml). The Surveyor Assay (Transgenomic #706025) was used to confirm the introduction of mutations into the PKR gene. The cells were clonally selected by limiting dilution, and loss of PKR expression in individual clones was determined by Western blot.

Results

HCMV pTRS1 binds to the 5' mRNA cap To identify viral factors that might regulate protein synthesis, we used a mass spectrometry based approach to identify proteins co-purifying with m^7G sepharose in HCMV-infected cells. A stringent washing protocol was used to minimize false positive interactions. Elution of the bound proteins with free m^7GTP added an additional degree of specificity to the purification protocol. As a further specificity control, we also identified host proteins that co-purify with sepharose beads without m^7GTP. Very few proteins purified with the beads in the absence of m^7GTP (Fig. S5). Proteins co-purifying with sepharose only beads are listed as likely contaminants in Table S1. Mass spectrometry identified many host proteins previously shown to associate with the mRNA cap including components of the eIF4F translation initiation complex, the cap binding complex (CBC), and gemin 5 [25-27] (Table S1). In addition peptides from two HCMV proteins were identified with high confidence, pTRS1 and pIRS1.

We confirmed the capture of both pTRS1 and pIRS1 on m^7G sepharose from infected cell lysates by Western blot assay. pTRS1 and pIRS1 bound to the m^7G resin throughout a time course of infection (Fig. 1*A*). pTRS1 and pIRS1 are identical in their first two thirds and appear to be functionally redundant [7, 9, 10], therefore we chose to focus our work on pTRS1. Free m^7GTP inhibited the retention of pTRS1 on the m^7G resin (Fig. 1*B*), demonstrating that pTRS1 recognized the m^7G moiety rather than non-specifically binding to the resin. pTRS1 expressed

outside the context of infection in HeLa cells also bound the m^{7}G resin (Fig. 1*C*), demonstrating that additional viral proteins were not required for the interaction. pTRS1 binding to m^{7}G was resistant to micrococcal nuclease digestion (Fig. 1*D*), indicating that the association was not brokered by an RNA intermediate. In fact nuclease treatment increased the pTRS1 binding to m^{7}G resin. Partially purified, nuclease-treated pTRS1 also co-purified with the m7G resin (Fig. 1*E*). The partially purified pTRS1 preparation did not contain detectable levels of the host eIF4E cap binding protein or the eIF4G component of the eIF4F complex, suggesting that pTRS1 can directly associate with the mRNA cap. Consistent with previous reports that pTRS1 binds RNA [8, 28], pTRS1 was captured from transfected cell lysates with oligo-(d)T sepharose in an RNA-dependent manner (Fig. S1*A*), demonstrating the efficacy of the nuclease treatment. The addition of excess free m^{7}GTP did not affect pTRS1 capture by the oligo-d(T) sepharose. This suggests that the association of pTRS1 with the mRNA cap and the remainder of the mRNA are separable interactions.

Figure 1

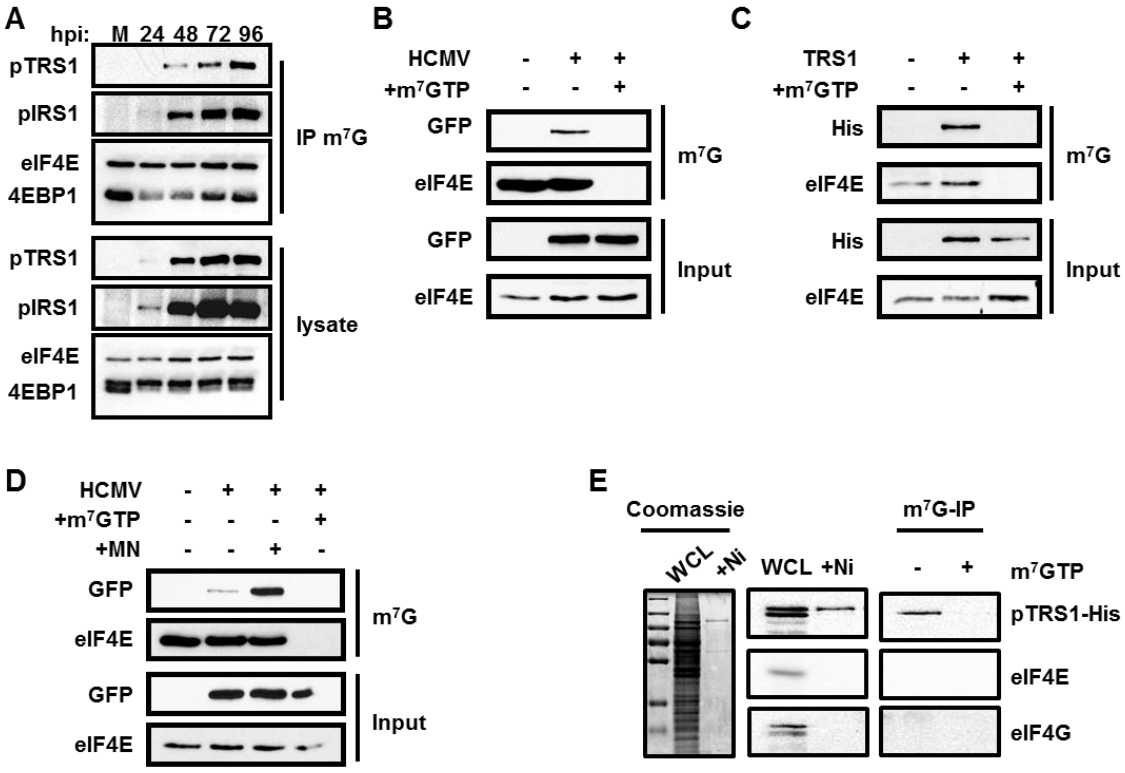


Figure 1 HCMV pTRS1 associates with the m7G mRNA cap. (A) Cells were infected with BADinGFP (3 IU/cell) and proteins bound to m7G sepharose were analyzed by Western blot with the indicated antibodies. Samples were harvested at the indicated time after infection. (B) Lysates from cells infected with BADinTRS1GFP (72 hpi) were incubated with m7G sepharose in the presence of free m7GTP (+m7GTP). (C) HeLa cells were transfected with a expression vector encoding pTRS1 fused to the his epitope and analyzed as in B. (D) Same as in B, except that one sample was treated with micrococcal nuclease (+MN) prior to incubation with m7G sepharose. (E) Partially purified pTRS1 incubated with m7G sepharose and analyzed as in B. (Left) Coomassie stained acrylamide gel of Whole Cell Lysate (WCL) and partially purified pTRS1 (+Ni) (Right).(Middle) Western blot of WCL and partially purified pTRS1 (Right) Western blot of m7G-associated proteins following incubation with partially purified pTRS1.

pTRS1 associates with mRNAs undergoing translation initiation The association of pTRS1 with the mRNA cap suggested that pTRS1 might interact with the translation machinery during infection. We first determined if pTRS1 co-sedimented with ribosomal subunits in HCMV infected cells. Infected cells were treated with puromycin to dissociate ribosomes into 40 and 60S subunits, and cytoplasmic lysates were then subjected to centrifugation in linear sucrose gradients to separate the ribosomal subunits. pTRS1 co-sedimented with the 40S ribosomal protein rpS6 and the 18S rRNA in a 10 to 20% sucrose gradient (Fig. S1*B*), arguing that pTRS1 associates with a very large structure, presumably the 40S ribosomal subunit, during infection. To determine if pTRS1 associates with active initiation complexes during infection, we asked if pTRS1 co-localized with stress granules in HCMV infected cells. Stress granules contain stalled translation initiation complexes bound to polyadenylated mRNA [21]. Translation initiation factors, the 40S ribosomal subunit, and mRNA all localize to stress granules upon exposure to stress-inducing agents that limit mRNA translation. However, 60S ribosomal subunits and active 80S ribosomes are excluded [29]. Stress granules were absent from cells infected with HCMV variants expressing a pTRS1-GFP or pIRS-GFP fusion protein as determined by the diffuse

localization of the stress granule marker G3BP1 (Fig. 2*A,B*) [30]. However, stress granules could be induced to form in infected cells by the addition of the stress-inducing agents, sodium arsenite and thapsigargin, as judged by the re-localization of G3BP1 into discrete cytoplasmic puncta (Fig. 2 *A,B*). While pTRS1 displays a diffuse cytoplasmic localization in untreated cells, treatment with arsenite (Fig. 2*A*) or thapsigargin (Fig. 2*C-E*) induced pTRS1 re-localization into discrete puncta that co-stained for G3BP1. pIRS1 behaved similarly to pTRS1, displaying diffuse cytoplasmic localization in untreated cells, and co-localization with G3BP1 in discrete puncta in the presence of arsenite (Fig. 2*B*). The re-localization of GFP to stress granules was dependent on the fusion to pTRS1, as GFP did not co-localize with G3BP1 in cells infected with an HCMV strain expressing GFP that was not fused to a viral protein (Fig. 2*C-E*). We also found that host initiation factors including eIF4G, eIF4A, eIF4E, co-localized with pTRS1 in stress granules (Fig. 2*C-E*). Together these data suggest that pTRS1 associates with active translation initiation complexes in HCMV-infected cells.

pTRS1 stimulates translation We next tested if pTRS1 expression affected translation. Cells were transfected with increasing amounts of a vector expressing pTRS1 together with a fixed amount of the pGL3-Control luciferase reporter construct, which expresses an mRNA encoding a 33 base pair 5'UTR derived from the plasmid multi-cloning site upstream of the luciferase coding region. pTRS1 induced a dose-dependent increase in the amount of luciferase activity (Fig. 3*A*, top). At the maximal concentration of pTRS1 tested, we observed a 19-fold increase in luciferase activity. pTRS1 expression had a minimal effect on luciferase mRNA abundance (Fig. 3*A*). To measure the effect of the viral protein on the rate of global protein synthesis, cells were labeled briefly (30 min) with ^{35}S-labeled amino acids at 24 h after transfection with the pTRS1 expression plasmid or a control plasmid expressing GFP. pTRS1 increased the rate of protein synthesis in transfected cells by approximately 50% as compared to control cells expressing the GFP protein (Fig. 3*B* and Fig. S4). Based on these data we

conclude that pTRS1 expression is sufficient to stimulate the translation of a reporter gene and increase the rate of protein synthesis.

Figure 2

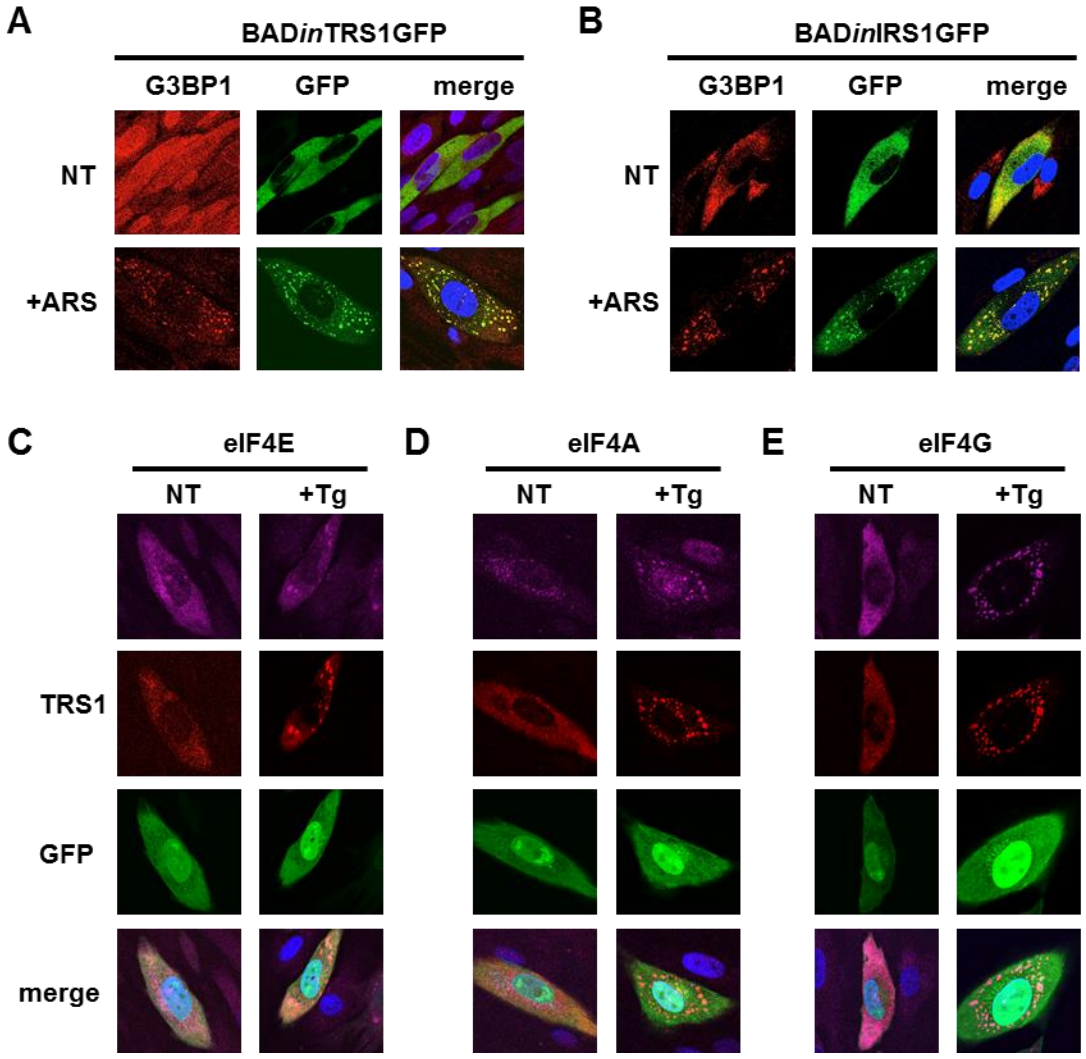


Figure 2 pTRS1 associates with active translation initiation complexes during HCMV infection. HFFs were infected with BADinTRS1GFP (A), BADinIRS1GFP (B) or BADinGFP (C-E) at a multiplicity of 0.5. (A & B) Cells were treated with sodium arsenite (+ARS, 0.5 mM) or vehicle for two hours at 72 hpi. Immunofluorescence confocal microscopy was used to visualize GFP (green), the stress granule marker protein G3BP1 (red) or nuclei (DAPI, blue). (C-E) Cells were infected with BADinGFP and treated with thapsigargin (+Tg, 2 µg/ml) for one hour before

fixation at 72 hpi. Confocal microscopy was used as above to visualize pTRS1 (red) together with eIF4E, eIF4G or eIF4A (purple). DAPI (blue) was used to visualize nuclei, and GFP (green) was used to identify infected cells.

The presence of a non-canonical RNA binding domain (RBD) in pTRS1 [8] suggested that pTRS1 might preferentially affect the translation of mRNAs containing specific sequences. We therefore compared the effect of pTRS1 expression on the translation of reporter constructs in which the 5'UTR of host or viral mRNAs were cloned upstream of the luciferase coding region. pTRS1 stimulated translation of mRNAs containing either host or viral 5'UTRs (Fig 3*C*) to varying extents. pTRS1 increased expression of the host 5'UTR reporters 5-7 fold compared to cells expressing GFP. However, pTRS1 preferentially stimulated translation of the viral UL99 5'UTR (>15 fold), and to a lesser extent the UL44 5'UTR (Fig. 3*C*). To rule out an effect of pTRS1 on transcription from the reporter constructs we measured the abundance of luciferase mRNA in control or pTRS1 expressing cells. pTRS1 did not alter the abundance of luciferase mRNA from any of the reporters, except the UL44 5'UTR containing reporter which was slightly increased (Fig. 3*D*). Thus, pTRS1 increased expression from two reporters containing HCMV 5'UTRs to a greater extent than the control or cellular 5'UTRs.

These data suggested that pTRS1 might preferentially stimulate translation of the pUL99 5'UTR by recognizing specific sequences or structures in the RNA. We therefore determined if truncation of the UL99 5'UTR ameliorated the preferential effect of pTRS1 on UL99 5'UTR translation. pTRS1 stimulated expression of the full length UL99 5'UTR approximately 16 fold (Fig. 3*E*). pTRS1 also stimulated translation of a truncated UL99 5'UTR lacking the first 200 nucleotides, but to a lesser extent which was comparable to that of the luciferase reporter lacking the viral 5'UTR (7 fold). Similar results were obtained over a range of pTRS1 concentrations (Fig. S2). pTRS1 expression did not increase the abundance of the luciferase mRNA transcribed from either reporter (Fig. 3*E,* S2). Together these data demonstrate that

pTRS1 preferentially stimulates the translation of mRNAs containing specific sequences or features.

Previous work has shown that pTRS1 inhibits the double-stranded RNA-dependent protein kinase R (PKR) [7, 9]. Therefore an explanation for the preferential effect of pTRS1 on the translation of the viral 5'UTR reporter could be that the full-length UL99 5'UTR activated PKR, while mRNAs transcribed from the pGL3-Control vector and the truncated viral 5'UTR vector did not. We therefore measured the effect of each reporter on PKR T446 auto-phosphorylation, a measure of its activation [31] in the presence or absence of pTRS1. Similar levels of phosphorylated PKR were observed following co-transfection of each reporter with a control plasmid expressing GFP, and in each case pTRS1 efficiently blocked PKR auto-phosphorylation (Fig. 3*F*).

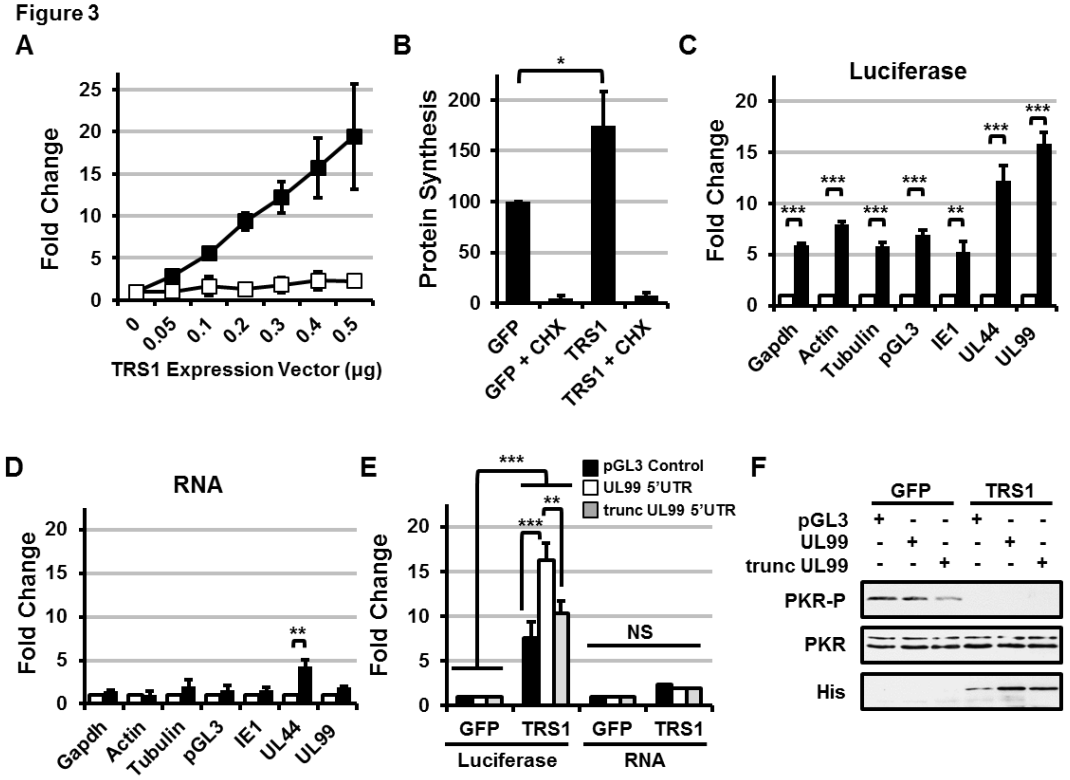


Figure 3 pTRS1 enhances translation of a reporter gene in a sequence dependent manner. (A) Increasing amounts of pTRS1 expression vector were co-transfected with a

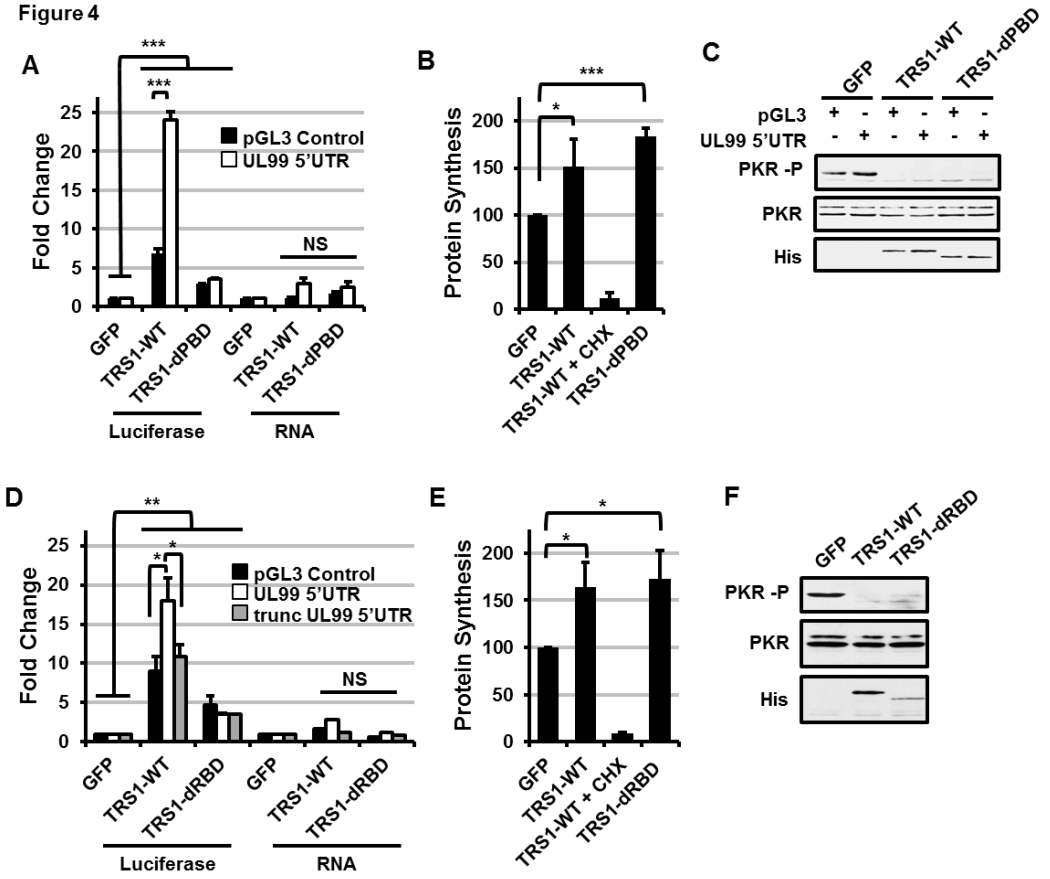
constant amount of pGL3-Control luciferase plasmid. The graph shows the fold change in luciferase activity (closed boxes) and luciferase RNA abundance (open boxes) compared control cells expressing GFP. (n=3) (B) pTRS1 or GFP expressing cells were incubated with radiolabeled amino acids for thirty minutes at twenty-four hours after transfection. The amount of radiolabel incorporated into acid-insoluble proteins was quantified using a scintillation counter. The rate of protein synthesis in control cells expressing GFP is set to 100. (CHX=cycloheximide; 100 µg/ml) (n=3) (C & D) A pTRS1 expression vector (0.2 µg) was co-transfected with reporters containing the 5'UTR from the indicated host (GAPDH, actin, tubulin) or viral (IE1, UL44, UL99) mRNAs upstream of the luciferase coding region. The graphs show the fold change in luciferase activity (C) or luciferase RNA abundance (D) in pTRS1-expressing cells relative to control cells expressing GFP. (n=3) (E) Cells were transfected with pGL3-Control (black bars), a reporter containing the 5'UTR of the HCMV UL99 mRNA (open bars), or a 5' truncation of the UL99 5'UTR (grey bars) together with a GFP (GFP) pTRS1 expression vector (TRS1). The graphs show the fold change in luciferase activity (left side) and RNA abundance (right side) in TRS1 expressing cells relative to the GFP control (n=3). (F) Cells were transfected as in E. Extracts were analyzed by Western blot using antibodies to PKR phosphorylated on T446 (PKR-P), total PKR, or the his epitope (TRS1). For all panels * = p-value < 0.05; ** = p value < 0.01; *** = p value < 0.001.

We further explored this result by determining if the previously identified pTRS1 PKR binding domain was necessary for pTRS1 to stimulate translation and inhibit PKR activation. A pTRS1 mutant lacking its PKR binding domain (amino acids 679 to 795; TRS1-dPBD), [9] was less effective than wild-type pTRS1 at stimulating translation of the reporter mRNAs (Fig. 4*A*). A control experiment confirmed that RNA levels were not differentially affected (Fig. 4*A*). The pTRS1-dPBD mutant increased the overall rate of cellular protein synthesis similarly to wild-type pTRS1 (Fig. 4*B*), and bound the m^7G mRNA cap as well as wild type pTRS1, if not better (Fig. S3). In addition PKR auto-phosphorylation was inhibited equally as well by wild type pTRS1 or

the pTR1-dPBD mutant (Fig. 4*C*). We conclude that in this setting the PKR binding domain is dispensable for inhibition of PKR auto-phosphorylation and increased levels of protein synthesis. However, the carboxyl-terminus of pTRS1 is necessary for the preferential stimulation of a reporter containing an HCMV 5'UTR.

We also assessed the ability of a pTRS1 mutant lacking its RNA binding domain (amino acids 84 to 246; TRS1-dRBD) [8] to stimulate mRNA translation. In initial experiments we found the expression of the TRS1-dRBD mutant to be consistently lower than that of wild-type pTRS1. We therefore transfected higher amounts of TRS1-dRBD expression plasmid to achieve equivalent expression of the two proteins. Deletion of the pTRS1 RNA binding domain diminished the effect of pTRS1 on the translation of the full length and truncated UL99 5'UTRs, while RNA levels were not differentially affected (Fig. 4*D*). However the TRS1-dRBD mutant maintained the ability to stimulate translation of all reporters, albeit to a reduced level, and stimulated the overall level of protein synthesis to a comparable extent as wild type pTRS1 (Fig. 4*E*). TRS1-dRBD also retained the ability to co-purify with m^7G sepharose (Fig. S3) and efficiently inhibit PKR auto-phosphorylation (Fig. 4*F*). We conclude that the pTRS1 RNA binding domain is necessary to preferentially stimulate the translation of mRNAs containing specific sequences or structures, but is dispensable for increased levels of protein synthesis and inhibition of PKR auto-phosphorylation.

Figure 4 The pTRS1 PKR binding and RNA binding domains are dispensable to inhibit PKR auto-phosphorylation, but necessary to preferentially stimulate translation. (A) Cells were transfected as in figure 3 with either pGL3-Control (black bars) or the UL99 5'UTR luciferase construct (open bars) together with either a control vector (GFP) or the indicated pTRS1 expression constructs. The graph shows the fold change in luciferase activity (left side) and RNA abundance (right side) in pTRS1 expressing cells

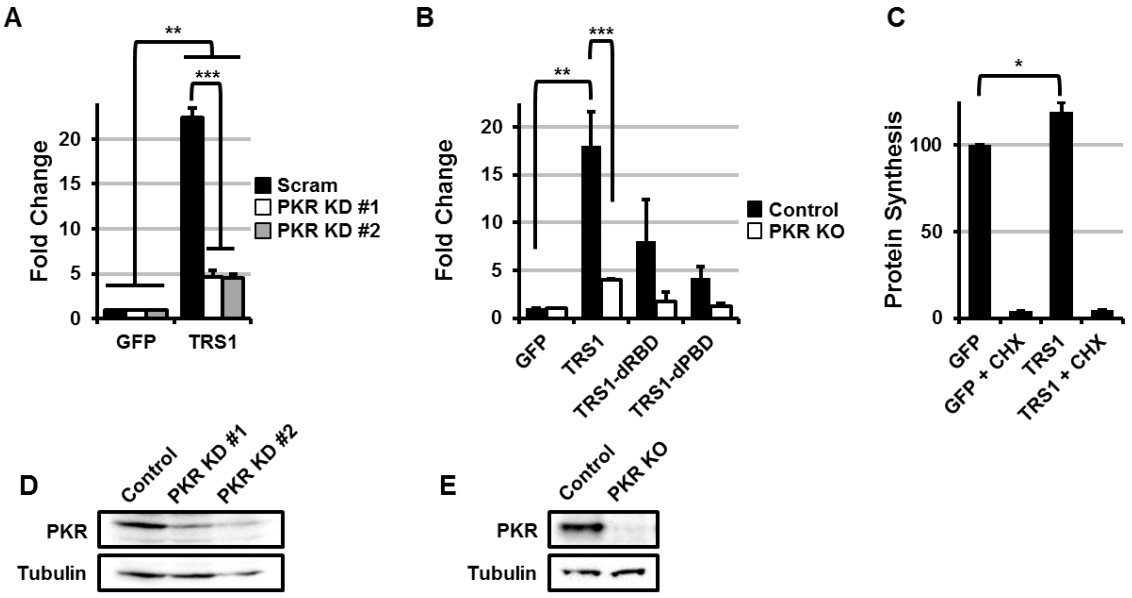


relative to control cells expressing GFP (n=3). (B) The rate of nascent protein synthesis in cells expressing wild type pTRS1 or pTRS1 lacking the PKR binding domain was measured as in figure 3B (n=3). (C) Cells were transfected as in figure 3 with pGL3-Control vector or the UL99 5'UTR reporter together with a control vector (GFP) or the indicated pTRS1 constructs. PKR phosphorylation (PKR-P) was measured by Western blot. (D) Same as in A, except a pTRS1 mutant lacking the RNA binding domain (333ng) and the truncated UL99 5'UTR pGL3-Control construct was included (n=3). (E) The rate of nascent protein synthesis in cells expressing wild type pTRS1 or pTRS1 lacking the RNA binding domain was measured as in figure 3B (n=3). (F) Cells were transfected with the UL99 5'UTR reporter together with a control vector (GFP) or the indicated pTRS1 construct. PKR auto-phosphorylation was measured by Western blot. For all panels * = p-value < 0.05; ** = p value < 0.01; *** = p value < 0.001.

We next determined if pTRS1 could stimulate translation in the absence of PKR. We first determined the effect of shRNA-mediated PKR depletion on pTRS1-stimulated reporter gene expression. In these experiments we used the UL99 5'UTR reporter, as the greatest of effect of pTRS1 was observed with this construct. pTRS1 expression resulted in a statistically significant increase in luciferase activity in PKR depleted cells (Fig. 5*A, C*). Interestingly wild type pTRS1 increased luciferase activity of all reporters tested to a similar extent in the absence of PKR (Fig. 5*B*), suggesting that a PKR-independent activity of pTRS1 results in a general increase in protein synthesis. In a second approach we measured the effect of pTRS1 on reporter gene expression and nascent protein synthesis in cells where CRISPR/Cas9-mediated genome editing was used mutate the PKR gene, and thus abrogate its expression (Fig. 5*F*). pTRS1 increased luciferase activity and the overall level of protein synthesis in PKR null cells (Fig. 5*D*,*E*). We also compared the ability of pTRS1-dPBD and pTRS1-dRBD to stimulate translation in PKR null cells. Neither mutant stimulated translation to a significant extent (Fig. 5*D*). This data demonstrates that pTRS1 is capable of stimulating translation independent of its ability to inhibit PKR.

Figure 5 pTRS1 stimulates translation independent of PKR inhibition. (A) HeLa cells were transduced with scrambled (Scram) or PKR-specific shRNAs, and then transfected with full length UL99 5'UTR luciferase reporter together with a GFP or pTRS1 expression vector. The graph shows fold change in luciferase activity in pTRS1 expressing cells relative to GFP control cells (n=3). (B) Cells expressing PKR-specific shRNAs were transfected with the indicated reporters together with GFP or pTRS1 and assayed as in panel A (n=3) (C) Western blot showing reduced PKR expression in cells

Figure 5



transduced with PKR-specific shRNAs. (D) The CRISPR/Cas9 system was used to generate HeLa cells lacking PKR or control cells lacking the AAVS gene. Cells were transfected with either GFP or the indicated pTRS1 constructs together with the full length UL99 5'UTR luciferase reporter. The graph shows fold change in luciferase activity in pTRS1 expressing cells relative to control (n=3). (E) The rate of nascent protein synthesis in PKR knockout cells expressing either GFP or pTRS1 was measured as in figure 3B (n=4) (F) Western blot showing PKR is not expressed in cells where the PKR gene was mutated using the CRISPR/Cas9 system. For all panels * = p-value < 0.05; ** = p value < 0.01; *** = p value < 0.001

Discussion

In this study we found that HCMV pTRS1 stimulates mRNA translation through both PKR-dependent and PKR-independent mechanisms. pTRS1 associated with the mRNA m^7G cap (Fig. 1*A*-*E* and Table S1), co-sedimented with 40S ribosomal subunits (Fig. S1*B*) and co-localized with stress granules in HCMV infected cells (Fig. 2*A*-*E*). Expression of pTRS1 alone increased the overall level of protein synthesis (Fig. 3*B*, 4B, *4E and* 5*D*) and stimulated the translation of a reporter gene expressing an mRNA with a short, non-structured 5'UTR (Fig. 3*A*). pTRS1 stimulated the translation of reporters expressing mRNAs with different 5'UTRs to

varying extents. In the comparison we studied, it increased expression from a reporter expressing an mRNA containing an HCMV 5'UTR to a two-fold (Fig. 3*C*, 3*E*, 4A and 4*D*) to 3-fold greater extent than the relatively short 5'UTR in a control reporter. Apparently, specific mRNA sequences or structures in viral transcripts influence responsiveness to pTRS1 expression. This preferential stimulatory effect requires the carboxyl terminal region of pTRS1 that contains its PKR binding domain (Fig. 4*A*) and a segment including the pTRS1 RNA binding domain (Fig. 4*D*). Inhibition of PKR activation contributed to the enhancement of translation by pTRS1, however pTRS1 also stimulated reporter gene expression and protein synthesis in cells lacking PKR (Fig. 5*A*, 5*C*, 5*D*). Thus pTRS1 enhances translation through both PKR-dependent and independent mechanisms.

pTRS1 was previously found to contain a non-canonical RNA binding domain capable of binding to uncapped, double-stranded RNA *in vitro* [8, 28]. Our results demonstrate that pTRS1 additionally interacts with the mRNA cap (Fig. 1*A*-*E*). Other viral proteins were not required for pTRS1 association with m^7G sepharose as the interaction occurred in transfected cells. The interaction was resistant to micrococcal nuclease digestion (Fig. 1*D*), and therefore was not dependent on the presence of mRNA. Our finding that partially purified pTRS1 co-purified with m^7G sepharose (Fig. 1*E*) suggests that pTRS1 may bind directly to the mRNA cap. This is consistent with our finding that the partially purified pTRS1 preparation did not contain detectable levels of the host eIF4E cap binding protein (Fig. 1*E*). However additional biochemical experiments are needed to fully explore this possibility. Our finding that pTRS1 co-purified with mRNA in the presence of excess m^7GTP cap analog (Fig. S1*A*) suggests that pTRS1 likely associates with both the body of the mRNA and the mRNA cap, perhaps increasing the stability of pTRS1 interaction with its cognate mRNAs.

Our finding that pTRS1 prevented PKR auto-phosphorylation (Fig. 4*B*) clearly supports previous studies demonstrating that pTRS1 is a potent PKR inhibitor [7, 9]. Binding to double-stranded RNA induces PKR homodimer formation, subsequent auto-phosphorylation and

activation [32, 33]. The ability of pTRS1 to inhibit PKR auto-phosphorylation therefore suggests that pTRS1 inhibits PKR dimerization. However, the mechanism by which pTRS1 prevents PKR auto-phosphorylation is unclear. pTRS1 mutants lacking either the RNA or PKR binding domain inhibited PKR auto-phosphorylation as efficiently as wild type pTRS1 (Fig. 4*C,F*), suggesting that a physical interaction with RNA or PKR may not be necessary for pTRS1 to inhibit PKR activation. However, it is possible that pTRS1 binds PKR through a region other than the previously defined PKR binding domain and thus prevents its activation. Additional biochemical experiments will be needed to determine the precise mechanism by which pTRS1 inhibits PKR activation.

We also found that pTRS1 preferentially stimulated the translation of reporter mRNAs containing certain sequences or structures (Fig. 3*E*, 4*A*, 4*D*). The preferential effect of pTRS1 was dependent on the presence of PKR, as pTRS1 induced the different reporters to a similar extent in PKR-depleted cells (Fig. 5*A*,*C*). The preferential stimulation of the viral 5'UTR required both the pTRS1 RNA and PKR binding domains (Fig. 4*A*,*D*). However, both the RNA and PKR binding domain mutants prevented PKR auto-phosphorylation, and presumably PKR activation (Fig. 4*C*,*F*). Thus the ability of pTRS1 to inhibit PKR auto-phosphorylation does not correlate with its ability to preferentially stimulate translation of specific mRNAs. Perhaps in this system PKR is activation does not require auto-phosphorylation. We find this possibility unlikely, as PKR mutants lacking the phosphorylation site measured in our assay have greatly reduced kinase activity [34]. We feel a more likely explanation is that an undetectable, but biologically significant, level of PKR phosphorylation occurs in the presence of the mutants, resulting in translation inhibition.

In either case, the fact that pTRS1 stimulates some reporters more than others in a PKR-dependent manner suggest that PKR preferentially inhibits the translation of specific mRNAs. Binding to double stranded RNA structures, rather than specific mRNA sequences, activates PKR [32]. Once activated PKR globally suppresses mRNA translation by inactivating

the eIF2α initiation factor. Perhaps recruitment of PKR to structures in mRNA 5'UTRs results in the local activation of PKR, resulting in the preferential inhibition of PKR-bound RNAs. Such a scenario has previously been suggested for the preferential inhibition of specific cellular mRNAs by PKR [35, 36]. This possibility could explain how an undetectable level of PKR auto-phosphorylation might have a biologically significant effect. pTRS1 may bind similar RNA structures and prevent PKR recruitment, thus preventing local PKR activation. Additional studies to define the complement of RNAs bound by PKR and pTRS1 will be needed to clarify the mechanism of preferential effects of pTRS1 and PKR on mRNA translation.

We also found that pTRS1 stimulated mRNA translation in a PKR-independent manner. pTRS1 increased the expression of each of the tested reporters in PKR depleted or PKR deficient cells (Fig. 5*A*, *B*), and increased the overall level of protein synthesis in the absence of PKR (Fig. 5*C*). Thus pTRS1 stimulates translation in both a PKR-dependent and PKR-independent manner. How might pTRS1 stimulate translation independent of its antagonism of PKR? We propose that pTRS1 preferentially associates with viral mRNAs in a sequence-dependent manner and bridges their association with the translation machinery. Our data suggests that pTRS1 acts at the initiation step of mRNA translation, as pTRS1 associates with the m^7G mRNA cap (Fig. 1*A*-*E*), co-sediments with 40S ribosomal subunits (Fig. S1*B*) and co-localizes with stress granules containing stalled translation initiation complexes in infected cells (Fig. 2*A*-*E*). Perhaps pTRS1 acts in concert with host translation initiation complexes to stimulate translation. Alternatively, pTRS1 could be part of an uncharacterized initiation complex. Based on the known role of pTRS1 in limiting PKR activity this may also allow for the continued translation of viral mRNAs in the presence of the host antiviral response. However, our data do not exclude the possibility that pTRS1 may also regulate additional aspects of the antiviral response that might act to limit protein synthesis. For example pTRS1 could bind to dsRNA and limit OAS activation, and thus block RNaseL-dependent mRNA decay. Additional

biochemical studies will be needed to define the molecular interactions underlying PKR-independent pTRS1 activity.

In sum, we have identified an HCMV protein, pTRS1, which is sufficient to increase the overall level of translation in transfected cells. pTRS1 stimulates the translation of individual mRNAs to different extents, raising the possibility that it favors translation of HCMV mRNAs while simultaneously blocking the host antiviral response. Based on these functions pTRS1 antagonists have strong potential as antiviral drugs. Such drugs could antagonize the ability of TRS1 to stimulate translation and relieve the viral block to PKR activity, thereby reducing viral protein synthesis while activating a key aspect of the host innate immune system.

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Chapter 4: Human cytomegalovirus pTRS1 and pIRS1 antagonize PKR to facilitate virus replication[1]

Introduction

Human cytomegalovirus (HCMV), like all viruses, requires host ribosomes and translation factors for the synthesis of viral proteins. Consequently, upon sensing infection host antiviral defenses inactivate critical translation factors, leading to reduced viral replication. To circumvent these defenses, HCMV manipulates antiviral signaling pathways to allow for efficient viral protein synthesis. Thus the interface of HCMV with the host translation machinery lies at the front line of the battle between host and virus for control of the infected cell.

Perhaps the best-studied antiviral defense targeting viral mRNA translation is the RNA-dependent protein kinase R (PKR). PKR binds to double-stranded RNAs (dsRNAs) produced during viral infections, resulting in PKR dimerization and activating auto-phosphorylation (6, 23, 35, 41). Activated PKR in turn inhibits mRNA translation by phosphorylating its substrate the eukaryotic initiation factor 2 alpha (eIF2α) (25, 27, 37, 50). eIF2α plays a critical role in translation initiation as a regulatory subunit of the trimeric eIF2 complex, which mediates binding of the ternary complex, consisting of eIF2, GTP, and $tRNA^{Met}$, to the ribosome (21). eIF2α phosphorylation by PKR prevents recycling of the ternary complex after initiation, resulting in an overall decrease in translation initiation and diminished viral protein synthesis and replication (46).

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[1]This chapter has been submitted to the Journal of Virology and is currently under review. Written by: Ziehr, B., Vincent, H.A., Moorman, N.J.

Phosphorylation of eIF2α further limits protein synthesis by sequestering actively translating mRNAs into cytoplasmic ribonucleoprotein complexes called stress granules (2). During viral infection stress granules are most often induced by activated PKR, however additional virus-induced stressors such as the accumulation of unfolded proteins and/or nutrient depletion are also involved (4). Prolonged periods of stress lead to the degradation of stress granule-associated mRNAs, which further inhibits viral protein expression (45). Despite the induction of stress response pathways known to trigger stress granule formation, stress granules do not form in HCMV-infected cells (22, 38). This suggests that HCMV encodes viral proteins that inhibit stress granule formation. However a role for HCMV proteins in the inhibition of stress granule formation has not been described.

Many viruses generate dsRNA ligands recognized by PKR during infection, and thus viruses commonly encode PKR antagonists. Human cytomegalovirus (HCMV) encodes two PKR antagonists, the TRS1 and IRS1 proteins (pTRS1 and pIRS1, respectively). The amino terminal 550 amino acids of pTRS1 and pIRS1 are encoded by the short repeat regions of the viral genome, and are therefore identical, while the remainder of pTRS1 and pIRS1 are encoded by the unique short segment of the genome and thus diverge. However the unique regions of pTRS1 and pIRS1 are highly similar, sharing approximately 50% amino acid conservation. Both proteins limit PKR activation outside the context of HCMV infection (13), and the expression of either pTRS1 or pIRS1 is necessary for HCMV replication (31). Several functional domains have been identified in pTRS1 and pIRS1, including an RNA binding domain between amino acids 86-246 (18) and a PKR binding domain in the unique carboxyl-terminus (19). While both domains are necessary for PKR antagonism in heterologous systems and *in vitro*, the contribution of each domain to PKR inhibition during HCMV infection is currently unknown.

Efforts to study pTRS1 functions in the context of HCMV infection have been complicated by the functional redundancy of pIRS1. Expression of either protein is sufficient to support HCMV replication, although loss of both TRS1 and IRS1 results in a replication-deficient virus. As a result the majority of studies defining functional roles for pTRS1 and pIRS1 have been performed *in vitro* or in heterologous systems outside the context of HCMV infection. Both pTRS1 and pIRS1 complement the growth defect of vaccinia virus mutants lacking the E3L protein (5, 11-14, 18), a known PKR inhibitor, and pTRS1 complements the growth of HSV mutants lacking the ICP34.5 protein (10), which stimulates eIF2α dephosphorylation through the recruitment of a cellular phosphatase (15, 20). Similarly pTRS1 complements the replication of MCMV strains lacking the PKR antagonists m142 or m143 (9, 43). While these studies show that pTRS1 and pIRS1 act as PKR antagonists, the role of PKR in suppressing HCMV replication has not been defined. In addition the requirement for pTRS1 and pIRS1 to prevent PKR activation during HCMV infection has not been described.

In this study we used a combination of shRNAs and viral mutants to evaluate the role of pTRS1 and pIRS1 during HCMV infection. Our results confirm previous studies showing that either IRS1 or TRS1 is sufficient for HCMV replication. We found that expression of either pTRS1 or pIRS1 is both necessary and sufficient to suppress PKR and eIF2α phosphorylation and maintain overall levels of protein synthesis in HCMV infected cells. In addition we found that expression of either pTRS1 or pIRS1 is necessary to prevent stress granule formation during HCMV infection, and that pTRS1 alone is sufficient to prevent stress granule formation in transfected cells. Using a pTRS1 mutant lacking a portion of the unique carboxyl-terminus, we found that the pTRS1 PKR binding domain is necessary to prevent PKR activation during HCMV infection. Depletion of PKR restored virus replication and viral protein synthesis in the absence of pTRS1 and pIRS1, demonstrating for the first time that PKR inhibits HCMV protein expression and replication.

Materials and Methods

Viruses, Cells and Reagents MRC-5 fibroblasts, human foreskin fibroblasts (HFFs) and HeLa cells were grown in DMEM supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. MRC-5 fibroblasts and HFFs were used between passages 8 and 20. The AD169 strain lacking the TRS1 open reading frame was a generous gift of Dr. Adam Geballe (Univ. of Washington) and has been described previously (31). All mutations in the HCMV genome were generated in the AD169BAD*in*GFP bacterial artificial chromosome (BAC) using recombineering in the DY380 strain of *E.coli* as previously described (33, 34). To generate the PKR binding domain deletion mutant (HCMV∆PBD), a FLAG-FRT-KAN-FRT cassette was PCR amplified using primers FRT FWD and PBDFRT REV (sequence is listed in Table S1) containing 50 nucleotides of homology flanking the insertion site at nucleotide 228885 (corresponding to amino acid 679; accession: FJ527563.1). Following recombination, expression of the Flp recombinase was induced with arabinose to excise the kanamycin cassette. The resulting recombinant contains a FLAG epitope tag followed by a translation stop codon in frame with the 3' end of the TRS1 ORF. The gross integrity of the recombinant viral genomes was confirmed by restriction digest, and the sequence of the entire TRS1 open reading frame and the flanking 500 nucleotides were confirmed by Sanger sequencing. For each mutant two independent recombinants were isolated, sequenced and characterized to control for potential spurious mutations introduced during recombination. BAC DNA was purified using the Nucleobond Midiprep kit (Machery-Nagel) according to the manufacturer's directions. One microgram of BAC DNA together with one microgram of pCGN pp71 (3) was electroporated into MRC-5 fibroblasts to reconstitute infectious virus. Viral stocks were propagated on MRC5 fibroblasts and titered using the TCID50 method. Unless otherwise noted, all infections were performed at a multiplicity of infection of one in 300 µl of media. The amount of cell-free virus in the

supernatant was quantified by the TCID50 method on MRC5 fibroblasts as previously described (28).

The plasmid pcDNA-pTRS1 expressing the TRS1 protein with a carboxyl-terminal 6X His epitope tag was a kind gift of Dr. Adam Geballe (18). Cells were transfected with the indicated plasmids using PEI and analyzed at 24 hours after transfection unless otherwise noted.

siRNA and shRNA-mediated depletion siRNA targeting the PKR transcript (Dharmacon SMARTpool ON-TARGETplus EIF2AK2 siRNA) was transfected at a final concentration of 20 nM in 100 µl Opti-MEM using 6 µl MISSION siRNA transfection reagent (Sigma). Complexes were incubated at room temperature for 15 minutes prior to dropwise addition to 30-50% confluent MRC-5 cells. Cells were used in subsequent experiments at 72 hours after transfection. Efficient knockdown was routinely monitored by Western blot. MRC-5 cells stably expressing scrambled or IRS1-specific shRNAs were generated using the pSUPERretro system (Oligoengine). The sequence GGAGTTCATGTTTCGCGAACA targeting the unique 3' end of the IRS1 ORF was cloned into the pSUPERretro vector. Retrovirus stocks were generated by transfecting the Phoenix packaging cell line (40) with the pSUPERretro plasmids. Supernatants were collected at 48 hours post transfection, filtered through a .45 µm filter, and used to transduce cells in the presence of polybrene (4 µg/mL). Stable cell lines were obtained by selection in puromycin (1 µg/ml) for at least one week prior to use.

Western blot analysis Cells were collected by scraping at the indicated times and frozen as dry pellets at -80^{o}C until use. Cells were lysed in RIPA buffer (50 mM Tris-HCl:, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 1 mM EDTA) and the protein concentration determined by the Bradford assay (Amresco). Equivalent amounts of protein were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham). For monoclonal

antibodies, membranes were blocked for one hour at room temperature in 1% BSA in TBS-T (50 mM Tris-HCl: pH 7.4, 150 mM NaCl, 0.1% Tween-20) before incubation with primary antibody in TBS-T with 1% BSA for one hour at room temperature or overnight at 4^{o}C. For polyclonal antibodies, membranes were blocked in 1% BSA followed by incubation with primary antibody overnight at 4^{o}C in 5% BSA in TBS-T. For Western blots using the phospho-eIF2α (Ser51) antibody, membranes were blocked in 5% BSA in TBS-T overnight at 4^{o}C, followed by overnight incubation at 4^{o}C with primary antibody diluted in TBS-T containing 5% BSA. Antibodies to the following proteins were used in this study: IE1 ((48), 1:10,000), pUL44 (Virusys; 1:1,000), pp28 ((39); 1:5,000), pTRS1 ((36); 1:100), pIRS1 ((36); 1:100), P99 antibody against pTRS1 and pIRS1 ((31); 1:10,000), tubulin (Sigma T6199; 1:50,000), total PKR (Santa Cruz sc-707; 1:1,000), phospho-PKR (Thr446) (Abcam ab32036; 1:1,000), total eIF2α (Cell Signaling #3072, 1:1,000), phospho-eIF2α (Ser51) (Cell Signaling #3398; 1:1,000).

Indirect Immunofluorescence Cells were seeded into wells containing glass coverslips. Where noted cells were infected at a multiplicity of infection of 0.5. At the time of harvest coverslips were washed three times for 5 minutes with 37°C PBS and then fixed in 2% PFA for 15 minutes at 37°C. The cells were washed three times in room temperature PBS and subsequently permeabilized with 0.1% Triton X-100. The cells were again washed three times with PBS containing 0.2% Tween-20 (PBS-T) at room temperature and then incubated overnight in blocking buffer (2% BSA in TBS-T). The cells were stained for one hour at room temperature with the following primary antibodies diluted in blocking buffer: IE1 (1:10); pTRS1 (1:10); G3BP1, (Santa Cruz sc-98561; 1:50). The coverslips were washed three times with PBS-T and then incubated with fluorescent secondary antibody (Invitrogen goat anti-mouse or rabbit; 1:500). The coverslips were again washed three times and mounted on glass slides in VectaShield containing DAPI to stain nuclei (Vector Laboratories) and sealed with nail polish.

Images were captured using a Zeiss 710 confocal microscope with the help of the UNC Microscopy Core.

Quantification of Stress Granule Formation Control or PKR-deficient HeLa cells were transfected with either a GFP or pTRS1 expression vector. Twenty-four hours after transfection, cells were treated with arsenite for 1 hour and then processed for immunofluorescence microscopy as above. Transfected cells (i.e GFP or pTRS1 positive) were identified, and the relative fluorescence intensity of the GFP or pTRS1 signal in each cell was quantified using ImageJ software. The relative pTRS1 or GFP expression level in each cell was scored on a scale of 1-100, with 100 representing the highest observed expression. The presence of stress granules in each transfected cell was then determined by measuring G3BP1 puncta formation. Transfected cells containing two or more G3BP1 puncta were considered positive for stress granules, while those with less than two puncta were considered negative. The range of fluorescence intensity for GFP and pTRS1 was divided into quartiles (1-25% of maximum, 26-50% of maximum, etc.) and the percentage of cells containing stress granules within each expression quartile was calculated. A minimum of 100 hundred GFP or pTRS1 positive cells were analyzed. The data is displayed as the percent transfected cells containing stress granules within each quartile of GFP or pTRS1 expression.

Metabolic Labeling of Nascent Proteins Nascent proteins were metabolically labeled and quantified as described previously (29). Briefly, cells were incubated in methionine and cysteine-free media (Sigma) for 15 minutes. ^{35}S-labeled methionine and cysteine (125 µCi; Perkin Elmer; EasyTag Express Labeling Mix) were added and allowed to incorporate for 30 minutes. Cells were then washed twice in ice-cold PBS, scraped and collected by centrifugation. Cell pellets were lysed in RIPA containing protease inhibitors (Roche) and protein concentrations were determined by Bradford assay (Amresco). Trichloroacetic acid (TCA) was added to a final concentration of 20%, and precipitated proteins were captured on

glass microfiber filters by filtration under vacuum. The filters were washed twice with 20% TCA, once with 100% ethanol and allowed to air dry. The filters were then transferred to vials containing scintillation fluid (EcoScint), and radioactivity was quantified using a scintillation counter. The amount of radioactivity was normalized to the protein concentration for each sample.

Results

HCMV mutant strains lacking either pTRS1 or pIRS1 replicate to similar levels as wild type virus (7, 47), while a mutant lacking both pTRS1 and pIRS1 replicates poorly (31). Previous studies of a pTRS1/pIRS1 double knockout virus were complicated by the inability to efficiently complement virus replication by expression of pTRS1 *in trans* (31). This likely reflected insufficient expression of the pTRS1 transgene, or improper timing of expression. We reasoned that the failure of the pTRS1 transgene to fully complement the replication of a pTRS1/pIRS1 double deletion virus would limit our ability to generate and study recombinant viruses lacking domains of pTRS1 required for virus replication. Thus we sought to develop a system that allowed us to control pIRS1 expression, and express pTRS1 mutants to wild type levels with wild type kinetics from the context of the viral genome. We therefore used a combination of viral genetics and shRNA-mediated gene silencing to manipulate pTRS1 and pIRS1 expression during HCMV infection. We first generated primary human fibroblasts expressing either a scrambled shRNA (control cells) or an shRNA specific for the pIRS1 transcript (shIRS1-HFs). Infecting the cells with wild type virus allowed us to study the contribution of pTRS1 to HCMV infection in the absence of pIRS1. Conversely infection of control cells with a pTRS1 deletion virus (HCMVΔTRS1 described in (31); kindly provided by Dr. Adam Geballe) provided a system to study specific roles for pIRS1, while infection of shIRS1-HFs with HCMVΔTRS1 allowed us to determine how the absence of both proteins affected HCMV replication. Importantly, in this system both pTRS1 and pIRS1 were expressed from their native location in the viral genome

under the control of their endogenous promoters, allowing wild type kinetics and expression levels of the pTRS1 and pIRS1 proteins.

Infection of shIRS1-HFs with wild type HCMV resulted in a significant reduction in pIRS1 expression at all times after infection (Fig. 1A). Importantly, pTRS1 expression was not affected by the IRS1-specific shRNA. Consistent with previous reports showing that pIRS1 is dispensable for HCMV replication (7), wild type virus replicated to equivalent titers in both cell types despite efficient depletion of pIRS1 in shIRS1-HFs (Fig. 1C). These data also demonstrate that the IRS1-specific shRNA did not target additional host or viral proteins necessary for HCMV replication. Both wild type and HCMVΔTRS1 replicated to similar levels following infection of control cells. However, HCMVΔTRS1 infection on shIRS1-HFs reduced viral replication by greater than two orders of magnitude (Fig. 1C). These data demonstrate that our system confirms previous results showing that expression of either pTRS1 or pIRS1 is necessary for efficient HCMV replication.

We next examined viral protein expression after infection of control or shIRS1-HFs with wild type or HCMVΔTRS1 virus. Similar levels of representative immediate early (IE), early and late proteins were expressed after infection of control or shIRS1-HFs with wild type virus. Viral proteins of each kinetic class were also efficiently expressed after infection of control cells with HCMVΔTRS1 virus (Fig. 1A,B), however HCMV early and late protein expression was reduced after infection of shIRS1-HFs with the HCMVΔTRS1 virus (Fig. 1B). While the immediate early protein IE1 was expressed equivalently in shIRS1-HFs and control cells, we observed decreased expression of the early protein pUL44 and the late protein pp28 after infection of shIRS1-HFs with HCMVΔTRS1 (Fig. 1B). From these data we conclude that the expression of either pTRS1 or pIRS1 is necessary for the efficient expression of HCMV early and late proteins.

Figure 1

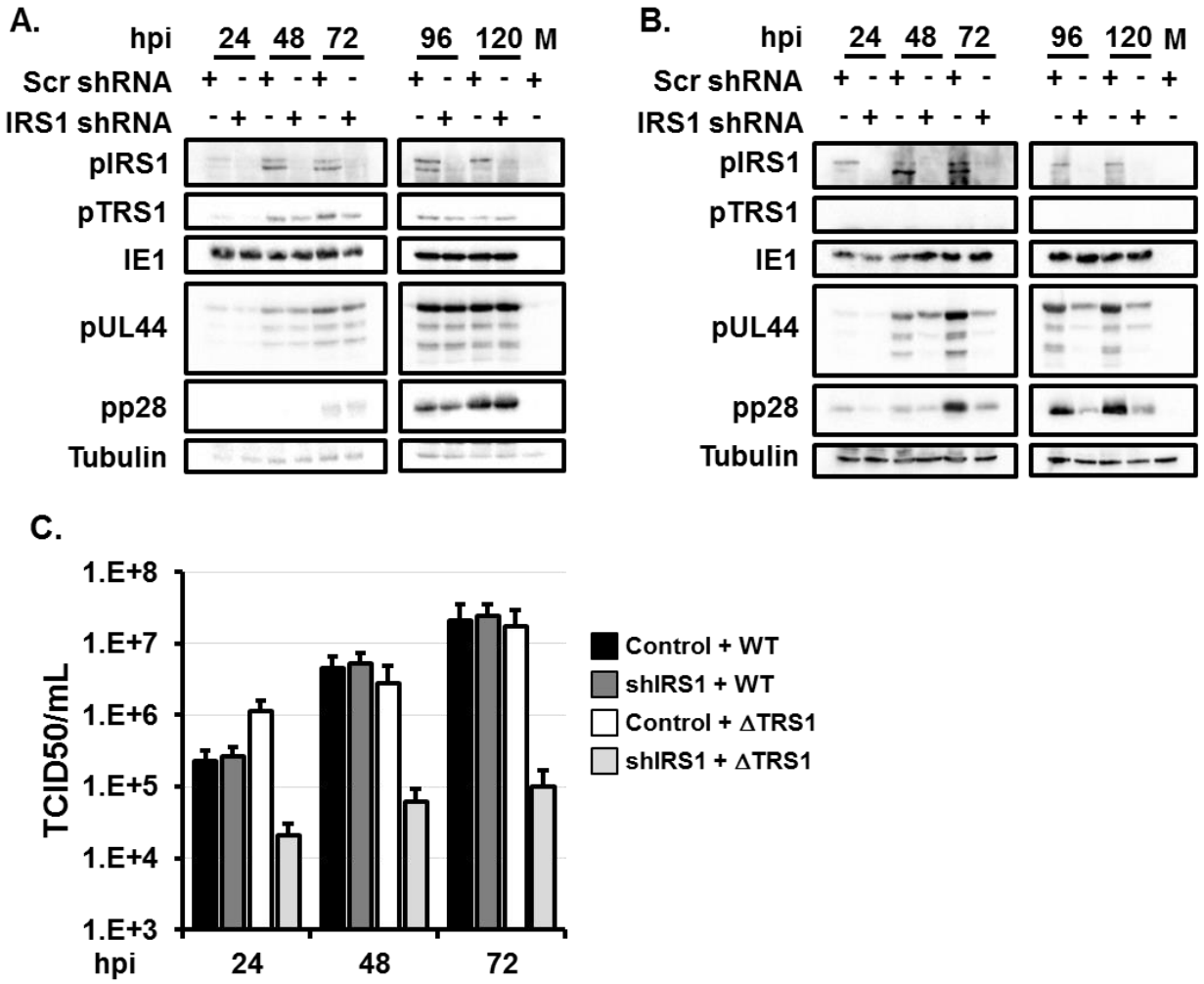


Figure 1 Expression of either pTRS1or pIRS1 is necessary for efficient HCMV replication. A) Control cells (Scr) or shIRS1-HFs were infected with wild type HCMV at a multiplicity of three. Cells were harvested at the indicated times and analyzed by Western blot analysis (n=3) B) Control and shIRS1-HFs were infected with HCMV lacking TRS1 (HCMVΔTRS1) at a multiplicity of three. Cells were harvested at the indicated times and analyzed by Western blot analysis (n=3). C) Cells were infected as in A, and the amount of virus in cell free supernatants was measured by the TCID50 method (n=3).

pTRS1 and pIRS1 inhibit the antiviral kinase PKR in the context of vaccinia virus infection and in transfected cells (13, 14, 49). To confirm that pTRS1 and pIRS1 similarly limit

PKR activation during HCMV infection, we measured PKR autophosphorylation and phosphorylation of the PKR substrate eIF2α after infection of control or shIRS1-HFs with wild type or HCMVΔTRS1 virus. Infection with wild type virus did not induce PKR or eIF2α phosphorylation in either cell type (Fig. 2A), demonstrating that the expression of pTRS1 is sufficient to limit PKR activation. Similarly, neither PKR nor eIF2α were phosphorylated in control cells infected with HCMVΔTRS1, showing that pIRS1 expression is sufficient to suppress PKR. However infection of shIRS1-HFs with HCMVΔTRS1 resulted in robust PKR and eIF2α phosphorylation (Fig. 2A). Consistent with the inhibition of protein synthesis induced by eIF2α phosphorylation, we observed an 80% decrease in the overall levels of protein synthesis when shIRS1-HFs were infected with HCMVΔTRS1 (Fig. 2B). These results demonstrate that the expression of either pTRS1 or pIRS1 is necessary to antagonize PKR activation, limit eIF2α phosphorylation and maintain protein synthesis in HCMV-infected cells.

eIF2α phosphorylation results in the redistribution of actively translating mRNAs into cytoplasmic puncta termed stress granules (2, 8). While stress granules do not form after infection with wild type HCMV (22), the increase in eIF2α phosphorylation after infection in the absence of both pTRS1 and pIRS1 suggested that pTRS1 and pIRS1 might limit stress granule formation during HCMV infection. In response to cellular stress, the G3BP1 protein localizes to cytoplasmic stress granules (42), therefore we used the formation of G3BP1-positive cytoplasmic puncta as a measure of stress granule formation. We found that G3BP1 puncta do not accumulate in control or shIRS1-HFs infected with wild type virus (data not shown). Similarly, few if any G3BP1 puncta formed after infection of control cells with the HCMVΔTRS1 virus (Fig. 2C). In contrast, infection of shIRS1-HFs with HCMVΔTRS1 resulted in the accumulation of G3BP1 puncta (Fig. 2C). Therefore the expression of either pTRS1 or pIRS1 is necessary to prevent stress granule formation during HCMV infection.

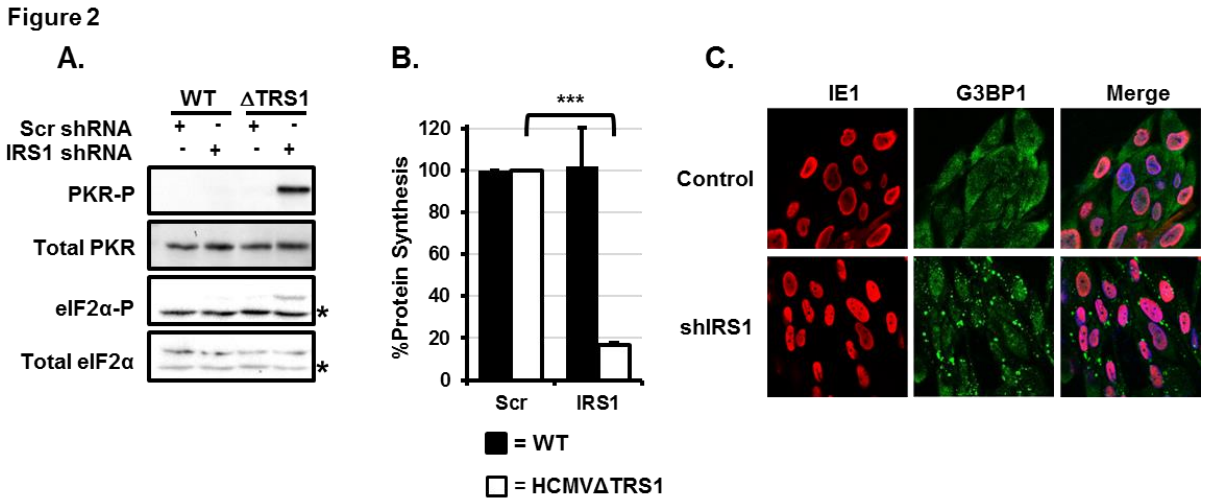


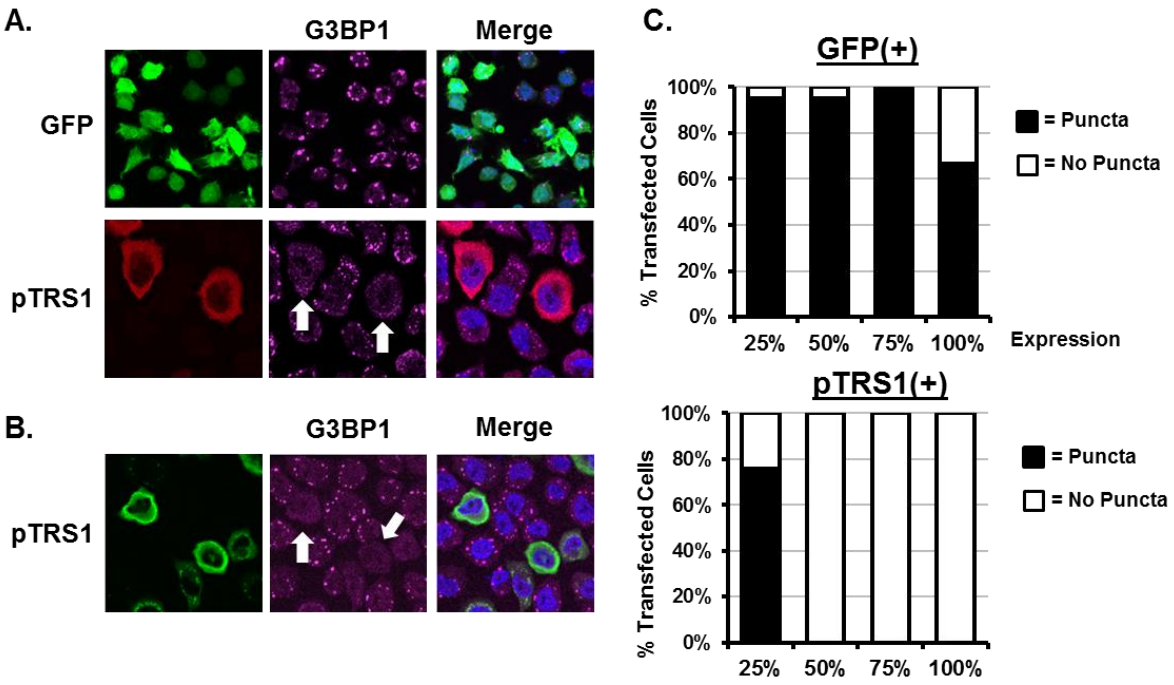
Figure 2 HCMV pTRS1 or pIRS1 is necessary to antagonize PKR, maintain infected cell protein synthesis and inhibit stress granule formation. A) Control (Scr) or shIRS1-HFs (shIRS1) were infected with either wild type virus or HCMVΔTRS1 at a multiplicity of three. Cells were harvested at 24 hours after infection and analyzed by Western blot (n=3). Asterisks indicate non-specific background bands. B) Cells were infected as in A, and the amount of radiolabeled amino acids incorporated into acid-insoluble protein in thirty minutes was quantified at 24 hours after infection (n=3). Filled bars indicate wild type infection and open bars indicate HCMVΔTRS1 infection. C) Control or shIRS1-HFs were infected with HCMVΔTRS1, and the formation of G3BP1 puncta was measured by indirect immunofluorescence at 24 hours after infection. A representative image from one of three independent experiments is shown.

To determine if pTRS1 was sufficient to prevent stress granule formation, we measured the accumulation of stress granules in cells transfected with a pTRS1 expression vector. Cells were transfected with either a pTRS1 expression vector or a control vector expressing GFP, and then treated with arsenite to induce stress granule accumulation. G3BP1 puncta readily accumulated in control cells expressing GFP. However, very few G3BP1-positive granules were found in cells expressing pTRS1 (Fig. 3A). Interestingly, arsenite induces stress granule formation through activation of the eIF2α kinase HRI (32), suggesting that pTRS1 could inhibit

stress granule formation induced by eIF2α kinases other than PKR. To confirm PKR was dispensable for arsenite-induced stress granule formation, we measured G3BP1 puncta formation after arsenite treatment in cells lacking PKR expression. The PKR gene in these cells was mutated using CRISPR/Cas9-mediated mutagenesis (49). Arsenite readily induced stress granule formation in PKR-deficient cells, and this induction was inhibited by pTRS1 expression (Fig. 3B). In order to measure the effect of pTRS1 expression on stress granule formation, we quantified the number of cells containing stress granules in cells expressing pTRS1 or GFP. Stress granules formed in > 90% of cells expressing GFP, regardless of GFP expression levels (Fig. 3C, top panel). In contrast, limited stress granule-positive cells (< 45%) were found in cells expressing low levels of pTRS1. Higher levels of pTRS1 expression were sufficient to completely inhibit stress granule formation (Fig. 3C, bottom panel). Together our results demonstrate that pTRS1 is necessary and sufficient to prevent stress granule formation, and suggest that pTRS1 also prevents stress granule formation induced by additional eIF2α kinases.

Figure 3 pTRS1 is sufficient to inhibit stress granule formation. A) HeLa cells were transfected with a GFP or pTRS1 expression vector. Twenty-four hours after transfection cells were treated with sodium arsenite (0.5 mM) for one hour, and G3BP1 puncta formation was measured by indirect immunofluorescence. B) PKR-deficient HeLa cells were transfected with a GFP or pTRS1 expression vector. Cells were treated with arsenite in A, and analyzed by indirect immunofluorescence as above. C) PKR-deficient cells were transfected and treated as in A. The presence of G3BP1-positive puncta in transfected cells was determined by indirect

Figure 3



immunofluorescence. The range of transgene expression was divided into quartiles (1-25% of maximum, 26-50% of maximum, etc.) and the results are shown as the percentage of GFP (top panel) or pTRS1 (bottom panel) expressing cells containing two or more G3BP1 puncta.

The increase in eIF2α and PKR phosphorylation observed in the absence of pTRS1 and pIRS1 during infection suggested stress granules might form as a consequence of PKR activation. To determine if PKR was necessary for stress granule formation during infection, we measured G3BP1 puncta formation in PKR depleted cells in the absence of pTRS1 and pIRS1. We found that PKR depletion reversed stress granule accumulation in shIRS1-HFs infected with HCMVΔTRS1 (Fig. 4A). Thus pTRS1 and pIRS1 expression counteract PKR-induced stress granule formation during HCMV infection. In addition, PKR depletion increased the expression of HCMV early and late proteins, and prevented eIF2α phosphorylation (Fig. 4B). The increased expression of viral proteins after PKR depletion correlated with an increase in the overall levels of protein synthesis in infected cells (Fig. 4C). Consistent with the increase in protein synthesis, PKR depletion partially rescued HCMVΔTRS1 replication in shIRS1-HFs (Fig. 4D). We conclude

that antagonism of PKR by pTRS1 and/or pIRS1 is necessary for efficient viral protein synthesis and replication.

Figure 4

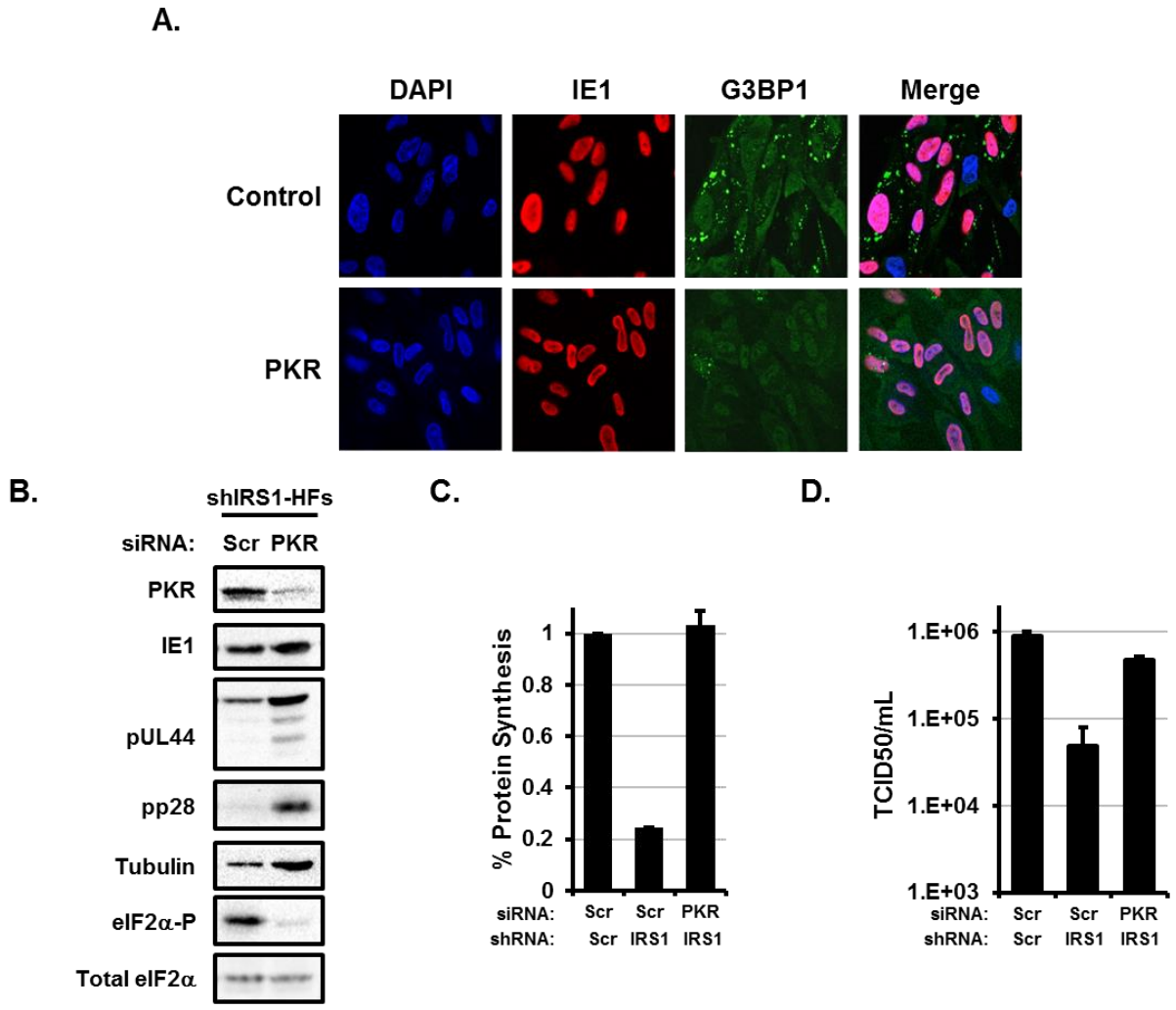


Figure 4 PKR inhibits viral protein synthesis and induces stress granule formation in the absence of pTRS1 and pIRS1. A) shIRS1-HFs were transfected with scrambled or PKR-specific siRNAs prior to infection with HCMVΔTRS1. The formation of G3BP1 (+) puncta was monitored by indirect immunofluorescence at 24 hours after infection. A representative image from one of three independent experiments is shown. B) shIRS1-HFs were transfected with scrambled (scr) or PKR-specific (PKR) siRNAs prior to infection with HCMVΔTRS1. Expression of the indicated

proteins was measured by Western blot at 96 hours after infection, except eIF2α-P and total eIF2α which were measured at 24 hours after infection (n=3). C) Cells stably expressing scrambled (Scr) or pIRS1-specific (IRS1) shRNAs were transfected with scrambled (scr) or PKR-specific (PKR) siRNAs and then infected with HCMVΔTRS1. The amount of radiolabeled amino acids incorporated into acid-insoluble protein in thirty minutes was quantified at 24 hours after infection (n=3). D) Cells were treated and infected as in C. The amount of virus in the culture supernatants at 96 hours after infection was determined by the TCID50 method (n=3).

As discussed above, several pTRS1 functional domains have been described outside the context of HCMV infection (5, 18) including a PKR binding domain in the carboxyl-terminal 116 amino acids of pTRS1 (19). As our system allows for the efficient reconstitution and propagation of pTRS1 mutant viruses, we next determined the role of the pTRS1 PKR binding domain in HCMV replication. We first generated a mutant virus lacking the pTRS1 PKR binding domain (HCMVΔPDB) (Fig. 5A). We used BAC-mediated recombineering to delete the final 116 amino acids of pTRS1, which contains the previously described PKR binding domain (19), and fuse a FLAG epitope to the pTRS1 carboxyl-terminus to allow us to monitor expression of the mutant pTRS1 protein. This mutant expresses pTRS1 from its native location in the viral genome under the control of its endogenous promoter allowing for wild type kinetics and levels of pTRS1 expression. Infectious virus was recovered after electroporation of the mutant genome into primary human fibroblasts, indicating that the HCMVΔPDB virus was viable. The truncated pTRS1 isoform mutant was expressed during infection, although to slightly lower levels than wild type pTRS1 (Fig. 5B). In addition the pTRS1 mutant displayed diffuse cytoplasmic localization, similar to wild type pTRS1 (data not shown).

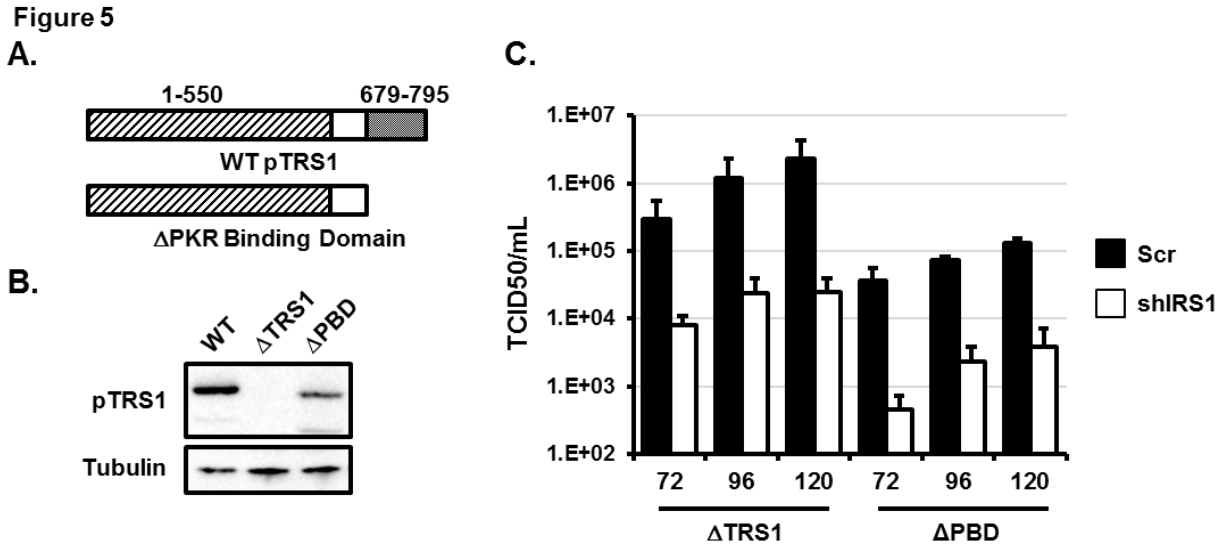


Figure 5 The pTRS1 PKR binding domain is required for efficient virus replication. A) Cartoon showing region of pTRS1 deleted in the HCMVΔPBD virus. B) Western blot showing expression of pTRS1 after infection with wild type HCMV or HCMVΔPBD virus at 72 hours after infection. C) Control (scr; filled bars) or shIRS1-HFs (open bars) were infected with HCMVΔTRS1 or HCMVΔPBD virus at a multiplicity of one, and the amount of cell free virus in the culture supernatants was quantified by the TCID50 method (n=3).

We then measured the replication of the HCMVΔPDB virus over a single round of virus replication. HCMVΔPDB replicated to lower titers than HCMVΔTRS1 on control cells, and virus replication was further reduced by tenfold in the absence of pIRS1 (Fig. 5C). While the HCMV early protein pUL44 and late protein pp28 were expressed in control cells during HCMVΔPDB infection, the expression of both viral proteins was significantly decreased in shIRS1-HFs (Fig. 6A). Interestingly the expression of the mutant pTRS1 protein itself was also significantly reduced in pIRS1-depleted cells. To determine if PKR activation might account for diminished viral protein expression, we measured PKR and eIF2α phosphorylation after infection of control or shIRS1-HFs with the HCMVΔPDB virus. We found no increase in PKR or eIF2α phosphorylation after infection of control cells with HCMVΔPDB (Fig. 6B), indicating that

truncation of the pTRS1 carboxyl-terminus did not result in PKR activation in the presence of pIRS1. However, the HCMVΔPDB virus induced robust PKR and eIF2α phosphorylation when pIRS1 was depleted (Fig. 6B), with a concomitant reduction in the overall levels of protein synthesis (Fig. 6C). In addition HCMVΔPDB infection induced stress granule accumulation in the absence of pIRS1, but not in control cells (Fig. 6D). We conclude that the carboxyl-terminus of pTRS1 is required for efficient virus replication, even in the presence of pIRS1. In addition our results suggest that the pTRS1 PKR binding domain is necessary to antagonize PKR during HCMV infection.

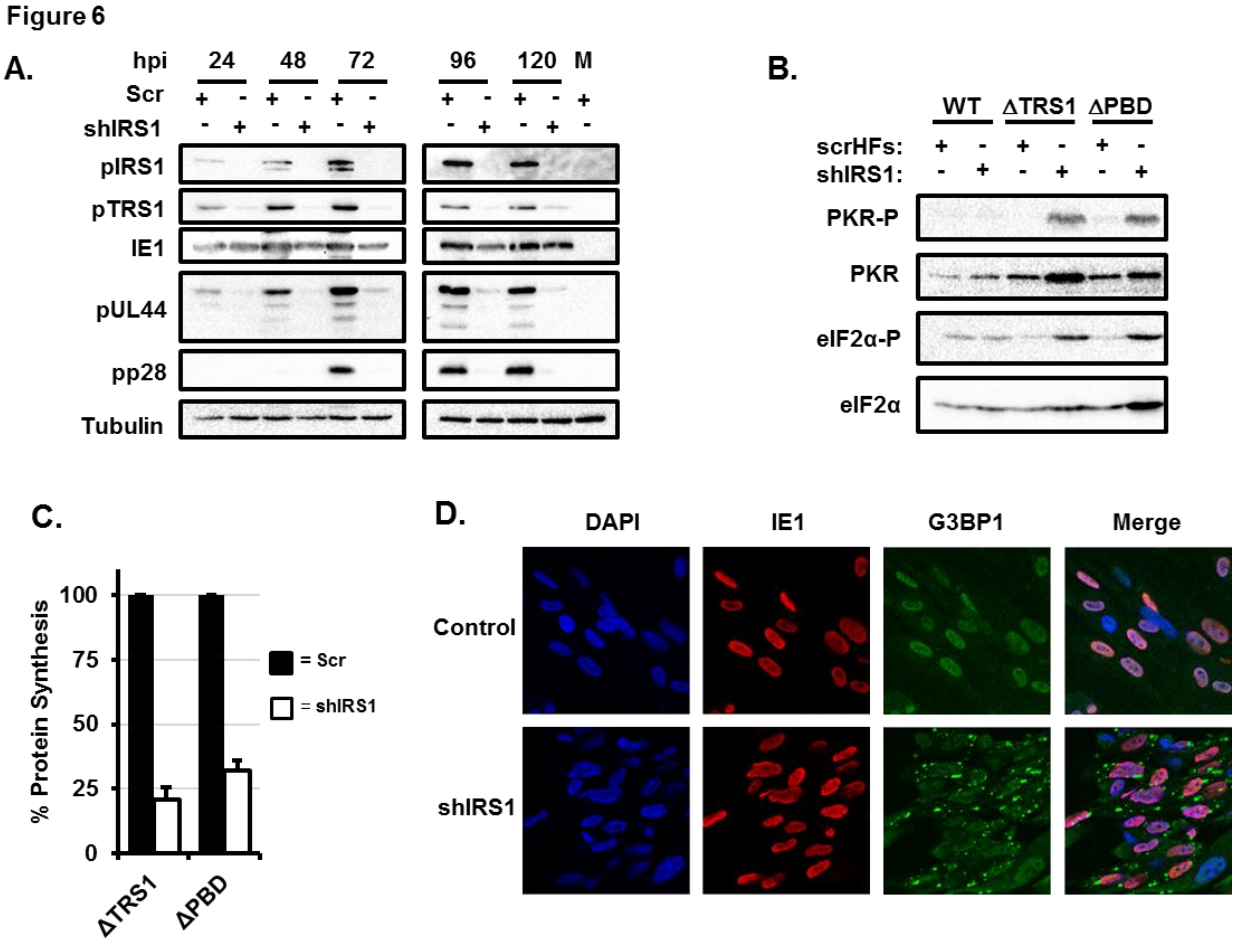


Figure 6 The pTRS1 PKR binding domain is necessary to antagonize PKR in the absence of pIRS1. A) Control (Scr) or shIRS1-HFs (shIRS1) were infected with the

HCMVΔPBD virus at a multiplicity of one. Cells were harvested at the indicated time after infection and the expression of the indicated proteins was measured by Western blot (n=3). B) Control or shIRS1-HFs were infected with the indicated virus as in A. Cells were harvested at 24 hours after infection and analyzed by Western blot (n=3). C) Control (filled bars) or shIRS1-HFs (open bars) were infected with HCMV□TRS1 or HCMV□PBD virus and the amount of radiolabeled amino acids incorporated into acid-insoluble protein in thirty minutes was quantified at 24 hours after infection (n=3). D) Control or shIRS1-HFs were infected with HCMVΔPBD and the presence of G3BP1 puncta was determined by indirect immunofluorescence at 24 hours after infection. Representative images from one of three independent experiments are shown.

Discussion

In this study we developed a novel system for the study of pTRS1 and its functional domains in the context of viral infection. Using this system we confirmed that expression of either pTRS1 or pIRS1 is necessary for efficient HCMV replication, viral protein synthesis and inhibition of the antiviral kinase PKR. We demonstrated that antagonism of PKR by pTRS1 or pIRS1 is critical for HCMV replication, as PKR depletion rescued virus replication and viral protein synthesis in the absence of pTRS1 and pIRS1. We also identified a novel role for pTRS1 as an inhibitor of stress granule formation. Expression of either pTRS1 or pIRS1 was sufficient to suppress PKR-dependent stress granule accumulation during HCMV infection, and pTRS1 alone was sufficient to prevent PKR-independent stress granule formation in transfected cells. The ability of pTRS1 to antagonize PKR and prevent stress granule formation during infection required the previously identified PKR binding domain, suggesting that a direct interaction between pTRS1 and PKR is critical for efficient virus replication.

Our data demonstrate for the first time that pTRS1 and pIRS1 inhibit PKR activation during HCMV infection, and that PKR antagonism is a critical function of pTRS1 and pIRS1

required for efficient viral replication. PKR autophosphorylation and substrate phosphorylation were increased in the absence of both pTRS1 and pIRS1 during infection (Fig. 2A), and overall levels of protein synthesis were greatly reduced (Fig. 2B). As a result virus replication and HCMV protein synthesis were significantly impaired (Fig. 1B,C). Depleting PKR prior to infection rescued HCMV replication (Fig. 4D), restored viral protein expression (Fig. 4B) and overall levels of protein synthesis in the absence of pTRS1 and pIRS1 (Fig. 4C). Therefore our data show that inhibition of PKR is a critical function of pTRS1 and pIRS1 during HCMV infection.

Our data also suggest pTRS1 may have additional roles in HCMV replication. The HCMV∆PDB virus replicated less efficiently than wild type HCMV or a mutant virus lacking the entire TRS1 open reading frame, even in the presence of pIRS1 (Fig. 5C). However the HCMV∆PDB mutant did not induce PKR or eIF2α phosphorylation or stimulate stress granule formation when pIRS1 was present (Fig. 6B,D), suggesting pTRS1 may have additional roles in HCMV replication. Previous studies found that replication of a HCMV pTRS1/pIRS1 double mutant virus was rescued by the vaccinia E3L protein, a known PKR antagonist (31). However E3L also suppresses additional aspects of the antiviral response (16, 17, 24, 26, 30, 44). Perhaps pTRS1 shares these PKR-independent activities with E3L. The pTRS1 truncation could also impact the ability of pTRS1 to facilitate packaging of viral DNA into nascent nucleocapsids, as previously described (1). Alternatively, the truncated pTRS1 isoform might partially inhibit pIRS1 function, resulting in low levels of PKR activation that are sufficient to limit viral mRNA translation. Additional studies in primary human cells completely devoid of PKR expression will be needed to determine what, if any, role pTRS1 plays in HCMV replication in addition to inhibiting PKR activation.

We also identified a novel role for pTRS1 in preventing stress granule formation. Previous studies found that stress granules do not form during HCMV infection (22, 49), despite the presence of dsRNAs (31). Our results provide a molecular mechanism for the lack of stress

granules during HCMV infection, namely the antagonism of PKR by pTRS1 and pIRS1. Stress granules did not form in infected cells expressing either pTRS1 or pIRS1, however the absence of both proteins from infected cells triggered robust stress granule accumulation (Fig. 2C). Thus expression of either pTRS1 or pIRS1 was necessary to prevent stress granule accumulation during HCMV infection. PKR depletion limited stress granule accumulation in infected cells in the absence of pTRS1 and pIRS1 (Fig. 4A), demonstrating that PKR activation triggers stress granule formation during HCMV infection. In addition, expression of pTRS1 outside the context of infection was sufficient to prevent stress granule accumulation in response to arsenite treatment (Fig. 3A), showing that additional viral proteins are not required for this activity. pTRS1 also blocked stress granule accumulation in response to arsenite treatment in PKR-deficient cells (Fig. 3B,C), suggesting that pTRS1 can prevent stress granule accumulation independent of its ability to antagonize PKR. Stress granules inhibit virus replication by sequestering viral RNAs and preventing their translation (4, 8, 45). While additional studies using depletion of specific stress granule components will be required to understand the impact of stress granule accumulation on HCMV infection, our results suggest that stress granules could similarly regulate HCMV protein synthesis.

Our data also provide new insight into the pTRS1 functional domains necessary for PKR inhibition during HCMV infection. The carboxyl-terminal 116 amino acids of pTRS1 contains a PKR binding domain that is necessary to complement growth in a vaccinia virus mutant lacking its major PKR antagonist, the E3L protein (18). Deletion of the pTRS1 PKR binding domain resulted in PKR activation, decreased viral protein expression and limited virus replication in the absence of pIRS1 (Fig. 6). It was previously suggested that pTRS1 inhibits PKR in part by competing with PKR for binding to dsRNA (5). Our finding that PKR was autophosphorylated after infection with the HCMV∆PBD virus does not contradict this conclusion, but rather suggests that during HCMV infection the ability of pTRS1 to bind dsRNA is not sufficient to

antagonize PKR. However we cannot rule out that these mutations alter pTRS1 folding to abrogate its function. Structural analysis of pTRS1 together with PKR will likely provide better resolution into how these domains contribute to PKR inhibition. Nevertheless, in light of the failure of the HCMV∆PBD pTRS1 mutant to antagonize PKR, it seems likely that pTRS1 binding to PKR is critical for PKR inhibition during HCMV infection. Confirmation of this hypothesis will require careful mapping of pTRS1 point mutants that specifically disrupt pTRS1 binding to PKR and the analysis of recombinant viruses carrying these mutations.

In summary, our work identifies inhibition of PKR as a critical function of pTRS1 and pIRS1 necessary for efficient virus replication and protein synthesis.

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Chapter 5: Discussion

Dissertation Summary

In this dissertation I investigated the control of translation during HCMV infection. Previous work detailed the manipulation of eIF4F complex members and eIF4F complex abundance during HCMV infection, but the role of eIF4F in the translation of viral mRNAs was unclear. (1,2) To address the role of eIF4F in the translation of viral mRNAs, I compared the efficiency with which viral mRNAs associated with ribosomes in the presence or absence of the eIF4F complex. I found inhibiting eIF4F complex formation during HCMV infection significantly limited the translation of many host mRNAs, but did not limit the translation of HCMV mRNAs. In addition, I tested the dependence of HCMV replication on eIF4AI/II helicase activity. Using an inhibitor of eIF4AI/II activity I showed that as infection progressed viral protein synthesis and replication became resistant to eIF4AI/II inhibition. These data suggest HCMV mRNAs translate through a non-canonical translation initiation pathway that is independent of the eIF4F complex and eIF4AI/II activity.

In the second chapter of this dissertation I sought to identify potential mRNA m^7G cap binding proteins that could direct eIF4F independent translation during HCMV infection. To identify proteins that interact with the mRNA m^7G cap during HCMV infection I performed a mass spectrometry screen, which identified two viral proteins bound to the mRNA m^7G cap. Both pTRS1 and pIRS1 bound the mRNA cap during infection. I confirmed that pTRS1 binds mRNA during HCMV infection and determined that pTRS1 can bind the mRNA m7G cap independent of the eIF4F complex or its members. In addition I showed that pTRS1 associates with the translation machinery and mRNAs that are undergoing translation during HCMV

infection. I found that pTRS1 stimulated mRNA translation outside the context of infection with preferential effects on specific mRNAs containing viral sequences. In addition, pTRS1 stimulated mRNA translation independent of its previously described capacity to antagonize PKR activation. My data suggests that pTRS1 may be capable of stimulating mRNA translation during HCMV infection in a manner that prefentially benefits viral mRNAs.

In the third chapter of this dissertation I describe for the first time the contributions of pTRS1 independent of pIRS1 in the context of HCMV infection. I built a system to allow the investigation of pTRS1 mutants independent of the confounding effects of pIRS1. In doing so I determined either pTRS1 or pIRS1 is necessary for efficient expression of early and late viral proteins as well as efficient viral replication. I demonstrated that the restriction of HCMV replication in the absence of both pTRS1 and pIRS1 is dependent on PKR. I also observed that during HCMV infection stress granules form as a result of PKR activation when both pTRS1 and pIRS1 are not present in infected cells. Outside the context of infection I confirmed that pTRS1 is sufficient to prevent stress granule formation and can do so independent of PKR. Finally I confirmed that the pTRS1 PKR binding domain is necessary for PKR antagonism during HCMV infection in the absence of pIRS1. My work with pTRS1 in the context of HCMV infection confirms that much of the pTRS1 work done *in vitro* and heterologous systems is relevant during HCMV infection. In addition my work demonstrates that pTRS1 can antagonize eIF2α activation by kinases other than PKR.

Significance of Dissertation

eIF4F Independent Translation of viral mRNAs In this dissertation I sought to determine whether the eIF4F complex is necessary to stimulate viral mRNA translation. To investigate the role of eIF4F in viral mRNA translation I limited eIF4F complex formation by inhibiting the cellular kinase mTOR. mTOR stimulates eIF4F formation and activity through

multiple mechanisms, one of the most robust mechanism by which mTOR maintains eIF4F activity is the phosphorylation of 4EBP1. Active mTOR phosphorylates 4EBP1, thereby limiting the binding of 4EBP1 to eIF4E. However, when mTOR is inhibited 4EBP1 binds eIF4E and prevents eIF4F formation. (See Introduction) When eIF4F was disrupted early in infection I observed a significant reduction in viral protein synthesis and viral replication. However, both HCMV protein synthesis and viral replication became resistant to eIF4F disruption as infection progressed. In fact, when I directly measured the association of viral mRNAs with ribosomes in the absence of the eIF4F complex I observed no defect in viral mRNA translation. In contrast the translation of host mRNAs known to be dependent on eIF4F for efficient translation decreased significantly in the absence of the eIF4F complex. The increasing resistance of viral mRNA translation and replication likely indicates a reduction in reliance on host factors that are translated in an eIF4F dependent manner. My data, therefore, suggests that the translation of viral mRNAs is independent of the eIF4F complex.

To confirm that viral mRNAs do not utilize the eIF4F complex for translation initiation I also suppressed the activity of eIF4AI/II and measured the efficiency of viral mRNA translation in the absence of eIF4AI/II activity. Inhibiting the eIF4AI/II RNA helicase limits the ability of eIF4F to scan through RNA secondary structure and thereby reduces eIF4F dependent translation. (3) Similar to preventing eIF4F formation I observed a significant reduction in HCMV replication when eIF4AI/II is inhibited at immediate-early and early times post infection. However, when eIF4AI/II is inhibited at late times post infection the translation of viral mRNAs and HCMV replication is unaffected. This supports my hypothesis that eIF4F activity is not necessary for the translation of viral mRNAs.

Through two different approaches I have shown that eIF4F activity is not necessary for the translation of viral mRNAs. I therefore, suggest that a unique complex specific to viral mRNAs directs the translation initiation of HCMV mRNAs. I argue that the mechanism of

translation initiation is specific to viral mRNAs because host mRNAs that normally rely on eIF4F for efficient translation showed a reduced rate of translation in the absence of eIF4F. Therefore the mechanism promoting the translation of HCMV mRNAs is not a general modification of translation initiation, but rather a process that can differentiate between host and viral mRNAs.

A translation initiation process with specificity for viral mRNAs argues that viral proteins are necessary to direct the process, but how would these proteins affect translation initiation? I envision two possible mechanisms that may account for the specific recognition and translation of viral mRNAs. First, there may be viral proteins that recognize viral mRNAs and stimulate their association with ribosomes. In this model a viral protein would exhibit greater affinity for viral mRNAs as compared to host mRNAs. Therefore, a protein with the ability to recognize specific viral sequences or structures seems likely. For a viral protein to be a candidate to fulfill this model it must also associate with the translation machinery, as binding viral mRNAs is not sufficient to promote translation initiation. In order to recruit a ribosome viral proteins could bind translation initiation factors at a variety of stages leading to 48S formation. For example, following binding of viral mRNAs a viral protein could then interact with eIF3 to recruit the 43S preinitiation complex, alternatively, a viral protein could directly bind the 40S ribosomal subunit in a 43S preinitiation complex to stimulate translation. Based on my data I argue that this process would not recruit the entire eIF4F complex. Unless the viral protein directing translation initiation nucleates an eIF4F-like complex that includes 4EBP1 thus accounting for continued translation despite signaling that stimulates 4EBP1 to suppress translation initiation.

My second model to explain the translation of HCMV mRNAs independent of the eIF4F complex relies on host factors that recognize viral mRNAs. Perhaps instead of a viral protein that specifically recognizes HCMV mRNAs, a viral protein could hijack a host pathway that already specifically binds viral mRNAs. For example, host proteins that sense viral RNA could be redirected to stimulate translation initiation instead of suppressing viral replication. One

potential host factor that could act in this way is PKR. PKR recognizes viral RNAs in order to activate host defenses that suppress viral replication. However, a viral protein that binds PKR could block PKR signaling as well as preferentially associate with viral mRNAs. Following binding to and suppression of PKR the hypothetical viral protein could interact with translation initiation factors to stimulate mRNA translation.

Roles of pTRS1 Independent of PKR Antagonism In chapter two I identified a viral protein, pTRS1, that interacts with both viral mRNAs and host translation machinery during HCMV infection. I then characterized the ability of pTRS1 to stimulate translation outside the context of infection. pTRS1 alone is sufficient to stimulate the translation of luciferase reporters and preferentially stimulates the translation of an mRNA containing specific viral sequences. pTRS1 is known to antagonize PKR activation, therefore I tested whether pTRS1 can stimulate translation in the absence of PKR. Importantly, pTRS1 stimulated reporter translation in cells where PKR was deleted from the cellular genome. This demonstrates that pTRS1 has pro-translational roles that are independent of PKR antagonism.

While I have not yet determined the mechanism by which pTRS1 stimulates translation, my data shows pTRS1 is in the proper context to directly stimulate translation. pTRS1 binds mRNAs and translation machinery in a manner that may position pTRS1 to stimulate translation. In the context of infection and outside of infection pTRS1 binds mRNAs as well as the mRNA m7G cap. The interaction of pTRS1 with mRNAs may allow recruiting of translation initiation factors to stimulate mRNA translation. In addition pTRS1 co-migrates with the 40S ribosomal subunit in cytoplasmic extracts from infected cells that were resolved through sucrose gradients suggesting that pTRS1 and the 40S associate in a complex during HCMV infection. If pTRS1 binds the 40S ribosomal subunit and mRNA at the same time it may stimulate the recruitment of the 40S subunit directly to mRNAs.

These data are consistent with the pTRS1 stimulating translation as described in the first model in the previous section. pTRS1 is a viral protein that binds mRNA and preferentially stimulates translation of mRNAs containing a viral sequence. pTRS1 also associates with the translation machinery. Perhaps pTRS1 specifically binds viral mRNA and recruits initiation factors to stimulate mRNA translation. However, the mechanism by which pTRS1 stimulates mRNA translation remains unclear and requires additional investigation, as discussed below.

System to Evaluate pTRS1 mutants The functional redundancy of pTRS1 and pIRS1 has made investigation of pTRS1 and pIRS1 mutants in the context of HCMV infection difficult. Either pTRS1 or pIRS1 is necessary and sufficient for HCMV replication. (4) Therefore the effect of any mutagenesis in either protein is obscured by the other. If both genes are deleted the virus fails to grow and cannot be complemented by exogenous pTRS1 or pIRS1. (4) To address this problem I designed a system to conditionally knock down pIRS1 expression. Using cells stably expressing shRNAs targeted to IRS1 (shIRS1) I can specifically deplete infected cells of pIRS1. This approach allows me to use wild type HCMV and a strain of HCMV lacking TRS1 (HCMVΔTRS1) to study the contribution of either pIRS1, pTRS1 or both to HCMV infection. Infecting control cells with HCMVΔTRS1 allows the study of pIRS1 in isolation, while the infection of shIRS1 cells with wild type HCMV allows the study of pTRS1in isolation. I can also study the absence of both pTRS1 and pIRS1 by infecting shIRS1 cells with HCMVΔTRS1. Under normal conditions HCMV genomes lacking both TRS1 and IRS1 can't reconstitute infectious virus, however, one can now study infection in the absence of either TRS1 or IRS1 by preventing IRS1 expression during infection with HCMV strains that lack TRS1.

Furthermore, one can now study domain mutants of pTRS1. Traditionally constructing a deletion mutant in TRS1 would not yield a phenotype as IRS1 is sufficient for HCMV replication. However, now one can use the a TRS1 mutant genome to reconstitute a viral stock at wild type level due to the presence of IRS1 and then infect shIRS1 cells to prevent pIRS1 accumulation,

which reveals any phenotype specific to a mutant pTRS1. Therefore, the generation shIRS1 cells now permit the study of pTRS1 mutants in the context of HCMV infection rather than in heterologous systems.

To demonstrate the effectiveness of the shIRS1 system I detailed the replication of an HCMV strain expressing pTRS1 lacking a previously defined PKR binding domain (pTRS1ΔPBD). In chapter two of this dissertation I show that pTRS1ΔPBD is sufficient to antagonize PKR activation from transfection stress outside of HCMV infection. However, I wanted to determine if the PKR binding domain was necessary to antagonize PKR is a more relevant system, that of HCMV infection. Using shIRS1 cells I show in chapter three that pTRS1ΔPBD is unable to antagonize PKR during HCMV infection. This data is the first direct test of the domains necessary for pTRS1 to antagonize PKR during HCMV infection. In addition my work clearly shows that the functions of pTRS1 outside the context of HCMV infection do not necessarily correlate with the functions of pTRS1 during HCMV infection. Developing the shIRS1 system provides an opportunity to study pTRS1 in the relevant context and has already provided insight into the role of pTRS1 during HCMV infection.

Antagonism of PKR and eIF2α Activation by pTRS1 pTRS1 has been extensively studied in the context of vaccinia virus infection. Vaccinia virus lacking its PKR antagonist, E3L, replicates poorly in many cell types. (5) pTRS1 expression is sufficient to rescue E3L deficient vaccinia virus replication. (6) While pTRS1 antagonizes PKR during Vaccinia virus infection, the capacity of pTRS1 to antagonize PKR during HCMV infection has not been shown. Furthermore, E3L antagonizes other host innate immune pathways such as TLR signaling, interferon production and viral DNA sensing. (7) Therefore it is unclear whether pTRS1 antagonism of PKR is the function of pTRS1 necessary to rescue vaccinia virus lacking E3L.

In addition, the work done with HCMV strains lacking both pTRS1 and pIRS1 did not directly test the role of PKR in suppressing HCMV replication. (4) The authors attribute the defect in HCMV replication to a cessation of protein translation in infected cells. They demonstrate a profound inhibition of protein synthesis, which they attribute to the significant eIF2α phosphorylation observed. However, a role for PKR in activating eIF2α was not demonstrated. So while much is known about pTRS1 antagonizing PKR during vaccinia virus infection and HCMV replication in the absence of pTRS1, the contribution of PKR antagonism by pTRS1 to HCMV replication has not been determined. In this dissertation I sought to directly test the role of pTRS1 in PKR antagonism during HCMV infection and the domains of pTRS1 necessary to antagonize PKR.

To investigate the relevance of PKR antagonism during HCMV infection I infected shIRS1 cells with an HCMVΔTRS1 effectively generating a pTRS1 and pIRS1 deficient infection. Consistent with previously published work I observe a significant shut off of protein synthesis during HCMV infection lacking both pTRS1 and pIRS1. I also noted increased PKR autophosphorylation and eIF2α phosphorylation in shIRS1 cells infected with HCMVΔTRS1. Both PKR autophosphorylation and eIF2α phosphorylation are indicative of innate immune sensing of viral dsRNA and signaling that should limit protein synthesis. HCMVΔTRS1 infection of shIRS1 cells gave rise to reduced early viral protein accumulation and failed to express late viral proteins. Unsurprisingly, HCMVΔTRS1 failed to efficiently replicate and gave rise to reduced levels of cell free infectious virus when infecting shIRS1 cells.

To confirm that PKR restricts HCMV replication when pTRS1 and pIRS1 are absent I depleted PKR from shIRS1 cells prior infection with HCMVΔTRS1. HCMVΔTRS1 replicates in pIRS1 cells nearly as well as in control cells when PKR is depleted from shIRS1 cells prior to infection. Knocking down PKR rescued viral protein expression and replication of HCMVΔTRS1 in the absence of IRS1. Importantly, HCMVΔTRS1 infection of shIRS1 cells lacking PKR did

not lead to eIF2α phosphorylation. Preventing eIF2α phosphorylation by removing PKR from the system demonstrates for the first time that PKR is the relevant eIF2α kinase during HCMV infection. My results also clearly show that either pTRS1 or pIRS1 is necessary to prevent PKR activation and subsequent inhibition of protein synthesis. These data confirm for the first time that pTRS1 antagonism of PKR is necessary for efficient HCMV replication.

In the process of characterizing the translational shutoff I observed during HCMVΔTRS1 infection of shIRS1 cells I noticed the accumulation of stress granules. Stress granules are storage compartments for mRNAs that failed to initiate translation as a result of global inhibition of protein synthesis. (8) Stress granules can be induced by PKR activation and eIF2α phosphorylation. (9) Previous work by the Alwine lab has shown that stress granules do not normally form in HCMV infected cells despite dsRNA that should activate PKR. (10) Intrigued by the idea that pTRS1 antagonizes stress granule formation I investigated whether pTRS1 was sufficient to prevent stress granule formation outside the context of infection. Not only does pTRS1 block stress granule formation outside of infection, but pTRS1 can do so independent of PKR and in response to activation of eIF2α kinases other than PKR. Using HeLa cells deficient for PKR I found that pTRS1 can prevent stress granule formation in response to arsenite treatment. Arsenite activates another eIF2α kinase, HRI, as a result of reactive oxygen species leading to the phosphorylation of eIF2α and stress granule formation. (11) These data demonstrate that pTRS1 can either antagonize multiple eIF2α kinases or directly antagonize eIF2α. Alternatively, pTRS1 may directly interact with stress granule factors to prevent stress granule formation.

A role for pTRS1 in antagonizing multiple eIF2α kinases could also represent a redundant approach by HCMV to maintaining protein synthesis. HCMV infection induces multiple cellular stresses that should result in eIF2α phosphorylation. In particular late in infection the cellular unfolded protein response (UPR) is activated and is necessary for efficient

HCMV replication. (12) The UPR is a cellular feedback mechanism that is activated when the protein folding capacity of the endoplasmic reticulum is overwhelmed. (13) As a result of the UPR the eIF2α kinase PERK is activated to suppress translation through eIF2α phosphorylation. Despite UPR signaling eIF2α does not become phosphorylated. If pTRS1 can antagonize the UPR dependent eIF2α kinase PERK similar to its ability to antagonize PKR and HRI, pTRS1 may explain the lack of eIF2α phosphorylation in the face of UPR activation. Another eIF2α kinase that is likely activated by HCMV infection is GCN2, general control nonderepressible 2. GCN2 senses amino acid depravation and phosphorylates eIF2α to stall translation and allow accumulation of amino acids. While the state of GCN2 in HCMV infected cells has not been investigated the high rates of protein synthesis in HCMV infected cells makes it likely that GCN2 may be activated during infection. Here again pTRS1 may be necessary to prevent eIF2α phosphorylation.

While pTRS1 may antagonize other eIF2α kinases it is important to note that suppressing PKR activation is likely the dominant function of pTRS1 during HCMV infection. Depleting PKR from HCMV infected cells lacking both pTRS1 and pIRS1 restored the majority of HCMV replication. So any role of pTRS1 in antagonizing other eIF2α kinases is likely secondary to antagonizing PKR. However, the small defect in HCMV replication still observed in shIRS1 cells depleted of PKR may be the result of other eIF2α kinases suppressing HCMV replication. Testing the capacity of pTRS1 to antagonize PERK and GCN2 activation will be necessary to explore the hypothesis that pTRS1 can antagonize multiple eIF2α kinases.

Areas for Further Investigation

Translation of Viral mRNAs Independent of eIF4F While I have strong evidence that the host eIF4F complex is not involved in the translation of viral mRNAs at least late in infection, I have not determined the mechanism by which viral mRNAs associate with ribosomes.

Identifying the factors, host or viral, that direct ribosome recruitment to viral mRNAs would provide excellent targets for antiviral drug development. Inhibiting the viral mechanism by which HCMV mRNAs associate with ribosome would potently limit HCMV replication with limited off target effects as the mechanism of ribosome recruitment may be unique to viral mRNAs. In the following paragraphs I will discuss a number of potential mechanisms that viral mRNAs may utilize to recruit ribosomes.

Host mRNAs are not entirely reliant on eIF4F for the recruitment of ribosomes. Another mRNA m^7G cap dependent translation initiation pathway has been described, one that stimulates the pioneer round of translation. The first round of translation for an mRNA is termed the pioneer round of translation. (14) The pioneer round functions as a quality control mechanism for mRNA splicing. (15) One of the first steps in the pioneer round of translation is the association of the nuclear mRNA m^7G cap binding complex with mRNAs. (16) The nuclear cap binding complex is composed of CBP20 and CBP80, the CBC, and similar to eIF4F stimulates the association of a ribosome with mRNAs. A role for the CBC in promoting the translation of viral mRNAs has not been investigated. Following the pioneer round subsequent translation initiation events are stimulated by eIF4F.

While the contribution of the CBC or even the pioneer round of translation to the translation of viral mRNAs is unknown, the CBC and pioneer round may limit the reliance of HCMV mRNAs on the eIF4F complex. If viral mRNAs do undergo the pioneer round of translation perhaps they continue to utilize a CBC on the mRNA m^7G cap instead of switching to eIF4F for subsequent rounds of translation. Continued utilization of the CBC would likely require additional host or viral factors. One or more factors would be necessary to prevent eIF4F from binding to viral mRNAs. In addition to keeping the CBC in the cytoplasm, host or viral factors would also be necessary to stimulate CBC binding of viral mRNAs in the cytoplasm.

Alternatively, HCMV mRNAs may possess unique structures to recruit and stabilize an interaction with the CBC to promote CBC dependent translation.

Another possible eIF4F independent mechanism HCMV may use to recruit ribosomes to viral mRNAs could be the usage of eIF4F components separate from the eIF4F complex as a whole. The data in chapter one shows that eIF4F as a complex is not necessary for viral mRNA translation. In addition, I also show that the helicase activity of eIF4AI/II is not required. My data does not evaluate any role for eIF4E, eIF4G or a non-helicase role for eIF4AI/II in the translation of viral mRNAs. Therefore, it is possible that HCMV mRNAs may utilize eIF4F members to stimulate translation initiation. One possible mechanism that would fit a model where HCMV mRNAs use only eIF4G to stimulate translation initiation is the binding of viral mRNAs by a viral factor that then recruits eIF4G to direct translation initiation. In this model viral RNAs are preferentially bound by a viral protein (potentially pTRS1), that viral protein then recruits eIF4G to the mRNA. Following eIF4G binding, translation initiation could proceed as normal with 43S binding.

The majority of host mRNA translation occurs through eIF4F, but many viruses utilize only individual eIF4F components. In most cases where only a portion of eIF4F is used it is in combination with an mRNA m^7G cap independent translation initiation mechanism such as an Internal Ribosome Entry Site (IRES). (17) RNA viruses commonly utilize IRES driven translation to avoid host control of translation initiation by only relying on pieces of eIF4F rather than the eIF4F complex. The extent of eIF4F independent translation late in HCMV infection makes it unlikely that IRESs explain all of the eIF4F independent translation. Nearly every viral mRNA efficiently associated with polysomes despite eIF4F disruption. If each HCMV mRNA contained an IRES element that would increase the number of described IRESs greater than 10 fold. Such widespread IRES usage seems unlikely, considering only one IRES like element has been

described in the HCMV transcriptome. However, IRES driven translation may account for a portion of eIF4F independent translation during HCMV infection.

Another possible role for eIF4F complex members independent of the eIF4F complex may be through a unique mRNA binding complex specific to infected cells. Multiple HCMV proteins have been identified as RNA binding proteins. Viral proteins that bind mRNAs may nucleate a viral specific translation initiation complex. The HCMV proteins pUS22, pp71, pUL69, pIRS1 and pTRS1 all bind RNA during HCMV infection. (18) Only pUL69 is known to interact with host translation initiation factors, PABP specifically. pUL69 similarly to pTRS1 and pIRS1 also binds the mRNA m^7G cap. However if any HCMV protein interacts with both viral mRNAs and host translation initiation factors that protein could nucleate a viral specific translation initiation complex and thereby direct eIF4F independent translation initiation. A viral factor nucleating a translation initiation complex would also allow for specificity in directing which mRNAs translate independent of eIF4F. A unique complex with specificity for viral mRNAs is consistent with my data as viral mRNAs continued to translate following eIF4F disruption, but host mRNAs known to be dependent on eIF4F for their translation were inhibited. Therefore any HCMV protein nucleating an initiation complex likely specifically interacts with viral mRNAs.

Contribution of mTOR Signaling to HCMV Replication mTOR activity promotes a plethora of anabolic processes, all of which could be necessary for HCMV replication. mTOR stimulates glucose metabolism, lipid synthesis, limits autophagy, prevents apoptosis and stimulates eIF4F dependent translation. (19, 20) Previous work demonstrated that HCMV utilizes multiple mechanisms to maintain mTOR activity during HCMV infection indicating that mTOR activity is critical for efficient HCMV replication. (1,2) Indeed directly inhibiting mTOR kinase activity significantly limits HCMV replication. (21) The mechanism of the inhibition of HCMV replication in the absence of mTOR activity has not been identified. My work

demonstrates that mTOR signaling does not promote the translation of viral mRNAs, but previous work clearly indicates mTOR signaling is necessary for efficient HCMV replication. So how might mTOR signaling promote HCMV replication, other than through eIF4F dependent translation of viral mRNAs? mTOR activity stimulates breakdown of glucose, production of nucleic acids and lipid synthesis. Given the high rate of glucose utilization and lipid production in HCMV infected cells it seems likely HCMV requires mTOR signaling to promote metabolic processes. (22) In support of this hypothesis work in the Shenk and Munger labs has shown lipid synthesis to be necessary for efficient HCMV replication. During HCMV infection internal membranes are rearranged to support viral assembly in a process that is dependent on de novo lipid synthesis. (23) In addition synthesis of long chain fatty acids are necessary for efficient viral infectivity. As HCMV is a large dsDNA virus, the accumulation of nucleic acids necessary for HCMV genome amplification is also likely crucial for efficient viral replication. All these observations point to a role for mTOR in promoting a metabolic state optimized for HCMV replication.

Another, but not exclusive hypothesis is that mTOR activity is necessary for the translation of host factors essential for HCMV replication. Previously published data shows that HCMV induces the accumulation of eIF4F complex members and the activity of eIF4F in an mTOR dependent manner. (1,2) This suggests that the eIF4F complex is important for efficient HCMV replication. Consistent with this hypothesis my data show that host mRNAs remain dependent on eIF4F for mRNA translation. Host factors necessary for HCMV replication could play roles in viral transcription or viral genome amplification. If eIF4F dependent translation is necessary for the expression of host factors critical for HCMV replication, the host factors required likely act in the immediate early or early phases of the viral life cycle as inhibiting mTOR late in infection did not limit HCMV replication. While I have ruled out a role for mTOR

signaling in promoting viral mRNA translation the current data suggests eIF4F dependent translation likely contributes to HCMV replication through the translation of host mRNAs.

Roles for pTRS1 Independent of PKR Antagonsim pTRS1 is considered a PKR antagonist. (24,25) My work in chapter three has finally confirmed that PKR indeed restricts HCMV replication and that pTRS1 or pIRS1 is necessary to antagonize PKR during HCMV infection. However, considering my data in chapter two pTRS1 clearly is capable of other roles outside of antagonizing PKR. pTRS1 can preferentially stimulate the translation of reporters containing viral 5' UTRs as compared to reporters containing host sequences. If antagonizing PKR was solely responsible for the increase in translation I would not expect to observe a difference in the extent to which pTRS1 stimulated translation. In addition, pTRS1 can still increase the cellular rate of protein synthesis and stimulate translation of reporters in PKR deficient cells. I hypothesize that through interactions with the cellular translation machinery pTRS1 can directly stimulate the translation of pTRS1 associated mRNAs. I envision two potential mechanisms by which pTRS1 may promote mRNA translation.

My first potential mechanism to explain the PKR independent pro-translational roles of pTRS1 is that pTRS1 bridges an interaction between viral mRNAs and the 40S ribosomal subunit to initiate mRNA translation. There are two features of pTRS1 biology that I believe strongly support this mechanism. First, pTRS1 binds mRNA though a unique RNA binding domain in the amino half of pTRS1. While no specificity is attributed to this RNA binding domain my data indicates that pTRS1 can preferentially stimulate the translation of viral mRNAs as compared to constructs not containing viral sequences. Therefore, I propose that the RNA binding domain does in fact preferentially interact with viral mRNAs allowing pTRS1 to differentiate between viral and host mRNAs and through subsequent interactions with the translation machinery promote translation initiation.

The second piece of pTRS1 biology that supports a mechanism by which pTRS1 directly recruits ribosomes to viral mRNAs is that pTRS1 likely associates with 40S ribosomal subunits. I have not formally tested whether the co-migration I observed is a direct interaction or if pTRS1 binds one of the many co-factors associated with ribosomal subunits, such as eIF3 or eIF2. However, if pTRS1 can manipulate the positioning of the 40S subunit it is logical to conclude that pTRS1 could bring 40S ribosomal subunits to mRNAs, specifically viral mRNAs, to stimulate translation initiation.

My second potential mechanism by which the PKR independent pro-translational role of pTRS1 may be accomplished is that pTRS1 may be part of an mRNA m^7G cap binding complex that is unique to HCMV infected cells. While it is an attractive hypothesis that pTRS1 alone can bridge mRNAs and ribosomes I have no direct evidence for it and based on host translation initiation many functions are needed to efficiently stimulate translation. Therefore it is likely that host factors other than eIF4F assist in stimulating viral mRNA translation initiation. In other herpes viruses there is evidence of alternative translation initiation factor usage. For example, in HIV infected cells viral usage of non-canonical translation initiation factors has been described. The host RNA helicase DHX29 is utilized by viral mRNAs to avoid reliance on eIF4F and specifically eIF4AI/II. (26) It seems likely that HCMV mRNAs also utilize alternative translation initiation factors to translate independent of eIF4F and that pTRS1 through preferentially interacting with viral mRNAs could nucleate a unique translation initiation complex.

Another approach pTRS1 may take to stimulate mRNA translation is the nucleation of a translation initiation complex composed of viral proteins. The Shenk and Moorman labs have also shown that in addition to pTRS1, pUL44 and pUL69 associate with polysomes during HCMV infection. (unpublished observation) pUL69 associates with nuclear export factors to stimulate nuclear export of viral mRNAs as well as binding to PABP. (27,28) pUL44 plays roles in HCMV genome amplification, but has not been described to associate with mRNA translation.

(29) One intriguing mechanism by which pTRS1 and pUL69 may stimulate the translation of viral mRNAs is by bridging the mRNA m^7G cap and the mRNA poly A tail. As reviewed in the introduction host mRNAs are though to circularize through an interaction between eIF4G and PABP. This brings the mRNA m^7G cap in close proximity to the poly A tail, effectively circularizing the mRNA and promoting mRNA translation. Perhaps a similar mechanism occurs between pTRS1 and pUL69, such that pTRS1 binds the mRNA m^7G cap and pUL69 binds the poly A tail, through PABP. The two viral proteins could then interact to circularize the mRNA and stimulate mRNA translation similar to host mRNAs. I have not yet directly tested this hypothesis, but it provides a novel function to both pTRS1 and pUL69 and would limit the reliance of viral mRNAs on eIF4F.

Regardless of whether pTRS1 directly recruits ribosomes to viral mRNAs or is part of a virus specific cap binding complex, pTRS1 does associate with mRNAs that are undergoing translation initiation in HCMV infected cells. While artificially inducing stress granule formation I observe pTRS1 relocalizing to distinct cytoplasmic puncta that co-stain with markers of stress granules. Only mRNAs undergoing translation initiation relocalize to stress granules as stress granules form when eIF2α is phosphorylated and initiation is inhibited. Elongating ribosomes simply finish translating following eIF2α phosphorylation. No 60S ribosomal subunits or 80S ribosomes localize to stress granules. Therefore, my data indicates that pTRS1 binds mRNAs prior to translation elongation and that pTRS1 is likely modulating translation initiation.

While my data suggest pTRS1 promotes translation and is in the proper context to stimulate translation initiation there are alternative explanations for the pro-translational activities of pTRS1. The most likely alternative explanation is that pTRS1 antagonizes another host antiviral pathway that restricts HCMV mRNA translation. Such roles for pTRS1 have been proposed before, but not described to date. (24) If pTRS1 inhibits a cellular pathway that restricts HCMV mRNA translation that pathway likely influences mRNA transcription, stability or

association of mRNAs with ribosomes. One of these effects is necessary to explain the increase in translation I observe following pTRS1 expression. If the host pathway pTRS1 antagonizes regulates mRNA transcription or mRNA stability I would expect to see an increase in the abundance of reporter mRNA following pTRS1 inhibition of that pathway. To control for changes in transcription in the presence of pTRS1 I monitored the abundance of reporter mRNA and did not detect any significant increase in reporter mRNA abundance when pTRS1 was co-transfected. Therefore it seems unlikely pTRS1 blocks a host pathway that limits mRNA transcription or mRNA stability.

Another host pathway that could restrict translation is interferon-induced proteins with tetratricopeptide repeats (IFIT). IFITs are interferon stimulated genes that sense or inhibit viral infection. (30) At least one particular IFIT, p56, has known roles in suppressing mRNA translation. (31) Following binding of viral ligands, including dsRNA, p56 associates with eIF3 and limits 43S ribosomal subunit formation. Perhaps p56 is detecting my reporters containing viral sequences and subsequently suppressing translation. If pTRS1 is able to either; prevent sensing of dsRNA by p56, block association of p56 with eIF3 or execute a mechanism of translation initiation independent of eIF3, then pTRS1 inhibition of p56 may explain the PKR independent pro-translational role of pTRS1.

pTRS1 Inhibition of Additional eIF2α Kinases In chapter three I found that pTRS1 prevents stress granule formation after activation of the eIF2α kinase, HRI. Similar to PKR HRI phosphorylates eIF2α to limit protein synthesis. (11) This observation is crucial as it demonstrates the ability of pTRS1 to prevent the phosphorylation of eIF2α, independent of PKR. While I have not assessed the ability of pTRS1 to antagonize HRI or directly inhibit eIF2α phosphorylation I think it is likely pTRS1 either targets a conserved feature of eIF2α kinases to generally suppress eIF2α phosphorylation or directly affects eIF2α to reverse eIF2α phosphorylation.

eIF2α kinases share a similar protein structure with conserved features. (32) PKR, HRI and PERK all dimerize following activation, GCN2 is the exception in that it is always dimer. eIF2α kinases also share similar protein folds, notably they all contain an αG helix used to interact with eIF2α. (33) Rather than pTRS1 evolving to interact with multiple eIF2α kinases through unique mechanisms, it would appears more likely that pTRS1 would evolve to target a conserved feature in all eIF2α kinases. This approach to limiting eIF2α phosphorylation would be far more efficient and allow one viral protein to antagonize multiple cellular stresses that normally limit protein synthesis. Given that all eIF2α kinases share a conserved αG, I propose that pTRS1 binds to all eIF2α kinases through the conserved αG helix. This mechanism would allow pTRS1 to block the interaction of all eIF2α kinases with eIF2α and thereby maintain protein synthesis despite a myriad of cellular stresses.

Alternatively pTRS1 may directly interact with eIF2α to block or reverse its phosphorylation. pTRS1 has not been shown to interact with eIF2α, however, given the ability of pTRS1 to prevent eIF2α phosphorylation from multiple eIF2α kinases the simplest mechanism is that pTRS1 binds eIF2α. Similar to the argument above, binding to each of the individual eIF2α kinases would be far more difficult than interacting with eIF2α directly. If pTRS1 bound eIF2α it could simply prevent eIF2α phosphorylation by eIF2α kinases. Or through a mechanism similar to HSV-1 pTRS1 may recruit cellular phosphatases to reverse eIF2α phosphorylation. Cellular phosphatases do not have substrate specificity, but rather rely on scaffold proteins to target the phosphatase to the correct substrate. For example, ICP34.5 of HSV-1 directs dephosphorylation of eIF2α through an interaction with PP1. (34) Perhaps pTRS1 carries out a similar role. Interestingly, the activity and specificity of cellular phosphatases is altered during HCMV infection. (35) The Geballe group has shown that cellular phosphatases are more active during HCMV infection as increased doses of phosphatase inhibitors were required to suppress phosphatase PP1 and PP2A. Additionally the set of proteins being

dephosphorylated was substantially altered following HCMV infection. One hypothesis to merge both observations would be that pTRS1 interacts with cellular phosphatases to redirect them towards eIF2α and thus sustain protein synthesis despite cell stress that would normally phosphorylate eIF2α.

Future Directions

Investigating eIF4F Independent Translation I propose that viral mRNAs translate through a viral specific translation initiation pathway. To identify host and viral factors necessary for HCMV mRNA translation I plan to identify the complement of proteins associated with translating viral mRNAs during HCMV infection. To do so I will modify the HCMV genome to contain unique sequences that can be captured with RNA aptamers. I will tag the mRNA for a representative immediate early (IE1), early (UL44) and late (pp28) protein. This approach would allow me to also investigate the complement of proteins associated viral mRNA translation as the infection temporally progresses. Following infection with my tagged viruses I will affinity capture mRNAs from polysome gradients. I will centrifuge my gradients to create maximum separation between 40S ribosomal subunits and 80S ribosomes. This will allow me to capture mRNAs undergoing initiation (40S ribosomal subunits). As well as mRNAs that have begun elongation (80S ribosomes). The proteins co-purifying with my viral mRNAs will then be identified by mass spectrometry.

I should then be able to identify host and viral proteins that promote efficient translation of viral mRNAs. For example any protein identified in the 40S fraction, but not in the 80S fraction could represent a protein necessary for efficient translation initiation. Alternatively, any protein identified associating with the 80S fraction, but not the 40S fraction is likely necessary for efficient translation elongation. Following the identification of factors present on viral mRNAs under normal conditions I will then deplete eIF4F from the infected cells and compare the

proteins bound to translating viral mRNAs with and without eIF4F. Any viral proteins found in the 40S fraction in the absence of eIF4F become excellent candidates to promote mRNA translation independent of eIF4F. I may not find any viral proteins in the 40S fraction following eIF4F disruption, but host proteins could also promote the translation of viral mRNAs in the absence of eIF4F and in that case any host factor that continued to associate with viral mRNAs in the absence of eIF4F will be investigated.

Investigating pTRS1 Antagonism of PKR and eIF2α pTRS1 clearly antagonizes PKR and prevents eIF2α phosphorylation. pTRS1 is proposed to out compete PKR for binding viral dsRNA. However my data suggests a different mechanism. The PKR binding domain of pTRS1 is necessary for PKR antagonism during HCMV infection and pTRS1 can antagonize multiple eIF2α kinases. If pTRS1 is simply binding viral mRNA to block PKR activation the PKR binding domain of the protein would likely be dispensable. This assumes TRS1ΔPBD folds correctly, if not I may be observing the same effects as a TRS1 deletion virus. In addition, the ability of pTRS1 to antagonize multiple eIF2α kinases suggests a common mechanism to prevent eIF2α phosphorylation and binding dsRNA would do little to antagonize HRI activation as HRI does not recognize dsRNA.

My first hypothesis as to how pTRS1 antagonizes PKR and eIF2α is that pTRS1 binds and prevents the function of either PKR, eIF2α or both. To determine the mechanism by which pTRS1 antagonizes PKR and eIF2α I propose the following experiments. First I would like to identify the region of PKR necessary for pTRS1 binding. If pTRS1 binds PKR in the conserved αG helix of eIF2α kinases this may provide a mechanism to allow pTRS1 to inhibit all eIF2α kinases. To identify the region of PKR required for pTRS1 binding I will construct truncation mutants of PKR and test for pTRS1 binding in cells transfected with mutant PKR constructs and pTRS1. I will then use immunoprecipitation to detect binding of PKR mutants by pTRS1. Alternatively, pTRS1 may only bind PKR following activation and dimerization. PKR binds

dsRNA and then dimerizes. This dimerization is necessary for a PKR autophosphorylation event that licenses PKR to phosphorylate eIF2α. (36) Therefore, I will test whether pTRS1 can bind to constituently dimerized PKR molecules. By overexpressing PKR constructs with a FLAG epitope that have their dimerization domain replaced with either biotin or streptavidin I will force PKR to dimerize. I will then capture exogeneous PKR immunoprecipitating PKR with a FLAG antibody. Finally I will determine whether pTRS1 can bind the exogenous dimerized PKR by western blot analysis of the immunoprecipitation. Alternatively, pTRS1 may also interact directly with eIF2α. To evaluate this mechanism I propose to immunoprecipitate both pTRS1 and eIF2α. Then I will determine whether the reciprocal protein binds.

My alternative hypothesis is that pTRS1 recruits phosphatases to reverse eIF2α phosphorylation and thereby maintain protein synthesis. To test this hypothesis I will also use pull down experiments to determine whether pTRS1 bind PP1 or PP2A. Both PP1 and PP2A exhibit altered activity during HCMV infection suggesting viral manipulation, possibly by pTRS1. Additionally I have identified a phosphatase binding motif in the carboxy terminus of pTRS1. The motif, RVxF, is located within the region deleted in the TRS1ΔPBD mutant virus. Intriguingly the loss of a phosphatase binding site may account for the PKR and eIF2α activation in TRS1ΔPBD mutant virus when pIRS1 is absent if pTRS1 does recruit phosphatases to antagonize PKR and eIF2α. To confirm this hypothesis I will mutagenize the HCMV genome to replace the RVxF motif with alanines and then evaluate PKR and eIF2α phosphorylation as well as viral replication in the absence of pIRS1.

Investigating Pro-translational Roles for pTRS1 While a role for pTRS1 in PKR antagonism has been described before, a role for pTRS1 in stimulating mRNA translation apart from antagonizing PKR is novel. Given my interest in how viral mRNAs translate independent of eIF4F I will continue to investigate the pro-translational activities of pTRS1. My leading hypothesis on the mechanism pTRS1 uses to stimulate mRNA translation is that pTRS1 directly

recruits ribosomes to viral mRNAs. To investigate that mechanism further I will first define the complement of mRNAs bound by pTRS1. To do so I will capture pTRS1 and use next generation sequencing to identify the mRNAs bound by pTRS1. I hypothesize that this list will contain mRNAs with conserved sequences or structures as pTRS1 preferentially stimulated the translation of reporters containing viral 5' UTRs. Any conserved sequences will be identified by the sequencing and subsequent sorting. mRNAs with high likely hood of structure based on software analysis by mfold will be subjected to SHAPE to confirm RNA secondary structure. SHAPE is a unique next generation sequencing approach that identifies areas of RNA secondary structure. (37) Prior to sequencing subject RNAs are exposed to electrophiles that form adducts on the RNA. RNA sequences not in RNA secondary structures will interact with the electrophiles to form adducts. When the RNA is reverse transcribed these RNA adducts increase the rate of mutation. Following sequencing, areas with little mutation are inferred to be in RNA secondary structures. The data is then correlated with programs that predict RNA structures based on free energy to provide the most likely structure. Next I will confirm that the identified sequence or structure is necessary and sufficient for pTRS1 to stimulate reporters containing those elements. Finally I will screen a library of pTRS1 mutants to identify the region of pTRS1 necessary for recognizing the conserved RNA element and make HCMV strains deficient for binding. These mutants will be evaluated for HCMV replication in the absence of pIRS1.

The second arm of my hypothesis is that pTRS1 bridges mRNAs and ribosomes. I will investigate this by defining the interaction of pTRS1 and ribosomal subunits. To do so I will purify 40S ribosomal subunits and pTRS1 to determine if additional factors are necessary for their interaction. Should pTRS1 bind the 40S directly I will determine whether pTRS1 binds ribosomal protein or ribosomal RNA. Alternatively, if pTRS1 requires additional host or viral factors to associate with the ribosome I will purify 43S preinitiation complexes from infected cells

with and without TRS1 and identify the translation initiation factors that are necessary for pTRS1 to bind the 40S ribosomal subunit. I will do this by washing the 43S with increasing concentrations of salt and determine which fraction pTRS1 elutes with. The proteins in the fraction pTRS1 elutes with will be identified by mass spectrometry. Proteins eluting with pTRS1 will be depleted from HeLa cells lacking PKR and the capacity of pTRS1 to stimulate translation will be evaluated. The loss of any protein necessary for pTRS1 to recruit a ribosome to mRNAs should limit the increase in translation following pTRS1 expression. This approach will allow us to identify the protein or proteins necessary for pTRS1 to bind the 40S ribosomal subunit. However, if I am unable to cleanly elute the various translation initiation factors I will simply isolate the fractions of a polysome gradient containing both pTRS1 and the 40S ribosomal subunit and use mass spectrometry to identify the proteins contained in the fraction. In either case the regions of pTRS1 necessary for binding to the 40S ribosomal subunit will be identified. Those regions will then be mutagenized in the HCMV genome and the resulting virus evaluated as above.

Dissertation Impact

In this dissertation I investigated the mechanisms that control the translation of HCMV mRNAs. During viral infection host and viral mRNAs compete for access to host ribosomes as HCMV does not encode its own ribosomes. The fact that HCMV mRNAs are reliant on host ribosomes generates a critical interface for antiviral intervention. Developing therapeutics against interfaces of host and viral factors should limit of off target effects. To identify potential antiviral targets against HCMV mRNA translation we must first understand the host and viral factors that direct viral mRNA translation. The rate limiting step of mRNA translation is translation initiation. Therefore, antivirals targeting initiation will be the most effective. To investigate the mechanism by which HCMV mRNAs undergo translation initiation I evaluated the role of the eIF4F complex plays in viral mRNA translation initiation. Similar to previous

reports I observed a significant reduction in HCMV replication following eIF4F inhibition or disruption. This suggests that targeting eIF4F may be an effective intervention against HCMV replication. However, when I measured the efficiency with which viral mRNAs associated with ribosomes I did not detect any defect in the absence of eIF4F.

If HCMV mRNAs translate independent of eIF4F some other translation initiation complex must promote the translation of viral mRNAs. To identify potential host or viral proteins that may stimulate viral mRNA translation initiation I captured mRNA m^7G cap binding proteins from infected cells. The viral protein pTRS1 was found in this screen and I confirmed that pTRS1 binds mRNA during HCMV infection and outside the context of infection. pTRS1 is sufficient to stimulate mRNA translation outside the context of infection. In addition, pTRS1 preferentially stimulates the translation of mRNAs containing viral sequences. pTRS1 is known to antagonize PKR, but outside the context of infection I showed that pTRS1 can stimulate translation independent of PKR. The roles of pTRS1 in stimulating mRNA translation suggest that inhibiting pTRS1 may be an effective antiviral therapy.

To confirm that pTRS1 is necessary for efficient HCMV replication I constructed a novel system to evaluate the contribution of pTRS1 to HCMV replication. I determined that pTRS1 and the PKR binding domain of pTRS1 are necessary during HCMV infection to prevent PKR activation and maintain viral protein synthesis. In addition, I showed that HCMV replication lacking pTRS1and its homolog pIRS1 can be rescued by depleting PKR from infected cells.

This dissertation highlights pTRS1 as an excellent candidate for antiviral development. pTRS1 likely supports a unique viral translation initiation complex, therefore inhibiting pTRS1 would prevent the accumulation of viral proteins. pTRS1 also antagonizes PKR during infection and blocking the ability of pTRS1 to do so profoundly limits HCMV replication. Therefore, if inhibitors that prevent pTRS1 from binding mRNA or PKR could be developed they would likely be potent inhibitors of HCMV replication.

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