Essential Role of Protein Kinase R Antagonism by TRS1 in Human Cytomegalovirus Replication

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A dissertation submitted in partial

fulfillment of the requirements

for the degree of

Doctor of Philosophy

University of Washington

2015

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Portions of this dissertation were adapted from the following publication:

Virology, in press, 2015

Braggin, J.E., Child, S.J., Geballe, A.P. Essential Role of Protein Kinase R Antagonism by TRS1 in Human Cytomegalovirus Replication.

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Abstract

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Human cytomegalovirus (HCMV) lacking *TRS1* and *IRS1* (HCMV[ΔI/ΔT]) cannot replicate in cell culture. Although both proteins can block the protein kinase R (PKR) pathway, they have been reported to have multiple other activities and binding partners. It remains unknown which of these functions are essential for HCMV replication. To investigate this issue, we first identified a TRS1 mutant that is unable to bind to PKR. Like HCMV[ΔI/ΔT], a recombinant HCMV containing this mutant did not replicate in wild-type cells. However, HCMV[ΔI/ΔT] did replicate in cells in which PKR expression was reduced by RNA interference. Moreover, HCMV[ΔI/ΔT] and the recombinant mutant

replicated to similar levels as virus containing TRS1 in two different cell lines in which PKR expression was knocked out by CRISPR/Cas9-mediated genome editing. These results demonstrate that the sole essential function of TRS1 in cultured human fibroblasts is to antagonize PKR and that its other activities do not substantially enhance HCMV replication.

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ACKNOWLEDGEMENTS

I would like to thank my truly exceptional mentor, Dr. Adam Geballe, for advising me through the Ph.D. process and for teaching me how to think critically as a scientist. I would also like to thank the Geballe lab members, past and present, for their help during my time in the lab and for making each day a great day to be a scientist.

I am grateful for the support of both the faculty and the students in the UW Microbiology Department and at the Fred Hutchinson Cancer Research Center. My committee members Michael Lagunoff, Shiu-Lok Hu, Denise Galloway, and Brad Preston (GSR) were invaluable for helpful advice throughout my time as a graduate student. I am also thankful for the support granted by the Cell and Molecular Biology Training Grant that I received from 2011-2012 (National Institute of General Medical Sciences, Public Health Service, National Research Service Award, T32 GM007270).

In completing this work that led to a publication, we thank Denise Galloway and Harmit Malik (FHCRC), Elizabeth Gray and Dan Stetson (UW), and Bertram Jacobs (University of Arizona) for reagents. We also thank the Genomics Core facility (FHCRC) for technical assistance.

DEDICATION

This dissertation is dedicated to my wonderful husband, Dr. Alistair Russell, and to my family. Thank you for supporting me with great patience throughout my time as a graduate student.

CHAPTER 1: INTRODUCTION

Herpesviruses and HCMV

Herpesviruses and infections in humans

Herpesviruses are large, double-stranded DNA (dsDNA) viruses that have coevolved with animals for hundreds of millions of years (Davison, 2002). These viruses first cause a lytic infection within hosts, but become lifelong infections that quietly persist in a latent state (Baichwal and Sugden, 1988; Cohrs and Gilden, 2001). Reactivation of these viruses during the lifetime of the host, triggered by a variety of factors, can cause mild to serious disease. The family Herpesviridae includes three subfamilies—known as alpha-, beta-, and gamma-herpesviruses—nine of which commonly infect humans (Roizman and Baines, 1991). Alpha-herpesviruses include herpes simplex viruses (HSV) types 1 and 2 and varicella zoster virus (VZV), which primarily cause skin and mucosal lesions but sometimes affect visceral sites, especially the brain. When latent, each of these viruses reside in a quiescent state in the neurons of infected individuals but occasionally reactivate to cause recurrent skin lesions, such as cold sores and shingles. Beta-herpesviruses include human cytomegalovirus (HCMV), and HHV-6A, HHV-6B, and HHV-7, all of which are commonly acquired in early childhood. The exact sites of beta-herpesvirus latency are unclear but are likely include

circulating granulocyte precursor cells. Finally, Gamma-herpesviruses such as Epstein-Barr Virus (EBV) and Kaposi Sarcoma Herpesvirus (KSHV) are each capable of causing cancer; these viruses establish latent infection in B cells (Arvin et al., 2007).

In addition to these human herpesviruses, there are hundreds of others that infect a wide range of animals, even including invertebrates such as oysters (Davison, 2002). However, in this thesis, I will focus on HCMV.

Infection of humans by HCMV

Many people become infected with HCMV at a young age through contact with mucosal secretions (Staras et al., 2008). HCMV can also be transmitted sexually and by blood and organ transplantation (Boeckh and Geballe, 2011). At least 50% of the human population tests seropositive for HCMV (Ross and Boppana, 2005). In healthy people with functioning immune systems, acute HCMV infection can occasionally manifest as a mononucleosis-like disease; however, most individuals are unaware that they are infected. Within immunocompromised individuals, such as advanced AIDS patients or those undergoing immunosuppressive therapy for organ transplants, dormant HCMV can reactivate. In these situations, HCMV can cause diseases of multiple different organs, commonly manifesting as pneumonia, encephalitis, and retinitis (Griffiths et al., 2015). While causing many problems in patients lacking healthy immune systems, HCMV is also the leading viral cause of congenital birth

defects. HCMV can pass from an infected mother to the fetus and cause disease in newborn infants. In a small percentage of babies with active, symptomatic infections, HCMV can cause blindness, deafness, and mental retardation (Boeckh and Geballe, 2011). No vaccine is currently available to prevent HCMV infection, and treatment is limited.

Animal models of study

Understanding the biology and pathogenesis of HCMV has been complemented by studies of other CMVs from nonhuman primates and rodents and studied both *in vivo* and cultured *in vitro*. Current models include mouse (murine) and rat CMVs (MCMV and RCMV), guinea pig CMV (GPCMV), and rhesus macaque CMV (RhCMV). GPCMV is a useful small animal model for studying congenital HCMV disease, as it, but not mouse CMV, can cross the placenta during pregnancy (Schleiss, 2002). RhCMV has been under development as a model for pathogenesis, immune responses, latency and vaccine development (Powers and Fruh, 2008).

Cell culture systems have also been developed to study these CMVs. For the most part, each CMV will only replicate well in cells of its host species (Lafemina and Hayward, 1988). However, manipulation of these viral genomes has been useful to understand the species specificity of certain CMV genes, as we have demonstrated in our lab previously (Child et al., 2012). Cell culture models also enable us to manipulate the viral genome to understand gene

function and replication requirements for genes homologous to those encoded in HCMV.

While these systems are useful for studying CMVs both *in vivo* and *in vitro*, related CMVs have many genetic differences from HCMV and therefore investigation of HCMV replication and gene function in cell culture remains an important tool for understanding the function of HCMV genes.

Study of HCMV in cell culture

In this study, I examine the replication of a lab-adapted strain of HCMV (AD169) in human fibroblasts (HF). While HCMV infects a variety of cell types *in vivo*, infection in tissue culture is limited to a few cell types, including human fibroblasts, endothelial cells, glial cells, and hepatocytes (Sinzger et al., 2008). Replication of AD169 is limited to HF because repeated passage of clinical isolates results in the deletion or mutation of genes nonessential for viral replication in these cells (Grazia Revello et al., 2001; Hahn et al., 2004). Several of these genes are critical for viral spread, latency, or evasion of the adaptive immune system, and others are important for replication within human hosts (Wang and Shenk, 2005). We chose to use AD169 because lab-adapted strains grow to higher titers and replicate faster than clinical strains. We also have readily available systems to manipulate the AD169 viral genome. However, AD169 has multiple gene deletions and mutations; therefore, caution is necessary in drawing conclusions about gene functions when comparing this

virus in cell culture to infection with clinical strains in humans (Bradley et al., 2009).

In our study, manipulation of a Bacterial Artificial Chromosome (BAC) containing entire the HCMV genome is used to create viral mutants (Borst et al., 2007). This tool has been used to determine which genes are essential and which are dispensable during HCMV replication in tissue culture; for example, a previous study showed that only 45 ORFs were required for replication of the lab-adapted strain Towne in HF, while 117 ORFs were found to be nonessential (Dunn et al., 2003). Besides allowing gene knockout in HCMV, BACs can also be used for recombineering genes back into the HCMV genome. This has allowed for the study of how genes from other viruses complement HCMV replication when expressed directly from the virus. In this study, we generated several HCMV recombinant viruses in which certain genes have been manipulated and re-entered into the genome.

Innate Immune Evasion by Viruses

Double-stranded RNA (dsRNA)-activated Antiviral Pathways

Host cells express a gauntlet of intrinsic defense mechanisms that sense and impede viral replication. Several mechanisms become active through

sensing double-stranded RNA (dsRNA), which accumulates in cells as a hallmark of viral infection (Jacobs and Langland, 1996). While dsRNA genomes or replication intermediates of RNA viruses likely directly trigger these systems, DNA viruses, such as herpesviruses, also produce dsRNA during infection, possibly as a consequence of overlapping convergent transcription (Gantier and Williams, 2007). Regardless of their origins, these dsRNA activate antiviral factors, which promote an antiviral state within infected cells. For example, binding of dsRNA to retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated protein 5 (MDA-5), and toll-like receptor 3 (TLR-3) starts a signaling cascade that leads to activation of transcription factors inducing genes involved in the antiviral response, such as IRF-3 and NF-κB (Wilkins and Gale, 2010). RNase L, which is activated following dsRNA-binding to 2'-5' oligoadenylate synthetase, degrades rRNA and mRNA and thereby prevents viral replication (Sadler and Williams, 2008). My thesis work focused on Protein Kinase R (PKR), which is also activated through interaction with dsRNA, ultimately causing the shutoff of protein synthesis and thereby halting viral replication (Fig. 1) (Garcia et al., 2007).

The PKR Pathway

Protein Kinase R (PKR) is constitutively expressed in most cell types; however, its expression is enhanced during interferon induction. PKR becomes active by binding dsRNA, dimerizing, and autophosphorylating; active PKR then

interferes with translation initiation by phosphorylating an important component, the eukaryotic translation initiation factor 2, on the alpha subunit (eIF2α) (Fig. 1) (Garcia et al., 2007). Translation initiation requires a ternary complex composed of eIF2, guanosine triphosphate (GTP), and the initiating methionyl tRNA. Phosphorylation of eIF2α causes it to bind to and inhibit the guanine nucleotide exchange factor eIF2B, preventing the restoration of the ternary complex and halting translation initiation. Other stress-inducing stimuli can result in the same effect via phosphorylation of eIF2α by other kinases, including starvation, heme accumulation, and the presence of unfolded proteins (Dever et al., 2007).

Evasion of PKR by Viruses

Because protein synthesis is vital for viral replication, many viruses employ strategies to block the PKR pathway and prevent the shutoff of protein synthesis (Fig. 2). Mechanisms exist that interfere with the pathway at each step, including the degradation of PKR (by a protease recruited by poliovirus), the recruitment of proteins that promote the dephosphorylation of eIF2α (herpes simplex virus-1 γ34.5), the relocalization of PKR (human papillomavirus E6), and the mimicking of PKR substrates (vaccinia virus K3L). Many viruses also employ strategies involving direct binding to PKR to prevent its function. Examples include viral RNA (adenovirus VAI/VAII) and viral proteins (HIV-1 Tat and Kaposi's sarcoma herpesvirus vIRF-2). Notably, many viruses encode antagonistic proteins capable of binding to both PKR and dsRNA (HSV-1 Us11,

EBV SM, influenza NS1, HCMV TRS1 and IRS1, and vaccinia virus E3L) (Langland et al., 2006).

While many viruses encode one or more PKR antagonists, the deletion of an antagonist from a viral genome often reduces or prevents viral replication in cell culture. For example, deletion of vaccinia virus (VACV) E3L (VVΔE3L) causes a 1000-fold viral growth decrease in cell culture when compared to wild-type vaccinia virus. VVΔE3L replication increases by two logs in PKR knockdown HeLa cells, demonstrating that PKR is an important target of E3L (Zhang et al., 2008). In our lab, we use VVΔE3L as a tool for testing the ability of a heterologous given protein or virus to antagonize the PKR pathway.

In several cases, virulence *in vivo* is dramatically reduced in viruses lacking their PKR antagonists. For example, VVΔE3L is avirulent in mouse models (Brandt and Jacobs, 2001). As well, deletion of the HSV-1 PKR antagonist γ34.5 eliminates neurovirulence in wild-type mice. Notably, HSV-1 Δγ34.5 virulence is restored upon infection of PKR-null mice, establishing PKR antagonism as a key determinant of pathogenesis in this system (Leib et al., 2000). Thus, viruses strongly depend on their ability to block the PKR pathway for replication and pathogenesis.

Deletion of CMV PKR antagonists attenuates or prevents viral replication

Similar to other viruses, deletion of PKR antagonists from each of several cytomegaloviruses is also detrimental to viral replication. MCMV encodes two

PKR antagonists, m142 and m143, that function together to block PKR (Budt et al., 2009; Child and Geballe, 2009). Deletion of either of these genes from the MCMV genome prevents viral replication both in cell culture and in mice. However, mutant viral replication is restored to the same level as wild-type MCMV in PKR knockout mouse embryonic fibroblasts and PKR knockout mice (Ostermann et al., 2015). Thus, the only role of m142 and m143 in MCMV replication appears to be to block PKR. GPCMV was recently shown by our lab to encode a PKR antagonist, GP145 (Bierle et al., 2012). GPCMVΔ145 suffers a replication defect in both guinea pig cells and is attenuated in virulence *in vivo* (Schleiss et al., 2015). These data suggest that GPCMV also relies on a PKR antagonist for efficient replication and virulence.

We have previously shown that deletion of PKR antagonists TRS1 and IRS1 from HCMV (HCMVΔIΔT) prevents viral replication (Marshall et al., 2009). The two proteins appear to serve redundant essential function(s) since deletion of either gene alone does not eliminate HCMV replication. Notably, several different mutant HCMVs in which IRS1 has been deleted replicate as efficiently as wild-type HCMV in cell culture leading us to focus on TRS1 in the work describe below (Blankenship and Shenk, 2002; Dunn et al., 2003). Although evidence suggests that the PKR pathway becomes activated during infection with HCMVΔIΔT, TRS1 (and IRS1) have additional known functions, and it is unclear whether PKR antagonism is the essential role played by either protein, or if it is one of several essential functions. The work in the following chapters details my findings.

HCMV TRS1 and IRS1

Evasion of PKR by HCMV TRS1 and IRS1

HCMV TRS1 and IRS1 were first shown to function as PKR antagonists by the ability of either protein to complement VVΔE3L replication (Child et al., 2004). Our lab found that dsRNA binding by TRS1 is necessary for this function (Hakki and Geballe, 2005). However, dsRNA binding alone is insufficient for VVΔE3L rescue. To antagonize PKR in the vaccinia system, a C-terminal region of TRS1 is also required (Hakki et al., 2006). Deletion of the TRS1 C-terminal 35 amino acids (TRS1[1-738]) maintains the ability to rescue VVΔE3L, but truncation to residue 679 (TRS1[1-679]) does not. We then investigated the role of the C-terminal region in PKR binding co-immunoprecipitation assays. While TRS1[1-738] retained the ability to bind PKR, TRS1[1-679] did not. Thus, PKR antagonism correlates for the ability of TRS1 to bind both dsRNA and PKR.

While the mechanism by which TRS1 or IRS1 blocks PKR is unknown, either protein is capable of preventing the phosphorylation of PKR (Child et al., 2012). Ongoing studies by our lab aim to understand the structure of TRS1 and to determine how human and primate CMV TRS1 genes affect the PKR pathway in order to gain insights into the underlying mechanism.

TRS1 or IRS1 is essential for HCMV replication

Our lab found that the presence of either TRS1 or IRS1 is essential to viral replication, as deletion of both genes (HCMV[ΔI/ΔT]) results in a virus that is unable to replicate (Marshall et al., 2009). However, mutant viruses expressing only TRS1 (HCMVΔIRS1) or only IRS1 (HCMVΔTRS1) each replicate in cell culture (Blankenship and Shenk, 2002; Dunn et al., 2003). These observations suggest that TRS1 and IRS1 are redundant in providing the essential function(s) for HCMV replication. This conclusion is not unexpected, since the genes are encoded in part in the genomic repeats and thus the N-terminal two-thirds of TRS1 and IRS1 are identical. Furthermore, they remain 50% similar at the amino acid level through their C-termini (Weston and Barrell, 1986).

Insertion of either TRS1 or the VACV E3L into HCMV[ΔI/ΔT] restores viral replication. While HCMVΔIRS1, which expresses TRS1 growth kinetics are similar to wild-type HCMV, HCMVΔTRS1, which expresses IRS1, shows a modest decrease in titer after low MOI infection (Blankenship and Shenk, 2002); the defect in HCMVΔTRS1 replication was originally attributed to a viral packaging function defect (Adamo et al., 2004). However, it seems unlikely that TRS1 is specifically required for packaging, as complementation by VACV E3L—a protein not involved in vaccinia viral packaging and from a different virus—restored growth of HCMV[ΔI/ΔT] (Marshall et al., 2009). Although HCMV[ΔI/ΔT] enters cells and begins expression of several viral gene products, protein synthesis is shut off between 24 and 48 hours post-infection. An increase in the

abundance of phosphorylated eIF2α and the absence of RNase L activation suggests that the activation of PKR is the primary cause of protein synthesis shutoff (Marshall et al., 2009). However, it has not been demonstrated that the direct cause of HCMV[ΔI/ΔT] failure to replicate is due to the loss of PKR antagonism by TRS1. We hypothesized that an additional function performed by TRS1 may also be essential to HCMV replication.

Other known functions of TRS1 (and IRS1)

In addition to PKR antagonism, TRS1 and IRS1 have been reported to share other functions. Many of these other functions can be attributed to the N-terminal regions of TRS1 and do not require the C-terminal region that is necessary for PKR antagonism.

Early studies showed that TRS1 is able to block the activation of the OAS/RNase L pathway that occurs following VVΔE3L infection (Child et al., 2004). It is unknown as to whether TRS1's dsRNA binding activity alone or TRS1 is sufficient for this function. Surprisingly, RNase L function does not appear to be active in cells infected with HCMV[ΔI/ΔT], suggesting that TRS1 does not need to block RNase L during HCMV replication (Marshall et al., 2009). It is not yet known whether TRS1 affects other dsRNA-activated pathways, so it remains possible that the inhibition of one of these pathways is important for HCMV replication.

Other factors that have been reported to bind to TRS1 include the 7-methylguanosine (7-mG) mRNA cap (Ziehr et al., 2015), the viral DNA polymerase accessory subunit UL44 (Strang et al., 2010), and the essential autophagy host protein Beclin 1 (Chaumorcel et al., 2012). While no functional correlates have been established that require binding to the 7-mG cap and to UL44 binding during replication, the direct association of either TRS1 or IRS1 with Beclin 1 has been shown to block autophagy (Chaumorcel et al., 2012; Mouna et al., 2015). Notably, several other viral PKR antagonists are also capable of blocking autophagy by binding Beclin 1, including HSV-1 γ34.5 (Gobeil and Leib, 2012). TRS1 inhibits autophagy independently of antagonizing PKR, as this function was shown to occur in the absence of PKR following transfection of PKR knockout mouse cells. Furthermore, TRS1 mutants that cannot block autophagy retain the ability to block PKR, and mutants that cannot block PKR can still block autophagy (Mouna et al., 2015). Therefore, these functions are distinct.

TRS1 and IRS1 have also been attributed to be potent activators of transcription in combination with immediate-early (IE) viral proteins 1 and 2 (Stasiak and Mocarski, 1992). Furthermore, an alternate, truncated form of IRS1, $IRS1^{263}$, was reported to antagonize this function (Romanowski and Shenk, 1997). However, we have data strongly suggesting that these effects on reporter gene expression are a consequence of the inhibition of PKR rather than due to transcriptional activation. Transfection of TRS1 along with a reporter gene expressing secreted embryonic alkaline phosphatase (SEAP) and PKR results in

the increase of SEAP expression, whereas less SEAP is expressed in absence of TRS1. We interpret these data to suggest that the activation of PKR during transfection is preventing SEAP expression; addition of TRS1 antagonizes a PKR-mediated translational block, allowing increased SEAP expression. Most importantly, our lab recently found that in transfection of PKR knockout cells, TRS1 does not enhance reporter gene expression any more than when PKR is present (Carpentier et al., unpublished data).

Although TRS1 and IRS1 have each been characterized to perform several functions in addition to PKR antagonism, it remains unclear as to which function(s) is essential for viral replication. In the following chapter, I will outline how we engineered a mutant of TRS1 deficient in its PKR antagonism function and tested its ability to replicate in HF. I hypothesize that PKR antagonism is at least one essential function of TRS1 in HCMV replication.

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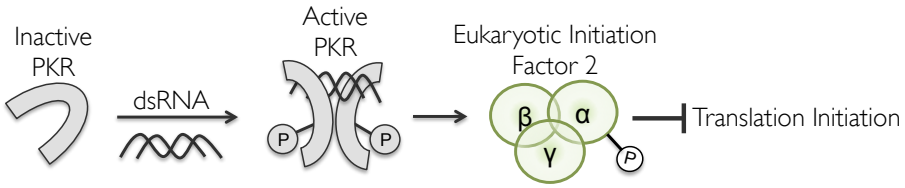

Fig. 1. The PKR pathway. PKR becomes active by binding dsRNA, dimerizing, and autophosphorylating. Active PKR then phosphorylates the eukaryotic initiation factor 2 on the alpha subunit (eIF2α), which prevents translation initiation and results in the shutoff of protein synthesis.

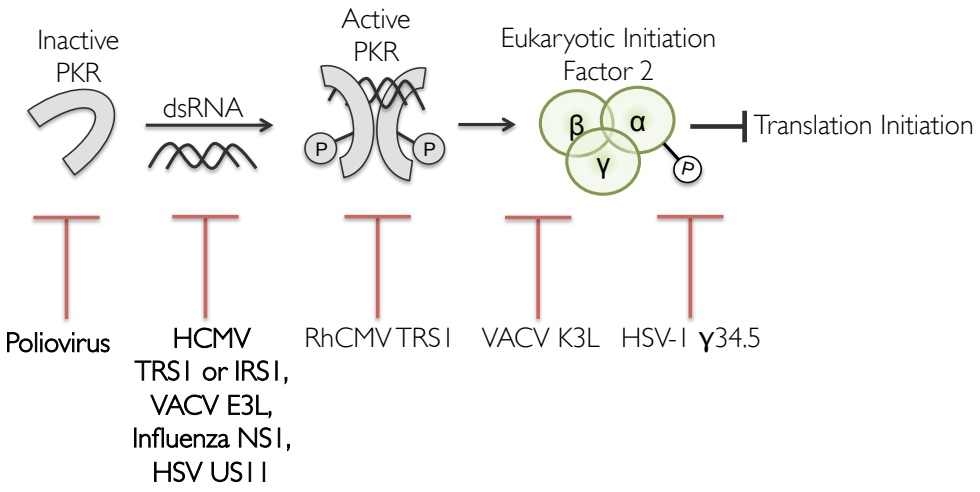

Fig. 2. PKR antagonists target the PKR pathway at various steps. A wide variety of viruses encode specific antagonists that block the shutoff of host protein synthesis; several examples are pictured above.

CHAPTER 2

ABSTRACT

The inability of HCMV[ΔI/ΔT] to replicate in cell culture indicates that TRS1 or IRS1 must play an essential role in viral replication. While TRS1 and IRS1 appear to have multiple functions, those that are essential for HCMV replication have not been established. In order to determine whether PKR antagonism is an essential function, I sought to identify a mutant of TRS1 that has lost the ability to antagonize PKR, yet retains other functions, and then to test the ability of this mutant to support HCMV replication. Since the C-terminus of TRS1 was known to be necessary for binding to PKR, I undertook several approaches to engineer mutations in this region. I identified one mutant that is unable to bind to PKR yet retains the ability to bind dsRNA, suggesting that its overall structure is preserved. Finally, I showed that this mutant cannot block PKR activation nor complement HCMV[ΔI/ΔT] replication, suggesting that PKR antagonism by TRS1 is an essential function during HCMV replication.[1]

[1]**Braggin, J.E., Child, S.J., Geballe, A.P.**, 2015. Essential Role of Protein Kinase R Antagonism by TRS1 in Human Cytomegalovirus Replication. Virology, in press.

Introduction

Human cytomegalovirus (HCMV) encodes two highly similar proteins, TRS1 and IRS1, each of which is able to inhibit the PKR pathway (Child et al., 2004). Infection of cells with a viral mutant lacking both genes (HCMV[ΔI/ΔT]) shuts off host protein synthesis and causes an increase in the level of phospho-eIF2α, indicating that the PKR pathway has been activated (Marshall et al., 2009). Unlike single mutant viruses that lack either TRS1 or IRS1, HCMV[ΔI/ΔT] does not replicate in cell culture (Blankenship and Shenk, 2002; Dunn et al., 2003; Jones and Muzithras, 1992; Marshall et al., 2009). These observations suggest that PKR pathway inhibition may be the essential role of TRS1 or IRS1 in HCMV replication. However, TRS1 and IRS1 have been reported to have several additional functions, one or more of which might be essential for viral replication. Both proteins have been reported to bind to Beclin 1 and inhibit autophagy (Chaumorcel et al., 2012; Mouna et al., 2015), to block the 2'-5' oligoadenylate synthetase (OAS)/RNaseL pathway (Child et al., 2004), and to have potential roles as transcriptional activators (Romanowski and Shenk, 1997; Stasiak and Mocarski, 1992). The *IRS1* gene has also been reported to encode a second product that acts as a transcriptional repressor (Romanowski and Shenk, 1997). Additional studies focusing on TRS1 have shown that it binds to the DNA polymerase accessory factor UL44 and may play a role in virion assembly (Adamo et al., 2004; Blankenship and Shenk, 2002; Strang et al., 2010). Finally, TRS1 was recently reported to have a role in translation stimulation following

mRNA cap binding (Ziehr et al., 2015). Thus, while TRS1 and IRS1 appear to have multiple functions, those that are essential for HCMV replication have not been established.

In order to determine whether PKR antagonism is at least one essential function of TRS1, I sought to decouple this function from TRS1's other capabilities. TRS1 was first characterized as a PKR antagonist as it is able to prevent the protein synthesis shutoff that normally occurs during infection of HF with vaccinia virus lacking one of its PKR antagonists, E3L (VVΔE3L) (Child et al., 2004). While the mechanism by which TRS1 antagonizes PKR remains unclear, TRS1 interacts with both dsRNA and with PKR. TRS1 interaction with dsRNA via an N-terminal domain is essential for rescue of VVΔE3L; dsRNA binding is independent of PKR binding (Bierle et al., 2013; Hakki and Geballe, 2005). TRS1 direct interaction with PKR requires a C-terminal region spanning 59 residues (Hakki et al., 2006). This region was also found to be necessary for TRS1 rescue of VVΔE3L, in conjunction with dsRNA binding (Hakki et al., 2006). In addition to dsRNA binding, several other functions attributed to TRS1—including binding Beclin 1, UL44, and self-dimerization—do not require this region and instead depend on the N-terminus (Chaumorcel et al., 2012; Strang et al., 2010).

In this study, I identified a C-terminal TRS1 mutant that does not bind to PKR and then inserted this mutant gene into HCMV[ΔI/ΔT]. I determined that this mutant could not rescue replication of HCMV[ΔI/ΔT]. Thus, blocking PKR appears to be an essential function of TRS1 during HCMV replication.

Use of the yeast system to study the C-terminal region of TRS1 required for PKR binding

In order to determine the role of PKR inhibition by TRS1 in HCMV replication, I first sought to identify TRS1 mutants that are unable to bind to and antagonize PKR. Previous data indicated that a C-terminal region of TRS1 is critical for these functions (Hakki and Geballe, 2005; Hakki et al., 2006). Because the exact boundaries and crucial residues that TRS1 requires to bind PKR remain unclear, I aimed to identify specific residues that mediate the interaction between TRS1 and PKR. The use of point mutants is preferable over existing truncation mutants because they are less likely to significantly alter the structure of TRS1.

There are several different ways to analyze protein-protein interactions, including the use of the yeast expression system (Fields and Song, 1989). In this system, two yeast expression plasmids are transformed into yeast. The prospective binding partners are each fused to fragments of a transcription factor, including a binding domain and an activation domain. If the proteins interact, the fragments of the transcription factor come within close proximity of each other, as the binding domain will bind to DNA and the activation domain will activate reporter gene transcription. I first chose to use the yeast system to analyze the interaction between TRS1 and PKR. Our lab previously used a yeast codon-optimized form of TRS1 in a study revealing the species-specific

interactions between TRS1 from different CMVs and host PKR derived from the animals these CMVs infect (Child et al., 2012). Additionally, this system is relatively quick and easy to use. TRS1 interaction with PKR in the yeast two hybrid stains I used drives the expression of histidine (for which this strain is auxotrophic) and β-galactosidase. Conversely, the reverse two-hybrid assay allows for the selection of a disrupted protein-protein interaction (Leanna and Hannink, 1996). A different yeast strain with URA3 reporter gene expression converts the added chemical, 5-fluoroorotic acid (5-FOA), to a toxic byproduct that kills yeast harboring positive protein-protein interactions.

I first aimed to use PCR-based random mutagenesis to create a library of TRS1 mutants and screen for the loss of PKR binding by reverse two-hybrid assay. However, preliminary data suggested difficulty in discerning between 5-FOA sensitive and 5-FOA resistant growth, and while a reduction in yeast growth correlated with increasing concentrations of 5-FOA, the system would need extensive tuning (data not shown).

Instead, I chose to narrow TRS1 domain for PKR binding with the yeast two-hybrid assay. Consistent with our previous report (Child et al., 2012), yeast codon-optimized TRS1 bound to kinase-dead (K296R) human PKR (Fig. 1). As prior investigation uncovered that a C-terminal region of TRS1 was required for PKR binding, I examined this region. Progressive deletions from the TRS1 C-terminus resulted in a gradual decrease in binding. One C-terminal TRS1 truncation (TRS1[1-679]), which was previously reported to not bind detectably to PKR in mammalian cell assays (Hakki et al., 2006), exhibited considerably

decreased but measurable PKR binding in this assay. Further deletion of codons upstream from residue 667 eliminated all detectable binding. Although these results did not define a discrete position at which binding is eliminated, they did corroborate the importance of the C-terminus of TRS1 for binding to PKR.

To attempt to further narrow the region of TRS1 that is sufficient for PKR binding, I also created and tested two N-terminal truncations in the yeast system, TRS1[45-795] and TRS1[106-795]. I did not detect binding between either of these truncations and PKR in this system (data not shown). However, one of the pitfalls in using the yeast two-hybrid assay is the difficulty in detecting the expression of proteins. In building these truncations into mammalian expression vectors, I found that these truncations failed to express, potentially due to protein instability caused by truncating the N-terminus (data not shown). Thus, I continued to focus on the C-terminus of TRS1, specifically within residues 690-700 (Fig. 1).

By aligning human TRS1 with several other homologs, I pinpointed five conserved residues in the region between residues 690-700 (Fig. 2a). Each of these five residues was mutated to alanine, creating TRS1[5-Ala]. Yeast two-hybrid analysis revealed that this TRS1[5-Ala] lost its PKR binding activity in comparison to wild-type TRS1 (Fig. 2b). I then created and tested each of the five point mutants represented in TRS1[5-Ala] individually and found that among each mutant, only TRS1[R697A] abrogated PKR binding in yeast. To verify this result in a more physiologically relevant system, I generated TRS1[R697A] in the

mammalian expression vector. I found that, as in the yeast system, TRS1[R697A] did not detectably bind to PKR in the mammalian cells (Fig. 2c).

Because I was attempting to identify a TRS1 mutant deficient in PKR binding but proficient in its other functions, I examined the ability of TRS1[R697A] to bind known N-terminal binding partners in the mammalian system as a way to assess its structural integrity (Chaumorcel et al., 2012; Hakki and Geballe, 2005; Strang et al., 2010). Surprisingly, TRS1[R697A] did not bind to dsRNA (Fig. 2d) or UL44 binding (data not shown), even though these two interactions were shown to occur with C-terminal deletion mutants. Furthermore, I found that TRS1[R697A] did not dimerize with TRS1[1-738], even though TRS1[1-450] is sufficient for the TRS1 its dimerization function. These data suggest that the [R697A] mutation caused a major structural disruption in TRS1.

Identification of TRS1 mutants unable to bind PKR through engineering of charged-cluster-to-alanine mutants

Because it is difficult to predict the effects of mutations on overall protein structure, I next chose to use charged-cluster-to-alanine (CCTA) mutagenesis in the mammalian system to try to identify a TRS1 mutant that is only deficient in PKR binding. This method, which has been used in studies of other HCMV proteins and in other systems (Schuessler et al., 2010; Schuessler et al., 2008; Schuessler et al., 2012), capitalizes on the propensity for charged clusters of amino acids to be surface-exposed (Zhu and Karlin, 1996). Therefore, charged

clusters are likely to be mediators of protein-protein interactions and to tolerate mutations without drastically impacting the overall protein structure. An alignment of HCMV TRS1 and IRS1 with closely related chimpanzee CMV homologs reveals several conserved charged amino acid clusters (Fig. 3a). I chose seven clusters that possess at least two conserved residues within a five-residue window and engineered alanine mutations at each of these positions to produce mutants TRS1-Mut 1 through TRS1-Mut 7.

I evaluated PKR binding to these mutants by co-transfecting plasmids that express kinase-dead PKR along with each of the TRS1 variants into 293T cells. The S2H-tagged TRS1 proteins were pulled down using Streptactin beads, after which bound proteins were separated by SDS:PAGE then subjected to immunoblot analysis with an anti-PKR antibody. Expression of the His-tagged transfected genes was also monitored by immunoblot analysis of the cell lysates. As shown previously, wild-type TRS1 and an N-terminal TRS1 mutant that cannot bind to dsRNA (Triple Mut; TRS1 featuring mutations R121A/R124A/K125A) both bound to PKR, while a C-terminal deletion mutant (TRS1[1-648]) and a GFP control plasmid did not (Fig. 3b, lanes 1-3, 10; (Bierle et al., 2013; Hakki et al., 2006)). Several of the CCTA mutants bound PKR (Fig. 3b, lanes 6, 8, 9, and 10), indicating that these residues are dispensable for this activity. However, TRS1-Mut 1 did not bind to PKR at all and TRS1-Mut 2 bound only weakly (Fig. 3b, lanes 4 and 5). Although TRS1-Mut 4 did not appear to bind to PKR, its expression was lower that the other TRS1 variants in this and other experiments (not shown).

These experiments identified PKR binding deficiencies of TRS1-Mut 1 and TRS1-Mut 2. I expected the CCTA mutants to retain other known TRS1 activities that do not require the C-terminal PKR-binding region (Hakki and Geballe, 2005). To test this prediction, I performed dsRNA-binding assays with each of the CCTA mutants. Immunoblot analysis of proteins bound to poly[I:C] beads revealed that wild-type TRS1 bound dsRNA, while Triple Mut did not (Fig. 3c). As in past experiments, I found considerable variation in the intensity of binding (Bierle et al., 2013). However, in contrast to the negative controls, Triple Mut and GFP, all of the CCTA mutants bound dsRNA to at least a detectable level. The one exception was TRS1-Mut 4, which again showed a reduced steady-state level of expression. These results suggest that these CCTA mutations, including those that disrupt PKR binding, retained dsRNA-binding activity and likely other functions that map to the N-terminus of TRS1.

PKR binding is required for VVΔE3L rescue by TRS1

Previous data showed that the C-terminal region of TRS1 that is required for PKR binding is also required to rescue replication of VVΔE3L, a vaccinia virus mutant lacking its own PKR antagonist, E3L (Hakki and Geballe, 2005). Therefore, to determine more specifically whether PKR binding is necessary for TRS1 to rescue VVΔE3L, I tested three of our CCTA mutants. HeLa cells were transfected with plasmids expressing TRS1-Mut 1, TRS1-Mut 2, or TRS1-Mut 3, which bind to PKR to varying extents (Fig. 3b). After transfection, cells were

infected with VVΔE3L (MOI = 0.1) and viral replication was measured. Wild-type TRS1 rescued VVΔE3L replication, whereas Triple Mut, TRS1[1-648], and GFP did not (Fig. 4), consistent with previous reports (Bierle et al., 2013; Hakki and Geballe, 2005). TRS1-Mut 1 failed to rescue VVΔE3L, while TRS1-Mut 2 rescued VVΔE3L to an intermediate degree and TRS1-Mut 3 rescued to wild-type levels. TRS1-Mut 5, 6, and 7, all of which bound to PKR as well as wild-type TRS1 did, also rescued VVΔE3L to a level similar to wild type TRS1 (data not shown). Thus, the ability of the mutants to rescue VVΔE3L replication correlated well with their ability to bind PKR, supporting the hypothesis that PKR binding by TRS1 is important for blocking PKR activity and enabling VVΔE3L replication.

Expression of TRS1 in *trans* during HCMV infection

I determined that TRS1-Mut 1 was incapable of rescuing VVΔE3L by transfection of HeLa cells followed by viral infection. However, in order to determine whether TRS1-Mut 1 was capable of restoring HCMV[ΔI/ΔT] replication, I considered and tested several different ways of expressing TRS1-Mut 1 in *trans*, as transfection of human fibroblast cells (used in HCMV infection experiments) is inefficient. To test whether TRS1 mutants were able to rescue replication of HCMV[ΔI/ΔT] in HF Tert cells, I transduced HF Tert with retroviruses expressing CMV promoter-driven wild-type TRS1 or TRS1[1-679] from the retroviral vector pLHCX. I chose TRS1[1-679] as a preliminary negative control that cannot rescue VVΔE3L (as a measure of inability to antagonize PKR)

(Hakki and Geballe, 2005). While each expression vector expressed detectable amounts of TRS1 or TRS1[1-679] after transfection of HeLa cells and only wild-type TRS1 rescued replication of VVΔE3L, HF Tert cell lines created by retroviral transduction with wild-type TRS1 did not express TRS1 and did not rescue VVΔE3L. Although sequencing of genomic DNA revealed the presence of integrated TRS1, the transgene appears to have been silenced, reducing TRS1 expression (data not shown).

I next attempted to use a lentiviral vector to create cell lines that express higher amounts of TRS1. The lentiviral vector pLVX-AcGFP1-N1 contains a CMV promoter and an N-terminal GFP gene preceding the polylinker. TRS1-S2H, TRS1[45-795], TRS1-Mut 1, TRS1-Mut 2, TRS1-Mut 3, or TRS1-Triple Mut were each cloned into this vector such that each gene was expressed with an N-terminal GFP fusion. I confirmed that the GFP fusion did not alter expression of each TRS1 construct via Western blotting of transfected HeLa cells and that each construct behaved as expected with regards to the ability to rescue VVΔE3L (data not shown). Following transduction of HF Tert, I probed cell lines by Western blotting and found that each cell line expressed easily detectable amounts of GFP-TRS1 (Fig. 5a; TRS1[45-795] data not shown). Furthermore, I was able to visualize GFP fusion protein expression and subcellular location via microscopy (data not shown). Each cell line behaved similarly to the plasmid vectors in terms of VVΔE3L rescue (data not shown).

To test cells for their ability to rescue HCMV[ΔI/ΔT] replication, I infected them in duplicate with either AD169 or HCMV[ΔI/ΔT]. Media was changed at

days 2, 6, and 10 prior to supernatant collection on days 3, 7, and 11. Virus present in collected supernatants was titered on TRS1-expressing, complementing cells. Wild-type HCMV replicated equivalently to equivalent amounts in each cell line. TRS1-S2H and TRS1-Mut 3 rescued HCMV[ΔI/ΔT] replication as expected, as these constructs also rescued VVΔE3L replication (Fig. 5b). Notably, TRS1[45-795] rescued HCMV[ΔI/ΔT] as well (data not shown); this construct is unable to block autophagy (Chaumorcel et al., 2012), suggesting that this function is not required for HCMV replication. The failure of TRS1-Mut 1 and TRS1-Mut 2 to rescue HCMV[ΔI/ΔT] was consistent with our hypothesis that PKR binding is required for TRS1 to antagonize PKR, suggesting that blocking PKR is essential for HCMV replication. Surprisingly, the cell line expressing Triple Mut rescued HCMV[ΔI/ΔT] (Fig. 5b). This was unexpected, as Triple Mut does bind PKR, but does not bind dsRNA nor does it rescue VVΔE3L replication (Bierle et al., 2013). It is possible that Triple Mut can block PKR in the HCMV system, but not in the vaccinia system.

While the VVΔE3L rescue assay measures the ability of a given protein to antagonize PKR in the vaccinia system, I was most interested in determining the impact of the TRS1 mutants on PKR in the HCMV system. Therefore, I mock-infected or infected each cell type with AD169 or HCMV[ΔI/ΔT] at an MOI of 3 and harvested cell lysates at 72hpi and monitored levels of phosphorylated PKR by Western blotting. I noted that all infected cell lines expressing any form of TRS1 resulted in an increase of phosphorylated PKR after HCMV[ΔI/ΔT] infection compared to mock or AD169 infection (Fig. 5c). As these cell lines were

not cloned, it is possible that TRS1 is only expressed in some cells and phospho-PKR is accumulating in the cells that do not express TRS1. At least half of the transduced cell population strongly expressed GFP, but many cells did not (or had weak GFP expression).

As another method for expressing high levels of TRS1, I explored the potential use of adenoviral vector with assistance from Jason Smith's lab at UW. I found that infection with several different GFP-expressing adenoviral vectors did not hinder the replication of wild-type HCMV. However, adenovirus genomes still contained PKR antagonists VAI and VAII, and it is unknown to how their activity against PKR might affect our experiment. Also, HCMV infection alone might rescue adenoviral replication. Ultimately, I was unable to create a TRS1-expressing adenoviral vector.

As another way of measuring the ability of each TRS1 construct to block PKR in a herpesviral system, I stimulated cells with interferon and infected each cell type with herpes simplex virus lacking one of its PKR antagonists, γ34.5 (HSVΔγ34.5), which normally does not replicate well in fibroblasts (Brown et al., 1994). Rescued replication of HSVΔγ34.5 would indicate that a TRS1 allele was capable of blocking PKR. However, the results were inconclusive in that the differences between HSVΔγ34.5 replication between control and TRS1-expressing cells were small (data not shown). Furthermore, repeated passage of these cells resulted in decreased expression of GFP, suggesting the transgene was being silenced. Thus, the TRS1-expressing cell lines did not prove to be useful for dissecting the essential function(s) of TRS1. Having tried

unsuccessfully to express a high amount of TRS1 *in trans* using several different approaches, I decided to continue testing TRS1 mutants in the HCMV viral expression system.

We constructed recombinant HCMVs expressing wild-type TRS1, TRS1-Mut 1, or Triple Mut using lambda red recombineering and Cre/Lox to remove bacterial sequences (see Chapter 5, Materials and Methods). The expected structures of the BAC DNAs were verified by restriction enzyme digestion (Fig. 6a). We reconstituted each virus by transfection into HF-TRS1. The correct location and identity of the TRS1 gene in each of the reconstituted viruses was confirmed by PCR using primers external to the TRS1 locus (Fig. 6b) and sequencing (data not shown). TRS1 protein expression following infection of HF-TRS1 cells (which express very low amounts of TRS1) was detectable from all viruses, with the exception of HCMV[ΔI/ΔT], as expected (Fig. 6c). While we also characterized and confirmed HCMV[Triple Mut] using these techniques (data not shown), we could not obtain stocks with a high enough titer for use in further experiments at this time.

To examine whether PKR binding by TRS1 is necessary for HCMV replication, I infected HF with each virus and measured the amount of virus produced in cell supernatants by titering on HF-TRS1 (Fig. 7a). TRS1[Triple Mut] did not replicate in HF (data not shown). AD169 and HCMV[TRS1-S2H] replicated in HF, while HCMV[ΔI/ΔT] did not, consistent with previous results (Marshall et al., 2009). I did not detect any HCMV[TRS1-Mut 1] replication in HF (Fig. 7a). These results suggest that the functions localized to the N-terminus of

TRS1 are insufficient for promoting HCMV replication and that PKR binding by TRS1 is essential.

TRS1-Mut 1 fails to block PKR activation

During infection of HF with HCMV[ΔI/ΔT], the abundance of phosphorylated eIF2α increases and protein synthesis levels are reduced, suggesting that the failure of HCMV[ΔI/ΔT] to replicate is due to the inability of this virus to block PKR activation (Marshall et al., 2009). To evaluate the impact of the inability of HCMV[TRS1-Mut 1] to bind to PKR on activation of the PKR pathway, I infected HF with HCMV[TRS1-Mut 1] or control viruses at an MOI of 3, collected lysates at 48 hpi, and evaluated the levels of phosphorylated and total PKR and eIF2α by immunoblot assays.

While infection with AD169 resulted in a very low amount of phosphorylated PKR, similar to the level detected in mock-infected cells, infection with either of two viruses that express TRS1 but not IRS1 (HCMV[TRS1-HA] or HCMV[TRS1-S2H]), resulted in a small increase of phospho-PKR compared to mock- and AD169-infected cells. In contrast, considerably more phospho-PKR accumulated after infection with either HCMV[TRS1-Mut 1] or HCMV[ΔI/ΔT] (Fig. 7b). In addition, the amount of total PKR was markedly reduced after infection with HCMV[TRS1-Mut 1] or HCMV[ΔI/ΔT]. This observation might reflect a reduction in PKR expression following HCMV[TRS1-Mut 1] and HCMV[ΔI/ΔT] infection due to the shutoff of protein synthesis that occurs following PKR

activation, as has been observed in other systems (Bierle et al., 2012; Child et al., 2012; Rothenburg et al., 2009).

Analyses of eIF2α in these same lysates revealed an increase in phosphorylated eIF2α during infection with HCMV[TRS1-Mut 1] or HCMV[ΔI/ΔT] compared to mock infection or infection with AD169, HCMV[TRS1-HA] or HCMV[TRS1-S2H] (Fig. 7b, compare lanes 4 and 5 to lanes 1 and 6). I also detected a decrease in the amount of total eIF2α, similar to that observed for total PKR, again suggesting an effect of shutoff of translation in cells in which TRS1 was absent or unable to block the PKR pathway. Cells infected with either of the two viruses that express TRS1 but not IRS1 caused an intermediate level of phosphorylated eIF2α to accumulate, but did not show any reduction in total eIF2α. These results are consistent with moderate but incomplete inhibition of PKR pathway activation by these viruses.

Most importantly, these data reveal that the PKR pathway is activated during infection with HCMV[TRS1-Mut 1] just as it is following infection with HCMV[ΔI/ΔT]. Therefore, TRS1-Mut 1 appears to be unable to prevent PKR activation during HCMV infection, supporting the hypothesis that blocking PKR activation is an essential function of TRS1.

Conclusion

In this chapter, I engineered and tested a C-terminal TRS1 mutation, TRS1[R697A], that appeared to abrogate PKR binding in the yeast two-hybrid

system. However, when this mutation was tested in the mammalian system, I found evidence that TRS1[R697A] disrupted both dsRNA binding and homodimerization, suggesting that the mutant was structurally damaged. Using an alternative method for identifying candidate sites for mutation that would minimize potential misfolding, I engineered a series of TRS1 mutants, some of which had diminished PKR binding ability (such as TRS1-Mut 1). I found that PKR binding by TRS1 was necessary to block PKR in the vaccinia system. However, my goal was to test whether TRS1 must block PKR to allow HCMV to replicate. Thus, I engineered several mutants into transgenic, TRS1-expressing cell lines. Although I found that cells expressing TRS1-Mut 1 did not rescue HCMV[ΔI/ΔT], I was unable to use these cells to determine whether TRS1-Mut 1 failed to block the PKR pathway (as we hypothesized).

We engineered recombinant HCMV to express this mutant (HCMV[TRS1-Mut 1]). Consistent with the results from infection of transgenic cells, HCMV[TRS1-Mut 1] did not replicate in HF. We also determined that, as during HCMV[ΔI/ΔT] infection, HCMV[TRS1-Mut 1] activated the PKR pathway. These data suggested that at least one essential function of TRS1 during HCMV replication is to block PKR. However, these data did not reveal whether TRS1 has more than one essential role in HCMV replication. In the next chapter, I will explore this possibility by engineering PKR-deficient cell lines.

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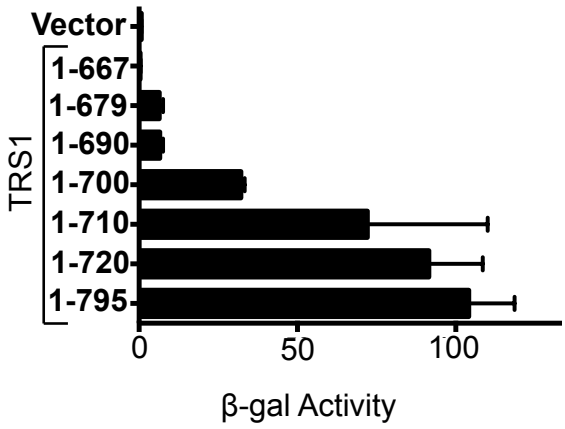


FIGURE 1. Yeast two-hybrid analysis of the interaction between PKR and TRS1. Plasmids expressing kinase-dead PKR fused to the GAL4 activation domain and the indicated regions of TRS1 fused to the GAL4 binding domain or an empty vector control were co-transformed into yeast. The strength of the protein-protein interactions was assessed by β-galactosidase activity assays.

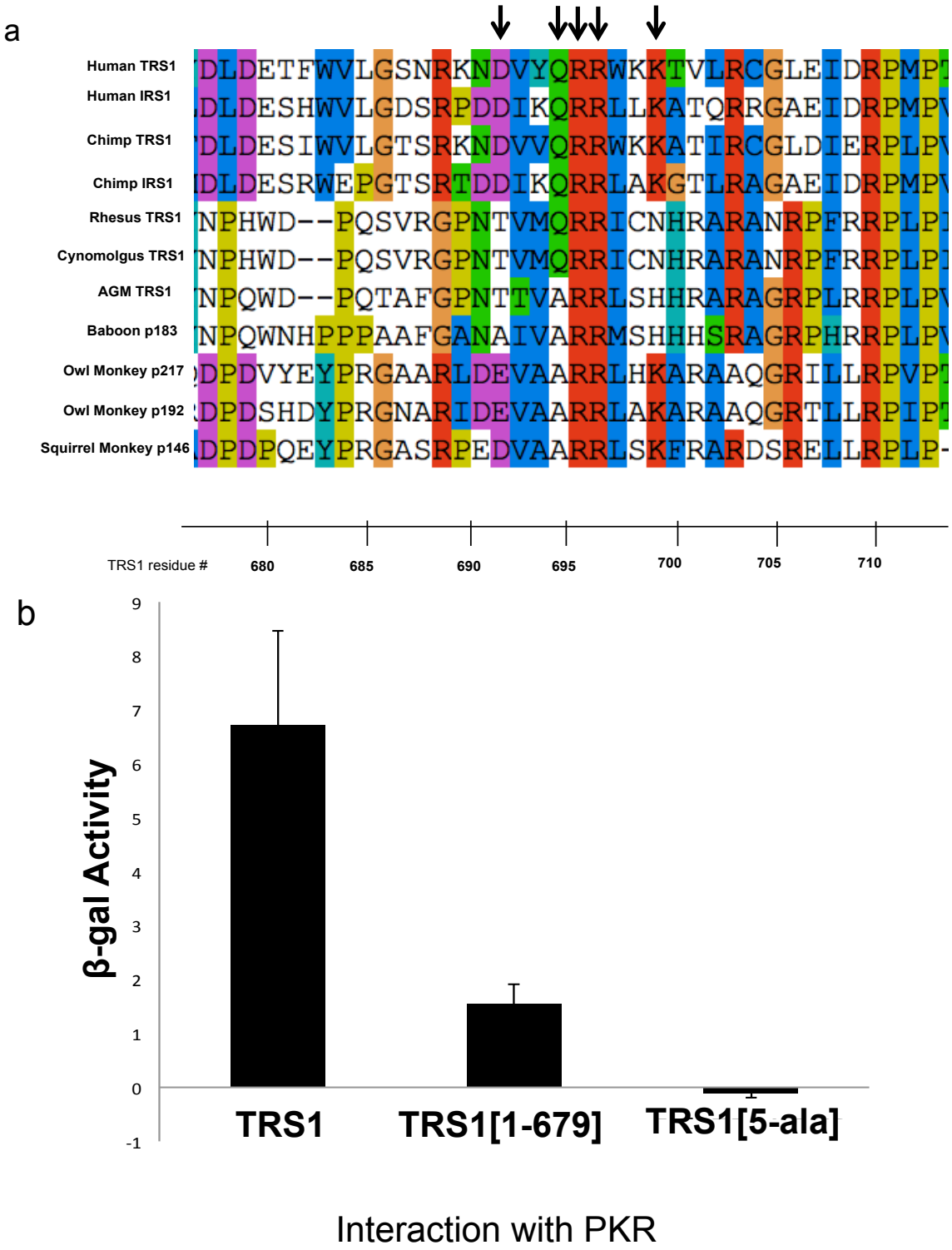
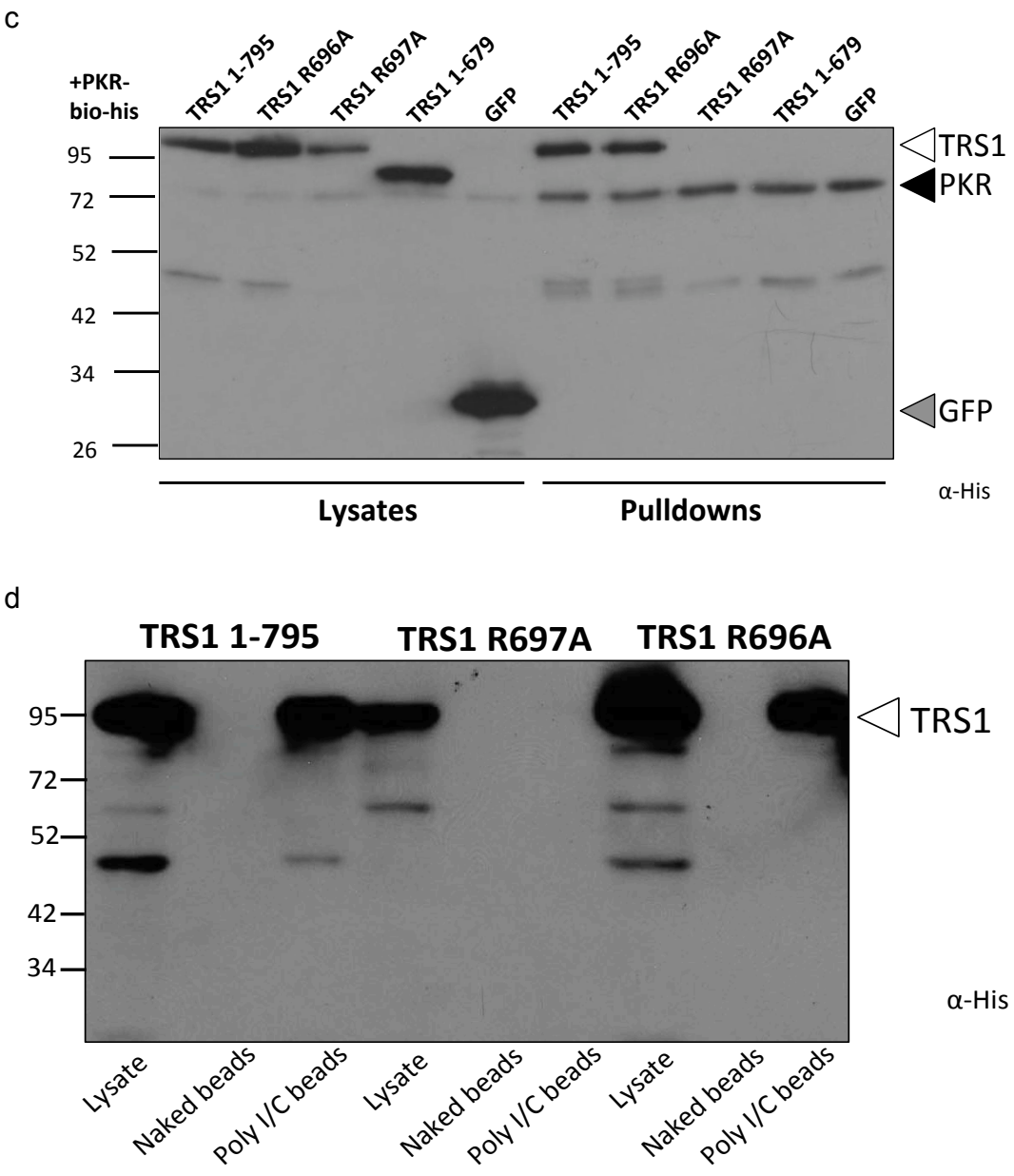


FIGURE 2. TRS1[R697A] does not bind PKR. (a) A ClustalX alignment of CMV US22 gene family members was used in choosing five conserved residues for alanine mutation within the region between amino acids 690-700, denoted by arrows. (b) TRS1[5-ala] did not bind to PKR in the yeast two-hybrid system.



(c) TRS1[R697A] did not bind to PKR in the mammalian transfection-based system. For the PKR binding assay in mammalian cells, COS7 cells were co-transfected with kinase-dead PKR-BH, biotinylase plasmid BirA, and the indicated TRS1-H expression plasmids. TRS1 R696A is another C-terminal mutant that we used as a positive control; this mutant rescues VVΔE3L and binds both PKR and dsRNA. At 48 hours post-transfection, lysates were harvested and pulldown assays performed with streptavidin beads as detailed in Materials and Methods. Input samples representing 5% of each lysate (left) and bound protein (right) were visualized using a His antibody. (d) TRS1[R697A] fails to bind to dsRNA. For dsRNA binding assays, 293T cells were transfected with the indicated TRS1-H constructs or a GFP-H control. At 48 hours post-transfection, input samples (lysate) and proteins that bound to Poly I:C Sepharose beads (Poly I/C beads) or control beads (naked beads) were analyzed by immunoblotting with His antibody.

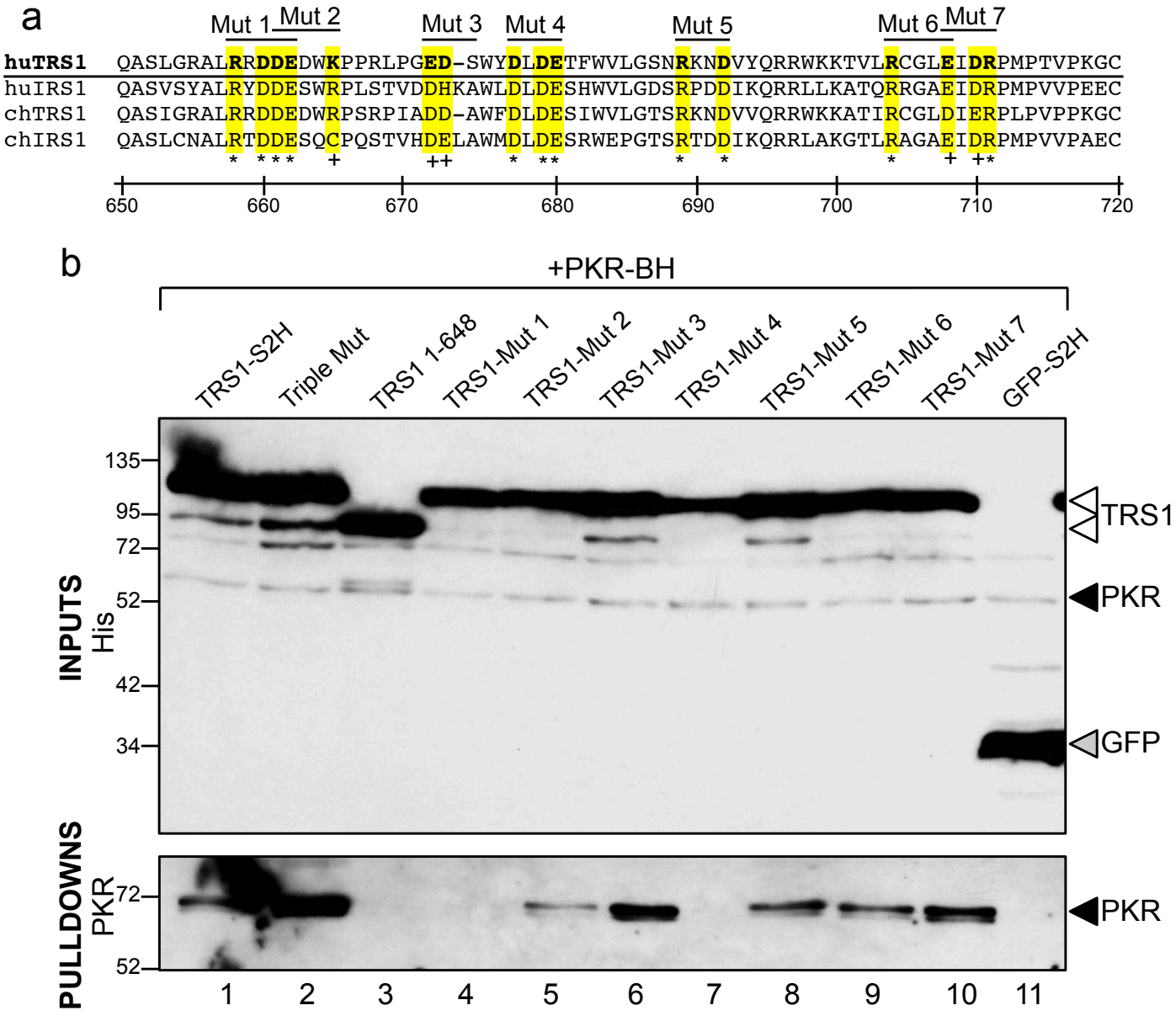
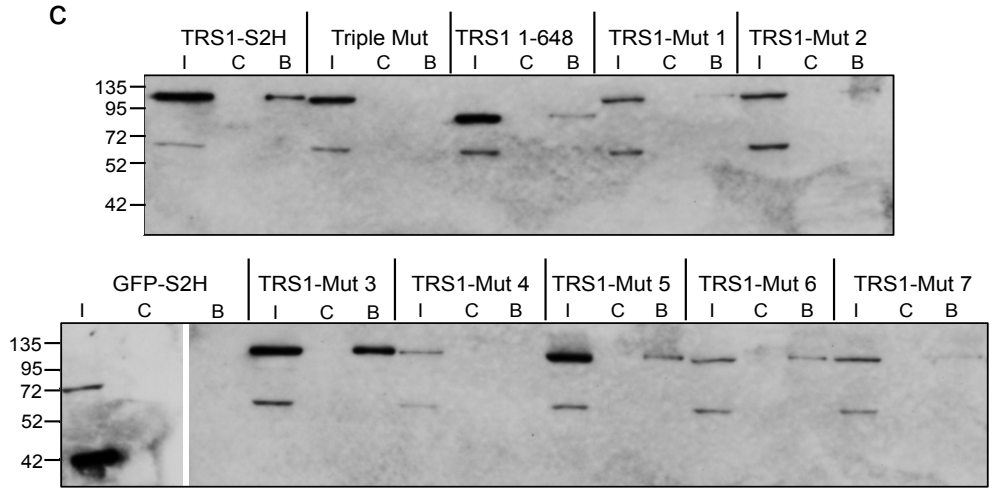


FIGURE 3. Binding properties of TRS1 charged-cluster-to-alanine mutants. (a) HCMV TRS1 and IRS1 (Towne strain) were aligned with Chimpanzee CMV TRS1 and IRS1 (Heberling strain). Clusters of two to five charged residues within five residue windows were identified (highlighted in yellow) near the C-terminus. Seven of these clusters were selected for mutation to alanine in human TRS1 (* denotes identical residues; + indicates conserved charge in at least two other genes). (b) For PKR binding assays, COS7 cells were co-transfected with kinase-dead PKR-BH and the indicated TRS1-S2H expression plasmids. At 48 hours post-transfection, lysates were harvested and pulldown assays performed as detailed in Materials and Methods. Input samples representing 5% of each lysate (top panel) were visualized using a His antibody. Following pulldown, bound PKR was detected by immunoblot analysis using the PKR B-10 antibody (bottom panel).



(c) For dsRNA binding assays, 293T cells were transfected with the indicated TRS1-S2H constructs or a GFP-S2H control. At 48 hours post-transfection, input samples (I) and proteins that bound to Poly I:C Sepharose beads (B) or control beads (C) were analyzed by immunoblotting with His antibody.

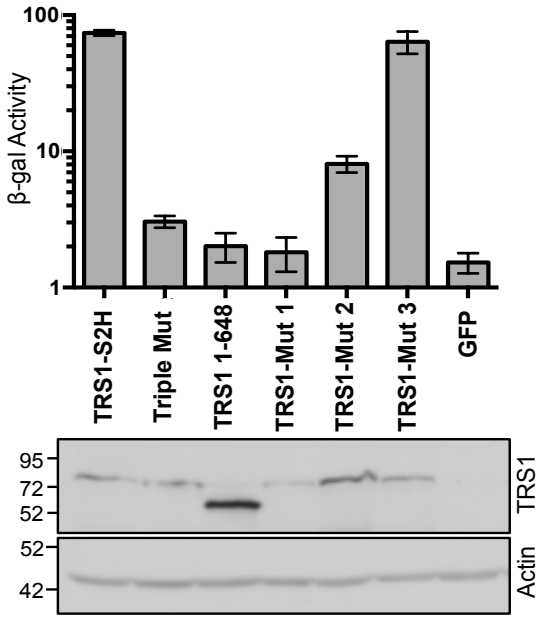


FIGURE 4. PKR binding is required for VVΔE3L rescue. HeLa cells were transfected with the indicated TRS1 constructs or a GFP control and infected with VVΔE3L (MOI = 0.1) at 48 hours post-transfection in triplicate. At 48 hpi, VVΔE3L replication was measured by β-galactosidase activity assay (top panel). Expression of TRS1 (and actin as a loading control) was monitored by immunoblot assay (bottom panel).

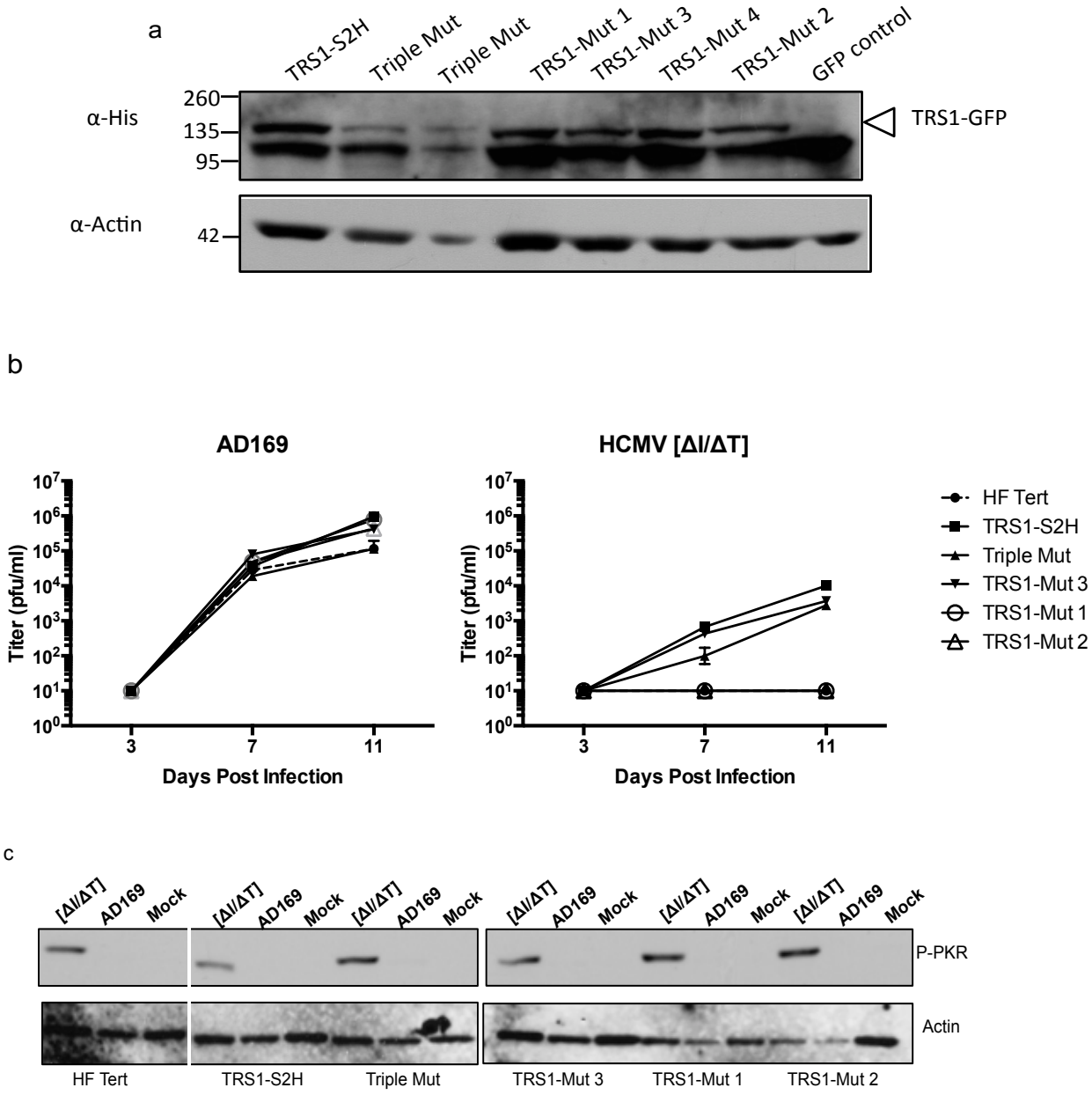


FIGURE 5. Expression of TRS1 *in trans* from transduced HF Tert cells. (a) Expression of GFP-TRS1 fusion proteins from transduced cell lines. Equivalent amounts of cell lysates were loaded onto an SDS-PAGE gel; however, lysate from Triple Mut-expressing cells was inadvertently loaded into two lanes instead of one. The size of these proteins is slightly greater than wild-type TRS1 due to the N-terminal GFP fusion. (b) Growth kinetics of AD169 and HCMV[ΔI/ΔT] in transduced cell lines. Cells were infected in duplicate with the either AD169 or HCMV[ΔI/ΔT] (MOI = 0.1). Virus present in the cell culture medium was collected at days 3, 7, and 11 and viral titers were determined on HF-TRS1 cells. Mean titers (and standard deviations) are shown. (c) PKR phosphorylation profile of transduced cell lines. Cells were infected with AD169 or HCMV[ΔI/ΔT] (or mock-infected) and lysates were harvested at 72hpi. Western blotting was used to probe for either phosphorylated PKR or Actin.

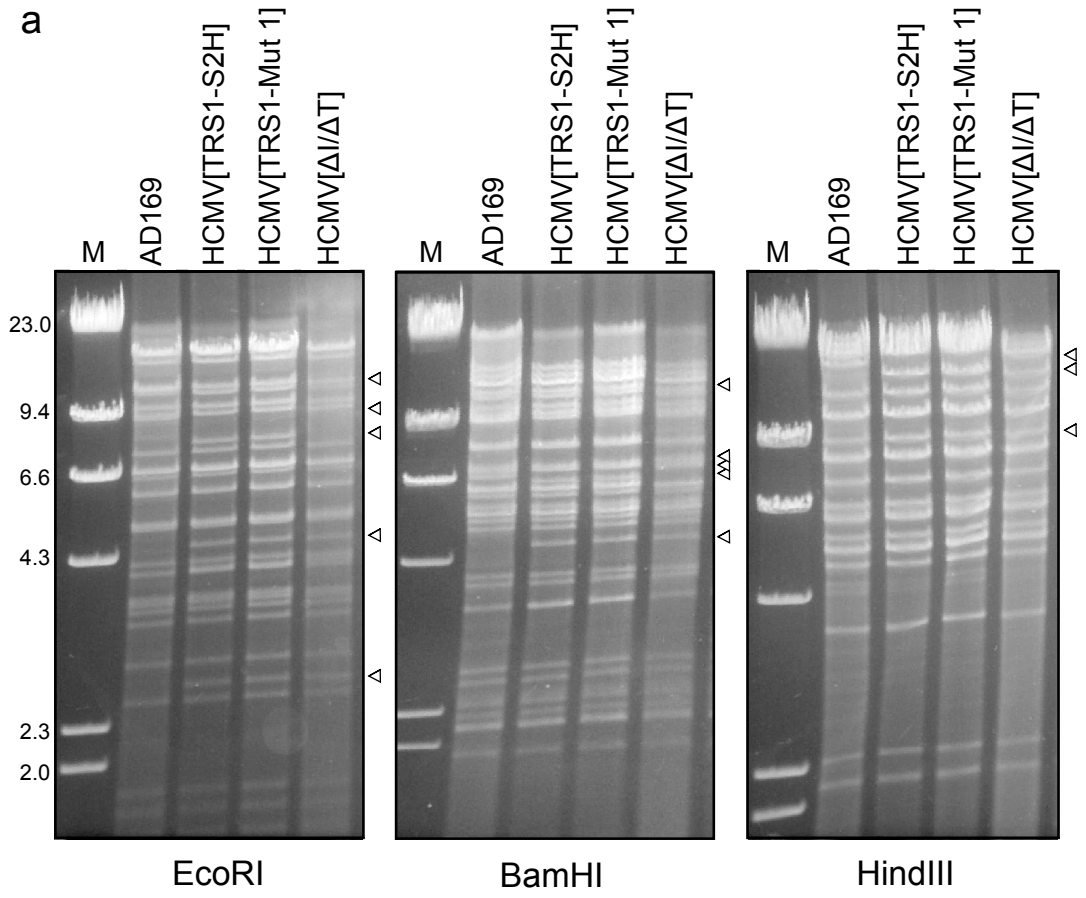
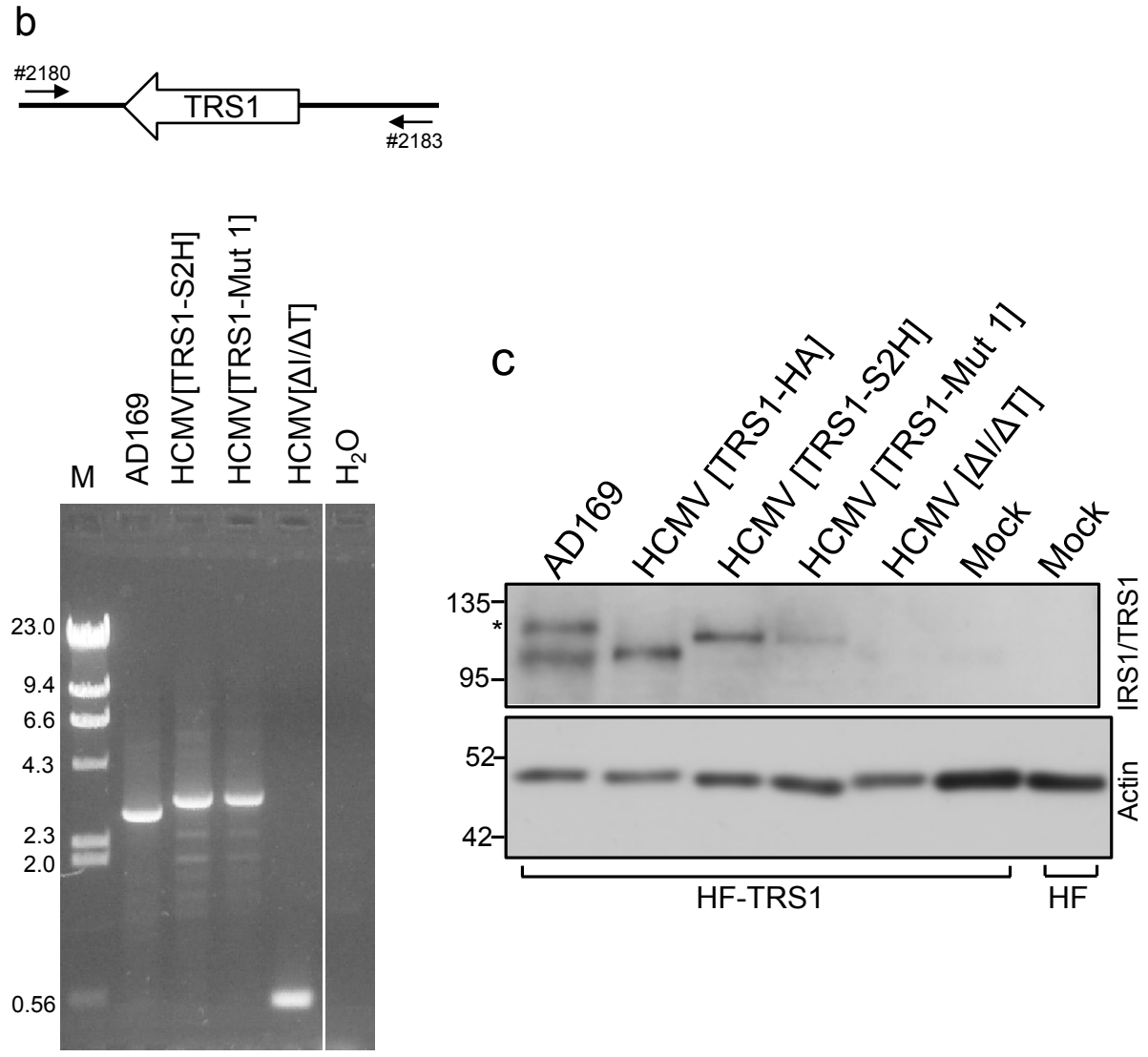


FIGURE 6. Analyses of the BACs and recombinant viruses. (a) BAC DNAs corresponding to the indicated recombinant viruses were digested with restriction enzymes and separated on agarose gels. Arrowheads point to bands expected to differ between the BACs.



(b) Primers external to the homology arms used for recombination into the HCMV[ΔI/ΔT] BAC were used to PCR-amplify this region from the indicated viruses. The HCMV[TRS1-S2H] and HCMV[TRS1-Mut1] products are ~ 0.23 kb larger than TRS1 from AD169 due to sequences encoding the epitope tags. Amplification of HCMV[ΔI/ΔT] DNA revealed the expected TRS1 deletion. (c) HF-TRS1 cell lysates were made at 72 hpi with the indicated viruses (MOI = 3). Immunoblot assays were performed with an antibody to the dsRNA-binding domain shared by TRS1 and IRS1. * indicates IRS1 expression from AD169.

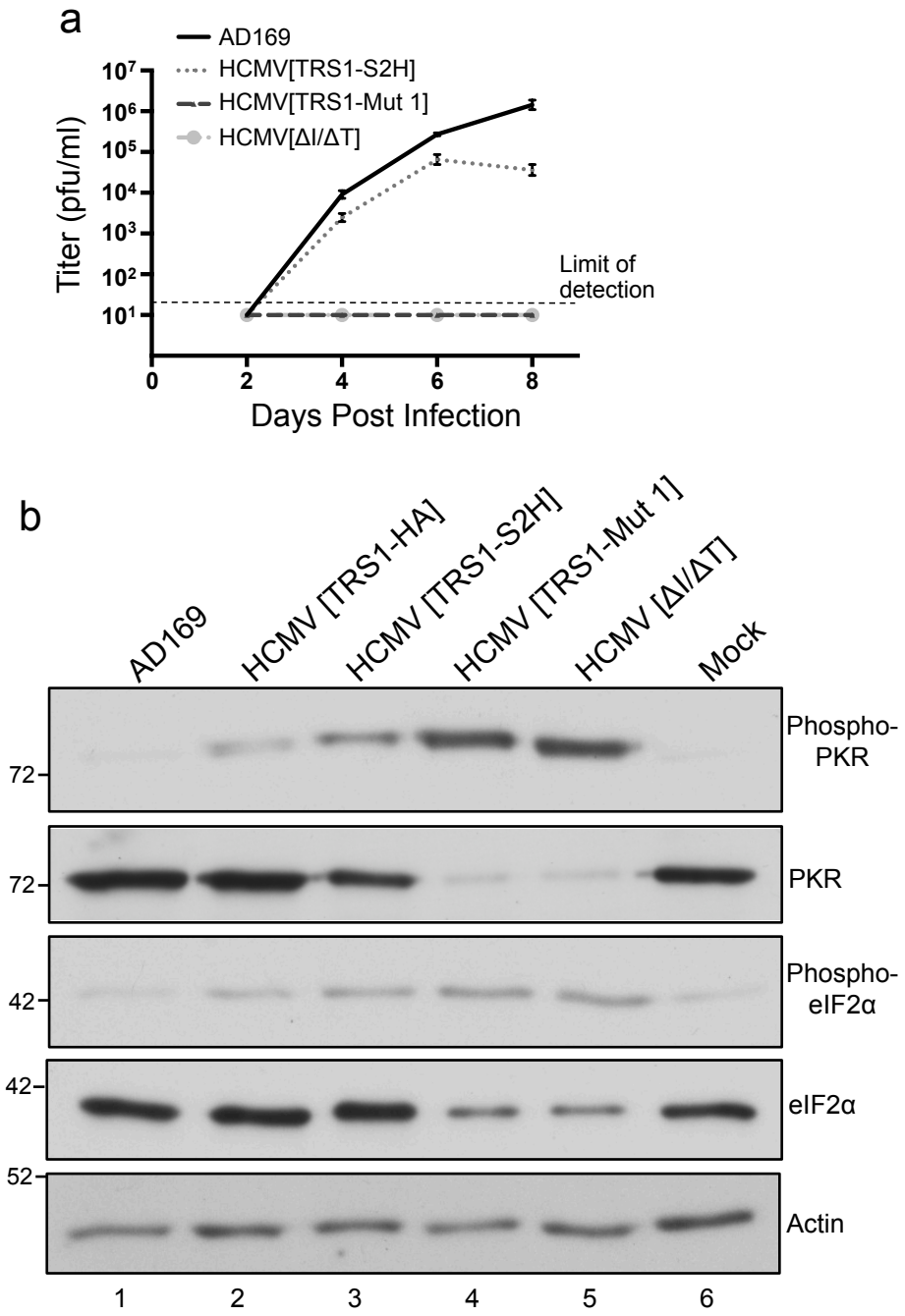


FIGURE 7. HCMV[TRS1-Mut 1] fails to replicate in HF. (a) HF were infected in triplicate with the indicated viruses (MOI = 0.1). Virus present in the cell culture medium was collected at two-day intervals and viral titers were determined on HF-TRS1 cells. Mean titers (and standard deviations) are shown. (b) Lysates from HF that were mock-infected or infected with each virus (MOI = 3) were collected at 48 hpi and the levels of total and phosphorylated PKR and eIF2α were examined by immunoblot analysis using the indicated antisera.

CHAPTER 3

ABSTRACT

In Chapter 2, I showed that HCMV[TRS1-Mut 1] did not replicate in HF and activated the PKR pathway, suggesting that blocking PKR is at least one essential function of TRS1 in HCMV replication. Other functions, such as the ability to bind dsRNA, are likely intact in TRS1-Mut 1, and one (or more) of these may be critical. In order to determine whether PKR antagonism is the only essential function, I created several cell lines in which PKR expression was reduced or eliminated and tested for the ability of HCMV[ΔI/ΔT] to replicate. I found that HCMV[ΔI/ΔT] replicated when PKR expression was reduced by shRNAs in HF. HCMV[ΔI/ΔT] did not replicate as well as HCMV[TRS1-S2H] or wild type HCMV in these cells, suggesting that residual PKR expression may be hampering replication or that another ancillary function of TRS1 is necessary for efficient replication. To distinguish between these possibilities, I used CRISPR/CAS9 gene editing technology to create PKR knockout cell lines in both HF and HF Tert. In these cells, I found that HCMV[ΔI/ΔT] replication was equivalent to HCMV[TRS1-S2H], strengthening the argument that the only essential function of TRS1 during HCMV replication is to block PKR. I also found that HCMV[TRS1-Mut 1] replication was also comparable to both HCMV[TRS1-

S2H] and to HCMV[ΔI/ΔT], suggesting other functions performed by TRS1 do not play a critical role in HCMV replication in cell culture.[1]

[1] **Braggin, J.E., Child, S.J., Geballe, A.P.**, 2015. Essential Role of Protein Kinase R Antagonism by TRS1 in Human Cytomegalovirus Replication. Virology, in press.

Introduction

In an earlier publication from our group, Marshall et al. found that HCMV[ΔI/ΔT] failed to replicate in HF and induced both protein synthesis shutoff and eIF2α phosphorylation (Marshall et al, 2009). These data led me to explore the hypothesis that TRS1 is required to block PKR to allow HCMV to replicate. In the preceding chapter, I created a C-terminal mutant of TRS1 (TRS1-Mut 1) that does not bind to or antagonize PKR, but retains some dsRNA binding ability. Although the data do not exclude the possibility that the mutation in TRS1-Mut 1 also weakens other TRS1 functions for which the C-terminal region is not absolutely required, they do suggest that PKR binding by TRS1 is most likely necessary for HCMV replication. Like HCMV[ΔI/ΔT], a virus expressing this mutant (HCMV[TRS1-Mut 1]) also failed to replicate in HF and activated the PKR pathway. Thus, these data suggest that at least one essential function of TRS1 is to antagonize PKR.

More evidence for this hypothesis stems from studies of a related virus, murine cytomegalovirus (MCMV). MCMV encodes m142 and m143, which complex together to block PKR (Budt et al., 2009; Child and Geballe, 2009). These genes, along with TRS1, are classified within the betaherpesviral US22 gene family (Mocarski Jr, 2007). Knockout of m142 and m143 prevents MCMV from replicating in mouse embryonic fibroblasts (MEF) and, like HCMV[ΔI/ΔT], activates the PKR pathway (Budt et al., 2009). Knockout of PKR in either MEFs or in mice restores replication of MCMVΔm142Δm143 (Budt et al., 2009;

Ostermann et al., 2015). These data suggest that the only essential function of these homologs to TRS1 encoded by a virus closely related to HCMV is to antagonize PKR.

However, because TRS1 has several additional functions, it is possible that one of these other roles is also critical during HCMV replication, in addition to PKR antagonism. Many viruses capitalize on encoding proteins that have multiple functions to conserve genetic space. Several of these multifunctional proteins are also PKR antagonists. For example, vaccinia virus (VACV) PKR antagonist E3L binds dsRNA and plays roles in interfering with other dsRNA-activated innate immune pathways, such RNase L, IRF3/IRF7, and ADAR1 (Gil and Esteban, 2004). As TRS1 and IRS1 can each bind dsRNA, it is not unlikely that they may also block several of these pathways, and they have been shown to block RNase L when complementing VVΔE3L replication (Child et al., 2004). Alternatively, TRS1 might be required to block autophagy or to bind UL44, among other functions.

To determine whether HCMV depends on TRS1 solely for PKR antagonism, I created and characterized PKR knockdown cells. I found that knocking down PKR in HF restored replication of HCMV[ΔI/ΔT], but not to wild-type levels. To understand whether residual PKR was responsible for this phenotype, I used CRISPR/Cas9 gene editing to engineer PKR knockout cell lines. I showed that HCMV[ΔI/ΔT] replication was restored to wild-type levels, strengthening the argument that the only essential function for TRS1 in HCMV replication is to block PKR. Finally, I found that HCMV[TRS1-Mut 1] did not have

a growth advantage over HCMV[ΔI/ΔT] in PKR knockout cells. These data suggest that TRS1 functions unrelated to PKR antagonism are nonessential during viral replication (Braggin et al., in press).

PKR knockdown by shRNA in HF Tert cells

We first transduced HF-Tert cells with a lentivirus expressing an shRNA targeting PKR and a scrambled control shRNA, as described in Materials and Methods (Chapter 5). We isolated two PKR knockdown clonal lines (HF Tert PKR-kd clones B and D), and one control clone (HF Tert Ctrl-kd) and assess the levels of total PKR by Western blotting. By comparison to varying amounts of parental cell lysates (Fig. 1), I estimated that PKR expression was reduced by ~95% in Clone D and ~80% in Clone B. Thus, I chose Clone D (referred to from this point on as HF Tert PKR-kd) for further studies. The control line, HF Tert Ctrl-kd), expressed similar amounts of PKR as HF Tert (data not shown).

Knockdown of PKR in HF Tert cells rescued VVΔE3L, as was previously shown in HeLa cells (Child et al., 2012; Zhang et al., 2008). Replication of VVΔE3L was nearly 1 log higher in HF Tert PKR-kd cells compared to either the parental cell line or the control cell line (data not shown). Thus, the knock down of PKR in HF Tert PKR-kd cells was sufficient to have a functional impact, at least as measured by VVΔE3L replication.

Knockdown of PKR in HF Tert cells does not rescue HCMV[ΔI/ΔT]

In order to determine whether the only essential function of TRS1 in HCMV replication is to antagonize PKR, I tested whether HCMV[ΔI/ΔT] could replicate in HF Tert PKR-kd. I infected both HF Tert PKR-kd and HF Tert Ctrl-kd cells with either HCMV[ΔI/ΔT] or wild-type AD169 HCMV at a low MOI. While AD169 formed plaques in both cell types, I saw no evidence of HCMV[ΔI/ΔT] plaque formation in either cell type in multiple experiments. To determine if a low concentration of HCMV[ΔI/ΔT] particles was indeed present, I titered viral supernatants from HF Tert PKR-kd cells at five days post-infection on TRS1-expressing complementing cells (HF-TRS1) and again did not detect any evidence of viral replication (Fig. 2). Thus, these cells were capable of rescuing VVΔE3L but were unable to rescue HCMV[ΔI/ΔT]. These results suggested two alternative hypotheses. First, the amount of residual PKR present in HF Tert PKR-kd cells was enough to block HCMV[ΔI/ΔT] replication, but not enough to block VVΔE3L replication. Second, TRS1 may have an essential function, unrelated to PKR antagonism, and thus HCMV[ΔI/ΔT] is unable to replicate in even in HF Tert PKR-kd cells.

To begin to distinguish between these hypotheses, I first determined whether residual PKR in HF Tert PKR-kd cells could be activated to inhibit translation. I measured protein synthesis by metabolic radiolabeling in three different cell types (HF Tert Ctrl-kd, HF Tert PKR-kd, and HF-TRS1) after mock infection or infection with AD169 or HCMV[ΔI/ΔT] (MOI = 3). Equivalent amounts of lysates harvested at 72hpi were separated on an SDS-PAGE gel, then

analyzed by autoradiography (Fig. 3). Infection of each cell type with AD169 resulted in a level of protein synthesis that is similar to mock infection (compare lanes 2, 5, and 8 with 3, 6, and 9). While infection of HF Tert Ctrl-kd cells with HCMV[ΔI/ΔT] resulted in a shutoff of host protein synthesis (lane 1), protein synthesis was restored in HF-TRS1 during HCMV[ΔI/ΔT] infection (lane 7), suggesting that TRS1 is necessary to maintain protein synthesis. Infection of HF Tert PKR-kd cells with HCMV[ΔI/ΔT] resulted in an intermediate level of protein synthesis (lane 4). The partial shutoff of protein synthesis could be a result of activated residual PKR; alternatively, another eIF2α kinase may be responsible. Thus, I next examined the level of phosphorylated PKR during HCMV[ΔI/ΔT] infection of these cells.

To evaluate whether PKR activation might explain the intermediate level of translation, we infected cells (MOI = 3) and examined the relative levels of both phosphorylated and total PKR by western blotting (Fig. 4). Very little phospho-PKR accumulated during both AD169 and mock infection of all cell types tested (compare lanes 2, 5, and 8 with 3, 6, and 9), whereas HCMV[ΔI/ΔT] stimulated higher levels of phospho-PKR in HF Tert Ctrl-kd cells. While the level of phospho-PKR accumulated during HCMV[ΔI/ΔT]-infected HF Tert PKR-kd cells was higher than during AD169 or mock infection, it was lower than in HF Tert Ctrl-kd cells. This intermediate amount of phospho-PKR might account for the intermediate level of protein synthesis during infection with HCMV[ΔI/ΔT].

Surprisingly, HCMV[ΔI/ΔT] infection of HF Tert Ctrl-kd and HF-TRS1 cells resulted in a similar level of phospho-PKR. Although HCMV[ΔI/ΔT] can replicate

in HF-TRS1 cells, replication is relatively inefficient and lags behind wild-type HCMV (Marshall et al., 2009). This may be due to the fact that TRS1 transgene expression from these cells is much lower than TRS1 expressed from the viral genome during infection. It is also unknown as to whether TRS1 expression is homogenous among the HF-TRS1 cells; TRS1 may be expressed at high levels in a some cells, which become permissive for HCMV[ΔI/ΔT], while others do not, resulting in detectable PKR phosphorylation. Thus, the high level of PKR phosphorylation in HF-TRS1 cells infected with HCMV[ΔI/ΔT] might be a consequence of partial inhibition of PKR.

Prevention of residual PKR activation in HF Tert PKR-kd cells

I sought to determine whether residual PKR in HF Tert PKR-kd cells might be responsible for preventing HCMV[ΔI/ΔT] infection. However, the data did not clarify this issue so I next attempted to further reduce the level of PKR in the HF Tert PKR-kd cells by use of siRNAs targeting PKR. I also attempted to use small-molecule inhibitors reported to block PKR function, such as 2-aminopurine (2-AP) (Jammi et al., 2003). I assessed the impact of each of these methods by monitoring VVΔE3L replication. Ultimately, neither the addition of siRNAs nor 2-AP treatment increased VVΔE3L replication in HF Tert PKR-kd cells (data not shown).

I also tested a different approach for blocking residual PKR by use of other viral PKR antagonists that target steps in the PKR pathway differing from the one

affected by TRS1. If TRS1 must perform another essential function (for example one that requires dsRNA binding), then use of a PKR antagonist—such as K3L, which does not bind to dsRNA—would not rescue HCMV[ΔI/ΔT] replication. Vaccinia virus E3L is the primary PKR antagonist active during infection of human cells, while K3L is not active in this system (Langland and Jacobs, 2002). However, a mutation of K3L (H47R) emerged during K3L gene expansion that allowed normally growth-deficient VVΔE3L to block PKR and replicate to a higher degree (Elde et al., 2012). To determine whether expression of $K3L_{H47R}$ could further reduce the effects of active PKR in HF Tert PKR-kd cells, I infected these cells as well as control cells with VVΔE3L expressing mutant K3L (VVΔE3L+$K3L_{H47R}$). In a similar manner, I tested the ability of the Toscana Virus NSs PKR-degrading protein for its ability to rescue VVΔE3L to a higher degree in PKR-kd cells than in Ctrl-kd cells (Kalveram and Ikegami, 2013). However, neither of these proteins increased VVΔE3L replication in HF Tert PKR-kd cells when compared to control cells (data not shown).

In conclusion, I was unable to determine whether the failure of HCMV[ΔI/ΔT] to replicate was due to residual PKR activation or because TRS1 was necessary for an additional function using HF Tert PKR-kd cells. In addition, in the course of these studies, I noted that wild-type HCMV replication was less efficient in HF Tert PKR-kd cells than in wild-type HF. Thus, I speculated that a difference in growth characteristics between HF Tert and primary HF cells might be masking replication of HCMV[ΔI/ΔT]. Thus, I decided to knock down PKR in primary HF and test replication of HCMV[ΔI/ΔT].

PKR knockdown in primary HF restores HCMV[ΔI/ΔT] replication

I used the same shRNA previously described and directed against PKR to knock down expression, this time in primary HF. However, because of the difficulty of cloning these cells, I characterized the mixed-population cell line. This HF PKR-kd cell line expressed a similarly low amount of PKR as the HF Tert PKR-kd described previously (Fig. 5). These cells also express much less PKR than HF cells containing a control shRNA as can be seen by immunoblot analysis (Fig. 6a, lanes 1 and 2). As well, VVΔE3L replication was again significantly increased in these knockdown cells (Fig 6b).

I infected the HF PKR-kd cells with AD169, HCMV[TRS1-S2H], and HCMV[ΔI/ΔT] to determine whether reduced PKR expression was sufficient to rescue HCMV lacking both TRS1 and IRS1. I quantified the amount of virus present in the medium after infection by titering the viruses on HF-TRS1. While replication of AD169 and HCMV[TRS1-S2H] in the HF PKR-kd cells and control HFs did not differ greatly, HCMV[ΔI/ΔT] was only able to replicate in the PKR-kd cells (Fig. 7). At 6 days post-infection, HCMV[ΔI/ΔT] titers in the PKR-kd cells were one to two logs lower than those of AD169 or HCMV[TRS1-S2H]. This could indicate that TRS1 might provide one or more auxiliary functions that aid replication. Alternatively, this reduction in HCMV[ΔI/ΔT] titer might simply be due to an inhibitory effect of the small amount of residual PKR remaining in these

cells. Regardless, these data confirm that PKR antagonism is the only function of TRS1 that is essential for HCMV replication.

PKR knockout via CRISPR/Cas9 gene editing

To determine whether the reduced replication of HCMV[ΔI/ΔT] compared to AD169 and HCMV[TRS1-S2H] in the PKR knockdown cells might be due to the presence of residual PKR, I next chose to eliminate PKR expression by creating PKR knockout cells. After considering several different gene editing technologies—such as zinc finger nucleases or TALENs—I chose to use CRISPR/Cas9 technology due to its low cost and ease of design (Mali et al., 2013; Trevino and Zhang, 2014). We first designed small guide RNAs guiding Cas9 to target a splice acceptor site upstream of the start codon (Fig. 8). Using lentiviral constructs to transduce both HF and HF Tert cells, we successfully reduced the amount of PKR expression when compared to control cells, as seen by Western blotting of mixed-population lysates (data not shown). These cells still expressed a detectable level of PKR. However, I found that the reduction in the level of PKR rescued VVΔE3L. This indicates that PKR expression is likely reduced to a level that permits VVΔE3L replication. I decided to propagate individual cellular clones to isolate cells expressing little to no PKR.

Using both limiting dilution and cloning ring methods, I isolated two control clones and six knockout clones from the mixed HF Tert cell populations. I tested each knockout clone for the ability to rescue VVΔE3L in comparison with a

control clone expressing Cas9 only (Fig. 9a). Western blotting confirmed expression of PKR in control cell lines and varying reductions in PKR expression in knockout clones (Fig 9b).

While cloning strategies aim to isolate and monitor the expansion of single cells, an impure population will arise from an expansion that began with more than one cell. This could explain why some “clones” retained PKR expression, yet rescued VVΔE3L. The partial reduction could also results from mutation in one but not both alleles in a clone and from nonhomologous end joining event that only partially reduce PKR expression. To identify bona fide clones, I isolated genomic DNA from each knockout clone and PCR-amplified a small region of DNA surrounding the gRNA target region. Fragments were TOPO-cloned and sequenced to reveal that several knockout “clones” contained more than two different sequences. Assuming that HF Tert cells only contain two PKR alleles, finding of more than two sequence variants indicates that the “clone” is really a mixed population. By these criteria, clones 2, 3, and 4 were impure.

Clones 1, 5, and 6 were pure populations as determined by sequencing (a maximum of two distinct sequences were found for each clone). Sequence analysis clones 1 and 6 revealed that while mutations occurred near the splice acceptor site, these mutations did not destroy it (data not shown). These clones also failed to rescue VVΔE3L, and continued to express PKR at levels equivalent to control clones (Fig. 9a and 9b).

Clone 5 contained one mutation that destroyed the splice acceptor site by deletion and one mutation that removed the original start codon via a very large

deletion and small duplication (Fig. 10). I determined *in silico* that a downstream start codon in good context could be used to make a truncated protein. Although clone 3 was found to be an impure population, I again isolated cells by limiting dilution and tested the new clones. I found one of these clones to be similar in mutation to clone 5; one allele had a destroyed splice site, while the other had large deletion that included the original start codon. This subclone also rescued VVΔE3L and expressed no detectable PKR (data not shown). I chose to ultimately study clone 5, which I will refer to as PKR KO A.

In addition to creating knockout cell lines via mutation of the splice acceptor site, we also engineered a small guide RNA targeting the first double-stranded RNA binding domain of PKR. We transduced primary HF to knock out PKR with the goal of creating a frameshift mutation, preventing the expression of PKR. Sequencing the genomic DNA from these cells revealed a one-basepair insertion that likely caused a frameshift (Fig. 10). However, we were not able to isolate individual clones from this HF. Thus, this cell line differs from PKR KO A in that it is a mixed population of HF, rather than a clonal population of HF Tert, and the mutations differ. I will now refer to this cell line as PKR KO B.

Both PKR KO A and PKR KO B cells express little or no wild-type PKR by western blot assays (Fig. 11). An extended exposure revealed what may be a background band as well as a truncated protein expressed in PKR KO A (lane 2), while a small amount of PKR was detected in PKR KO B (lane 4). In order to determine if any residual PKR present was capable of becoming active, I infected cells with VVdelE3L (MOI = 3) and harvested lysates at 24hpi. I found that in both

types of PKR knockout cells, no phosphorylated PKR was detectable (Fig. 12). (PKR KO C in lane 4 is another PKR knockout clone that I will not be discussing further.) Congruent with this finding, I showed that both PKR KO A and PKR KO B cells were able to rescue replication of VVdelE3L in comparison with paired control cells (Fig. 13).

PKR knockout restores replication of HCMV[ΔI/ΔT] and HCMV[TRS1-Mut 1].

To determine whether the reduced replication of HCMV[ΔI/ΔT] compared to AD169 and HCMV[TRS1-S2H] in the PKR knockdown cells might be due to the presence of residual PKR, I evaluated the impact of PKR knockout on the replication of AD169 and the HCMV recombinants. Both PKR KO A and PKR KO B lines and their paired control cells were infected with HCMV[TRS1-S2H], HCMV[TRS1-Mut 1], or HCMV[ΔI/ΔT] at an MOI of 0.1 and the amount of progeny virus in supernatants was determined by titering on HF-TRS1. As expected, HCMV[TRS1-S2H] replicated well and both HCMV[TRS1-Mut 1] and HCMV[ΔI/ΔT] failed to replicate in control cells (Fig. 14). In contrast, both of these viruses replicated as well as HCMV[TRS1-S2H] in each knockout cell line, again confirming that TRS1 is required to block the PKR pathway. This result also suggests that any ancillary functions performed by TRS1 are unnecessary and do not contribute substantially to viral replication in this system (Fig. 14).

Conclusion

In this chapter, I have shown that reduction or elimination of PKR rescues replication of HCMV[ΔI/ΔT], suggesting that blocking PKR is the sole purpose of TRS1 in a cell culture-based model of HCMV replication. My initial studies aiming to test whether PKR antagonism is the only essential function of TRS1 used a cloned, HF Tert PKR knockdown cell line. Result of these studies suggested that TRS1 might have an additional essential function besides blocking PKR. These initial results turned out to be misleading for unknown reasons. I speculate that perhaps the clone we isolated had or developed a mutation unrelated to PKR that unfortunately restricted viral replication. Nonetheless, subsequent studies of nonclonal HF PKR-kd knockdown cells and of clonal and non-clonal knockout cells revealed that PKR depletion was sufficient to rescue HCMV[ΔI/ΔT] replication.

Using HF that were neither cloned nor Tert-immortalized, I demonstrated that PKR knockdown in HF allows HCMV[ΔI/ΔT] replication, but at a diminished capacity in comparison to wild-type AD169. Next, I engineered two independent PKR knockout cell lines, each of which restored HCMV[ΔI/ΔT] and HCMV[TRS1-Mut 1] replication levels to that of HCMV[TRS1-S2H]. These data indicate that any functions performed by TRS1-Mut 1 are nonessential and in fact do not even aid in HCMV replication in these cells.

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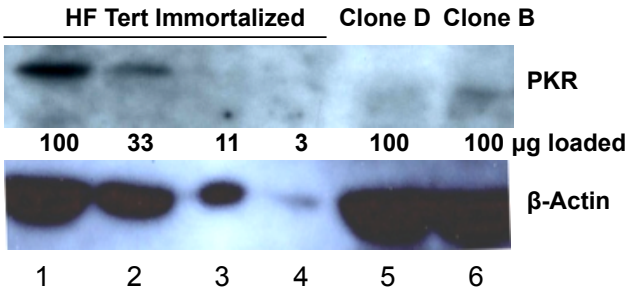


Figure 1. Total PKR levels in HF Tert PKR knockdown cells. Varying amounts of parental HF Tert cells lysates were compared to lysates of HF Tert PKR knockdown Clones D and B lysates after electrophorectic sepation by SDS-PAGE gel and western blotting.

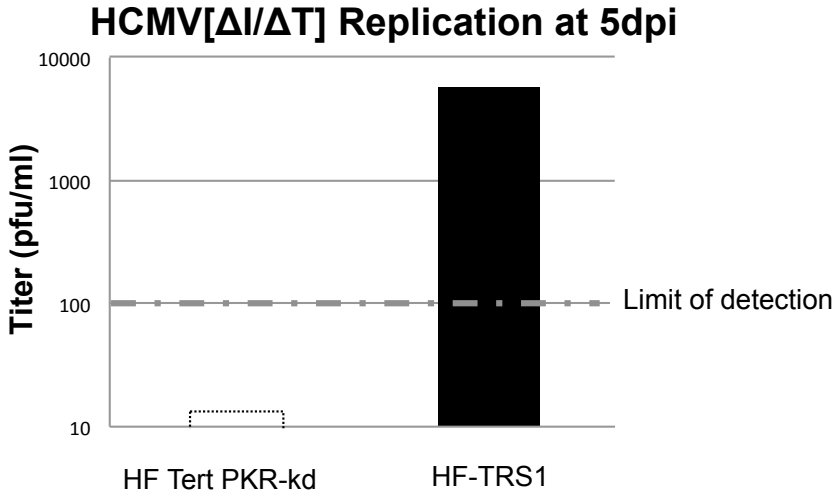


Figure 2. HCMV[ΔI/ΔT] does not replicate in HF Tert PKR knockdown cells. HF Tert PKR-kd cells or HF-TRS1 cells were infected with HCMV[ΔI/ΔT] (MOI = 0.1) and 5dpi supernatants were collected and titered on HF-TRS1. No plaques developed from infected PKR-kd cells, while HF-TRS1 supported HCMV[ΔI/ΔT] replication.

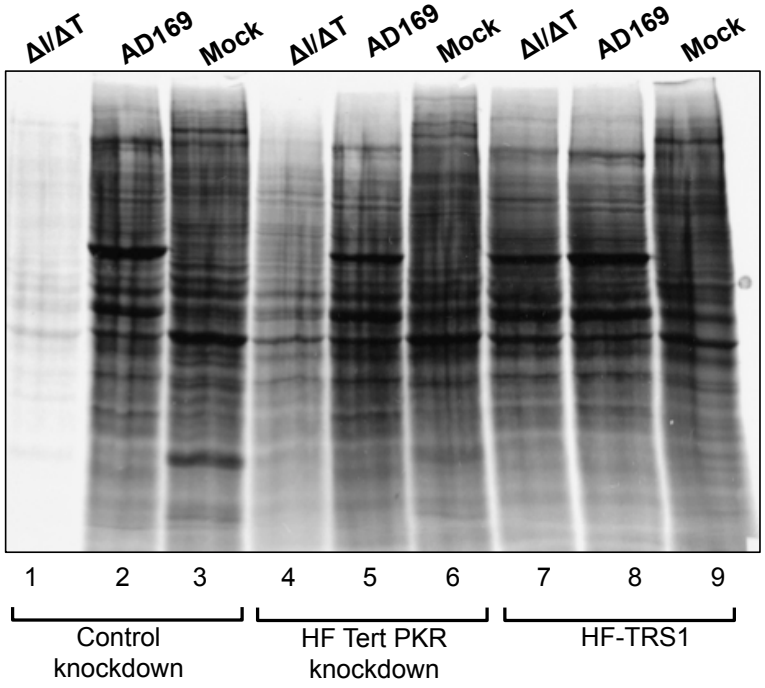


Figure 3. HF Tert PKR knockdown cells undergo a partial shutoff of host protein synthesis during HCMV[ΔI/ΔT] infection. Cells were infected with HCMV at an MOI of 3 for 72 hours and then pulsed with 35S-Met for 1 hour. Cell lysates were separated by SDS-PAGE and analyzed by autoradiography.

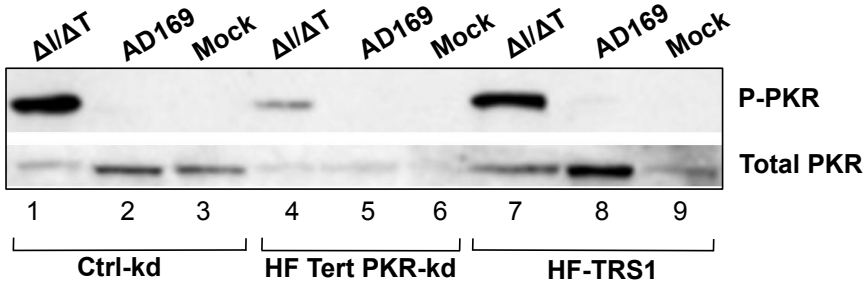


FIGURE 4. HCMV[ΔI/ΔT] activates residual PKR in HF Tert PKR knockdown cells. Lysates from the experiment resulting in Figure 3 were examined for the levels of phosphorylated and total PKR by immunoblot. PKR was phosphorylated in HCMVΔIΔT infection of either PKR-kd or HF-TRS1 cells but, the amount of phosphorylated PKR was much lower in PKR-kd cells.

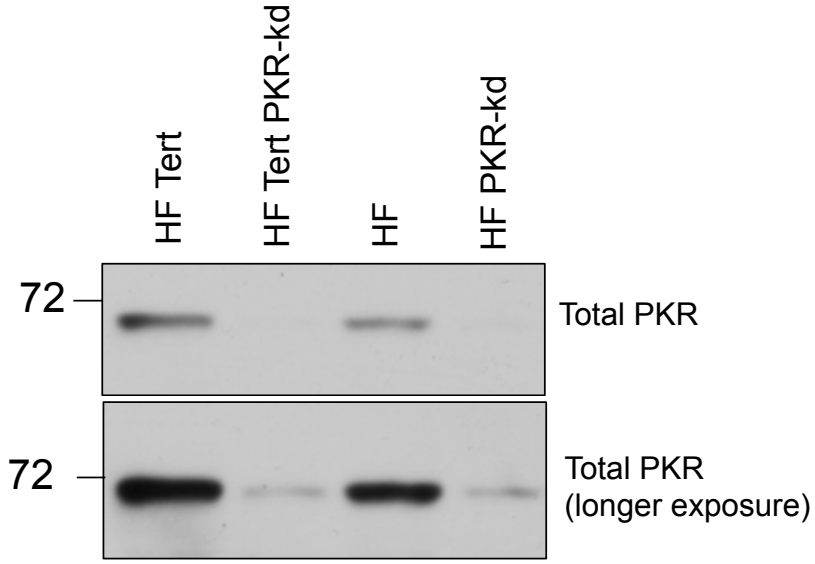


FIGURE 5. PKR expression in HF Tert PKR-kd vs. HF PKR-kd cells. PKR expression was measured by immunoblot assay in the indicated cell lines. The levels were equivalent between in the clonal line HF Tert and in non-clonal HF in which PKR has been knocked down using the same shRNA.

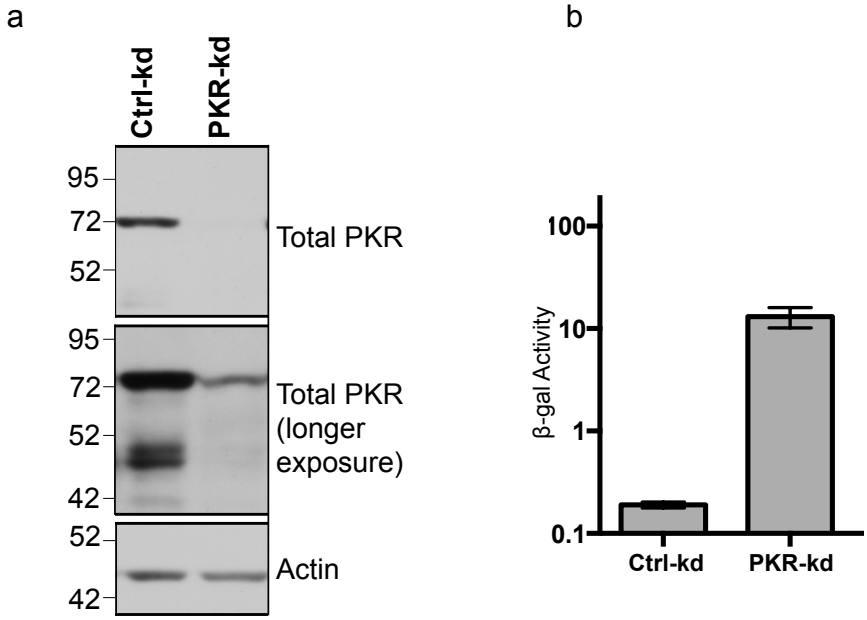


FIGURE 6. PKR expression and VVΔE3L rescue in HF PKR-kd cells. (a) The abundance of total PKR in HF PKR knockdown cell lines compared to that in control cells was measured by immunoblot assay. VVΔE3L replication in triplicate wells of (b) HF PKR knockdown cells was quantified by measuring β-galactosidase activity at 48 hpi as described in Materials and Methods.

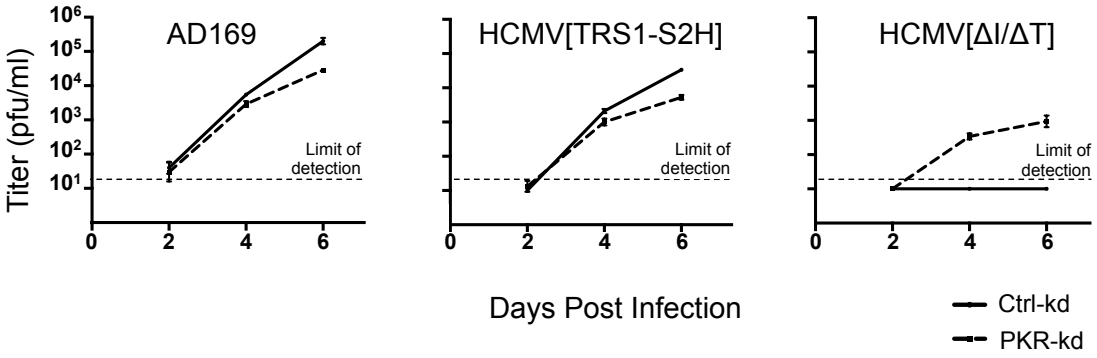


FIGURE 7. Knockdown of PKR in HF partially restores HCMV[ΔI/ΔT] replication. Triplicate wells of PKR knockdown and control HF were infected with AD169, HCMV[TRS1-S2H] or HCMV[ΔI/ΔT] (MOI = 0.1) Virus present in the cell culture medium was collected at two-day intervals and viral titers determined on HF-TRS1 cells. Mean titers (and standard deviations) are shown.

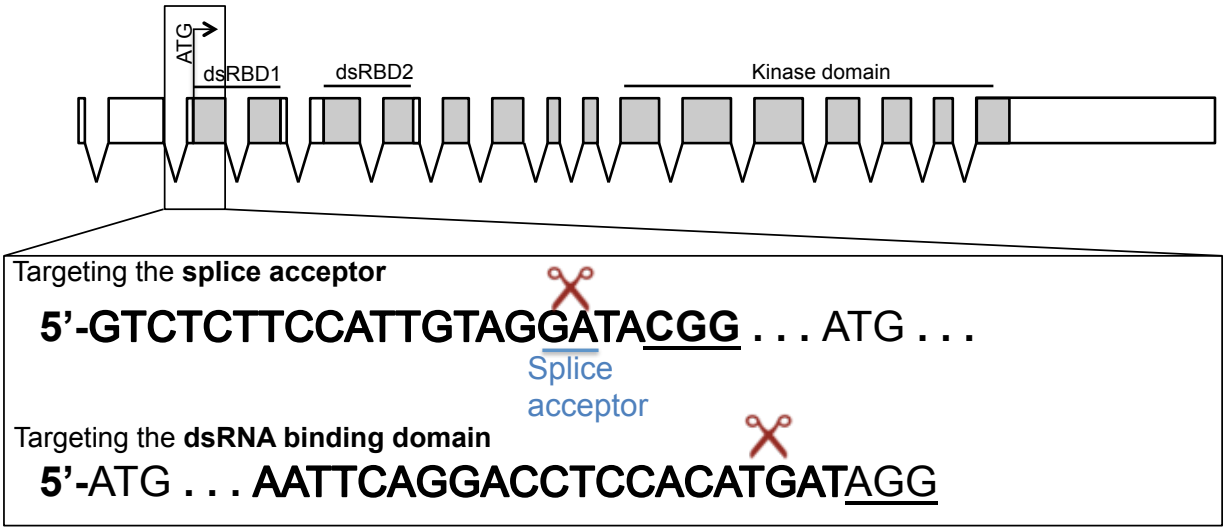


FIGURE 8. Design of small guide RNAs to knock out PKR. One sgRNA targets a splice acceptor site prior to the start codon of PKR within exon 3. The other sgRNA targets the beginning of the first dsRNA binding domain of PKR, also within exon 3. Pictured above is a schematic outline of the introns and exons of human PKR (adapted from Rothenberg et al, 2008).

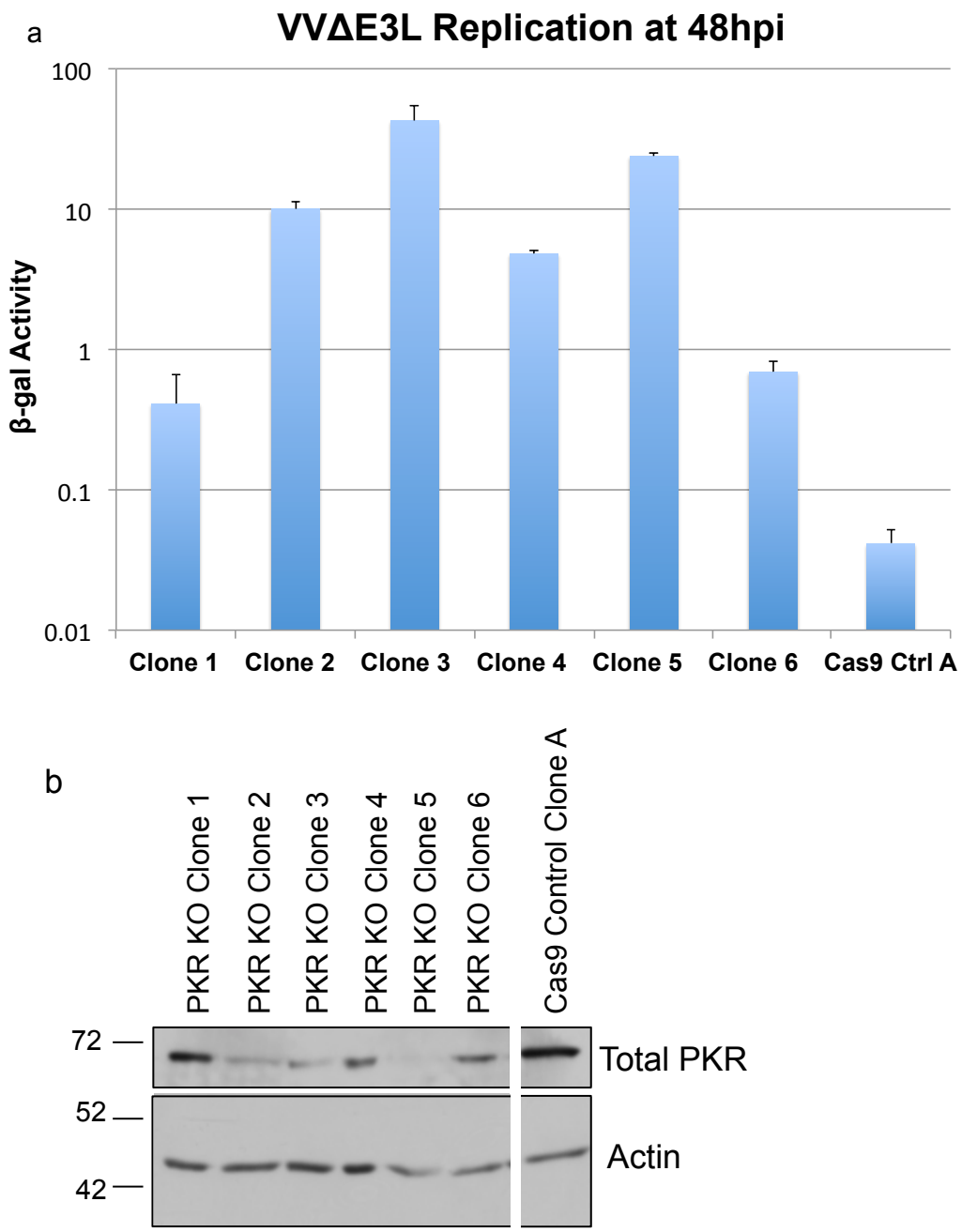


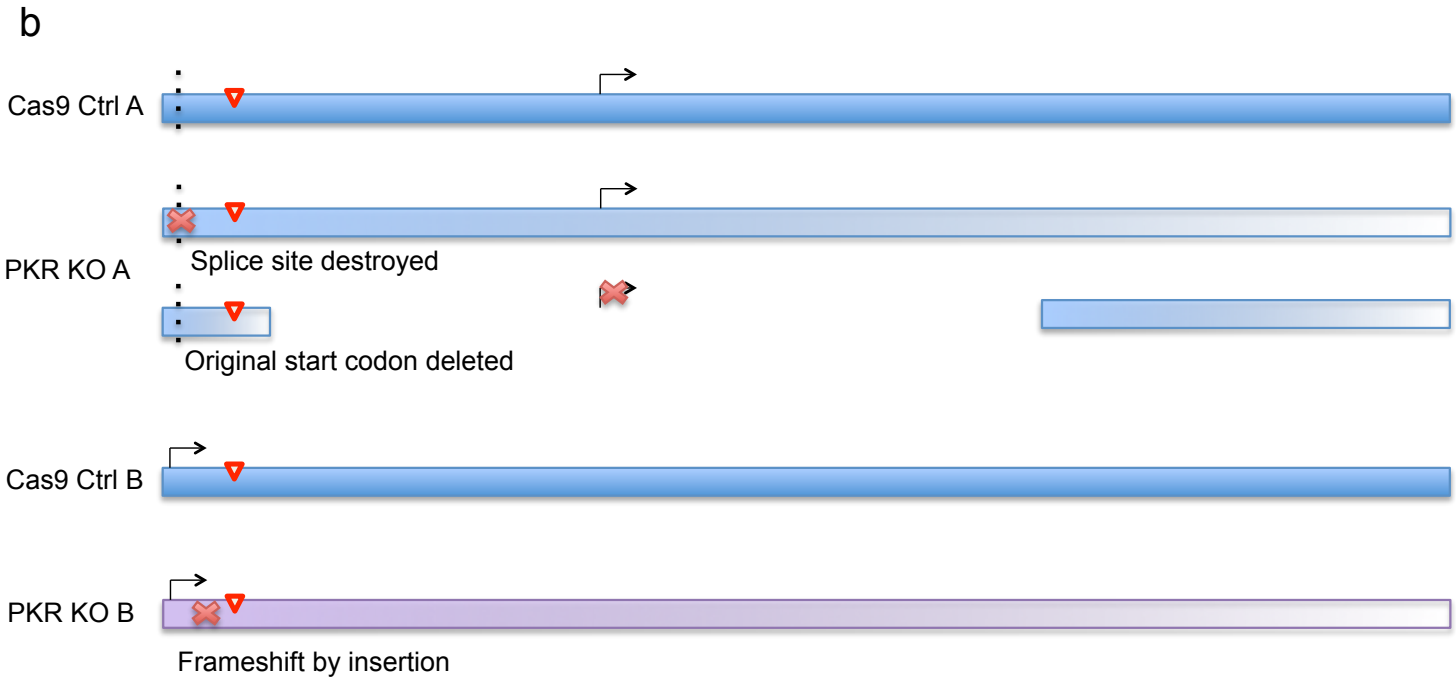
FIGURE 9. Individual PKR knockout clone analysis. HF Tert PKR cells were targeted at a splice acceptor site at the start of Exon 3 with a lentiviral-delivered sgRNA coupled to a Cas9 endonuclease. Six clones were isolated after selection with puromycin and after expansion, were analyzed for (a) VVΔE3L rescue and (b) total PKR by immunoblot.

a

Cell Line	Mutations in ORF
Cas9 Ctrl A	sgRNA 1 (splice site) GTCTCTTCCATTGT**AG**GATACGGGAAGAAGAA**ATG**GCTGGT
PKR KO A	GTCTCTTCCATTGT**A---**TACGGGAAGAAGAA**ATG**GCTGGT GTCTCTTCCATTGT**AG---**…Δ73bp +7bp duplication
Cas9 Ctrl B	sgRNA 2 (dsRBD 1) ATTCAGGACCTCCACATGATAGGAGGTAGGTTGCTATAAA
PKR KO B	ATTCAGGACCTCCACATTGATAGGAGGTAGGTTGCTATAAA

FIGURE 10. Sequence analysis and mutational consequences of CRISPR/Cas9-mediated PKR knockout cells. (a) For Cas9 Ctrl A and Ctrl B cells, the wild-type sequences are pictured. The red arrow indicates the predicted Cas9 cut site; the bold bases identify the splice acceptor site (for Ctrl A); the underlined bases identify the protospacer adjacent motif (PAM) site; and the arrow indicates the start codon (for Ctrl A). The two different alleles of PKR KO A cells are illustrated. PKR KO A features one allele with a mutation that destroys the splice site and one that retains the splice sites but has a large deletion that removes the original start codon. The top allele has a 3-bp deletion (3 dashes) and the bottom allele has a 73bp deletion and a 7bp duplication. PKR KO B features a 1bp insertion, likely in both alleles, that causes a frameshift at the start of the first double-stranded RNA binding region. (b) Schematic diagram of PKR KO A and PKR KO B mutational consequences is pictured. Red arrows indicate predicted Cas9 cut sites; dotted lines indicate the splice site; arrows indicate the start codon.

b



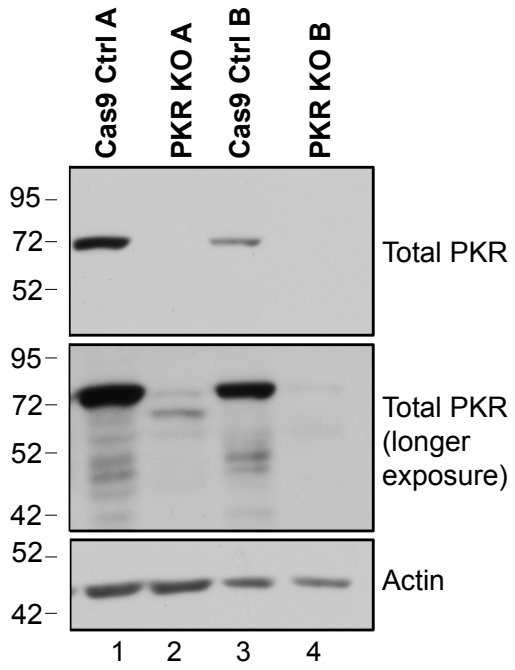


FIGURE 11. PKR expression in PKR knockout cells. (a) The abundance of total PKR in PKR KO cell lines A and B compared to that in paired control cells was measured by immunoblot assay.

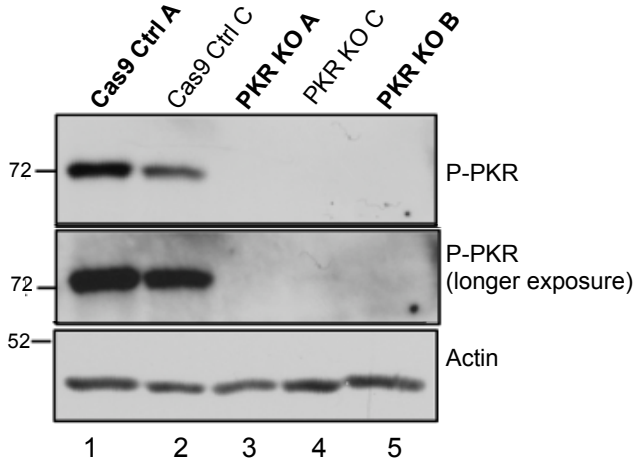


FIGURE 12. Phospho-PKR in VVΔE3L-stimulated PKR knockout cells. Cells were infected with VVΔE3L (MOI = 3) at 24hpi lysates were analyzed by immunoblot for the abundance of phospho-PKR.

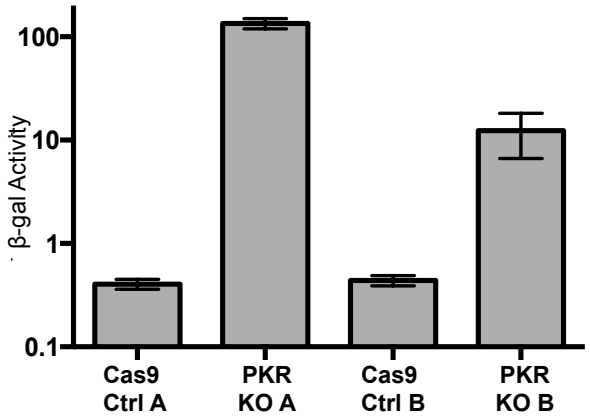


FIGURE 13. VVΔE3L rescue in PKR knockout cells. VVΔE3L replication in triplicate wells of PKR KO cells was quantified by measuring β-galactosidase activity at 48 hpi as described in Materials and Methods.

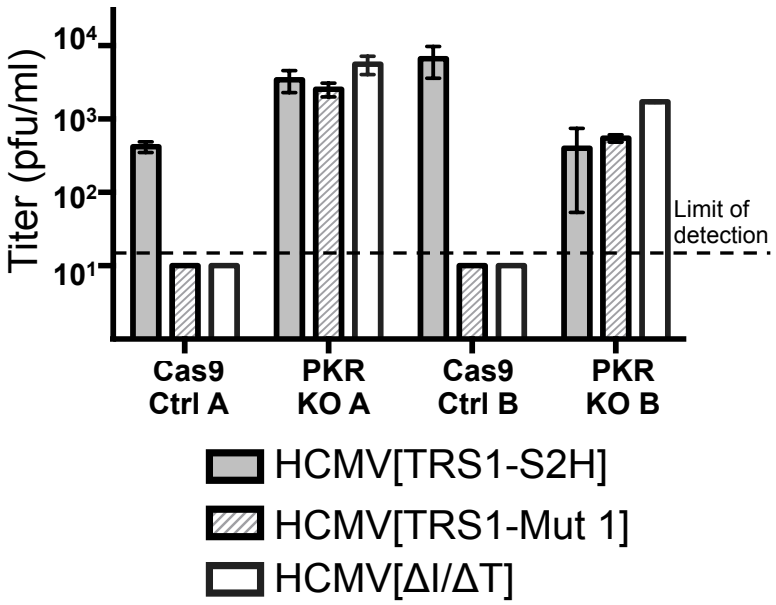


FIGURE 14. PKR knockout fully restores HCMV[TRS1-Mut 1] and HCMV[ΔI/ΔT] replication. Triplicate wells of PKR KO A and PKR KO B cells, along with paired control cells, were infected with HCMV[TRS1-S2H], HCMV[TRS1-Mut 1], or HCMV[ΔI/ΔT] (MOI = 0.1). Virus accumulating in the medium from days 6 to 8 post-infection was titered on HF-TRS1 cells. Mean titers (and standard deviations) are shown.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Summary

HCMV replication requires expression of either *TRS1* or *IRS1* (Marshall et al., 2009). While each of the proteins encoded by these genes has been reported to have multiple functions, which ones are essential for replication of HCMV were unknown prior to this study. I now provide strong evidence, based on analyses of mutant viruses and cell lines, that the sole essential function of TRS1 and IRS1 is their ability to inhibit the PKR pathway.

I first identified a TRS1 mutant, TRS1-Mut 1, that is unable to bind to PKR or to rescue VVΔE3L replication. Importantly, when inserted into HCMV[ΔI/ΔT], TRS1-Mut 1 was incapable of rescuing HCMV replication. In contrast, either of two wild-type TRS1 genes (TRS1-HA or TRS1-S2H) or the major PKR antagonist from vaccinia virus, E3L, expressed from the same virus backbone could each rescue viral replication ((Marshall et al., 2009) and Chapter 2). Although these results are consistent with the conclusion that the essential role of TRS1 is to block the PKR pathway, it remained possible that TRS1 had an additional critical function. In order to determine whether the only essential role of TRS1 was to block PKR, I constructed PKR knockdown and knockout cell lines. Our finding that HCMV[ΔI/ΔT] was able to replicate in each of these PKR deficient cell lines but not in control lines establishes that the only essential function of TRS1 (or IRS1) is PKR antagonism.

Effects of mutations of the TRS1 C-terminal domain on binding to PKR and to dsRNA

Despite considerable effort from our lab, the structure of TRS1 is still unknown. This limited our ability to predict whether mutation of the C-terminus would affect functions for which the N-terminus has been shown to be sufficient. The fact that prior studies using truncation mutants showed the C-terminus was not necessary for binding to dsRNA or to UL44, for homodimerization, and for blocking autophagy led us to predict that point mutations at the C-terminus would not perturb these functions (Chaumorcel et al., 2012; Hakki and Geballe, 2005; Strang et al., 2010). However, TRS1[R697A] disrupted both dsRNA binding and homodimerization, and even TRS1-Mut 1 appeared to have some reduction in dsRNA binding. One possibility is that TRS1 may really require the C-terminus to homodimerize or to bind dsRNA or UL44. TRS1 may need to undergo a conformational change in order to bind some of its N-terminal binding partners, and deletion of the C-terminus may obviate the need for this change. In contrast, mutation of the C-terminus may have perturbed the ability of TRS1 to undergo this conformational change.

To test this hypothesis, intramolecular fluorescence resonance energy transfer (FRET) is one way to probe for structural changes of TRS1 that may occur when binding to known partners (Truong and Ikura, 2001). Both the N- and C-terminus of TRS1 or C-terminal TRS1 mutants can be labeled with either a

donor or an acceptor probe. With proper probe design, labeled TRS1 should be able to retain the same behavior as wild-type TRS1 (for example, can still bind known partners and rescue VVΔE3L). Labeled TRS1 constructs will be *in vitro*-translated and exposed to poly[I:C] or PBS as a control. The change in fluorescence measured pre- and post-poly[I:C] exposure would suggest that TRS1 undergoes a conformational change during dsRNA binding, and a lack of change in TRS1 C-terminal mutants would suggest that this change does not or cannot occur.

More experiments are necessary to determine how TRS1 interacts with PKR. Previous work from our lab suggests that PKR binding by TRS1 can occur with weak or absent dsRNA binding (Bierle et al., 2013). This result supports a model that TRS1 must bind to both PKR and dsRNA in order to antagonize PKR. I hypothesize that TRS1 binding of PKR stabilizes the structure of TRS1 to allow dsRNA binding; this could also be explained by a PKR binding-induced conformational change of TRS1, as detailed in the previous paragraph. To test this hypothesis, I will transfect both HeLa cells and HeLa PKR knockout cells with wild-type and mutant forms of TRS1. I will then perform a dsRNA binding assay with the resulting lysates. Finding that wild-type TRS1 expressed in PKR knockout cells loses some dsRNA binding ability—similarly to TRS1-Mut 1 expressed in normal, PKR-expressing HeLa cells—would suggest that TRS1 needs to bind PKR to optimally bind dsRNA. It may be possible that TRS1 and PKR are simultaneously bound to the same molecule of dsRNA. However, we

are still working to purify TRS1 with the goal of isolating and sequencing bound dsRNA to uncover the source.

There are several limitations of the dsRNA-binding assay to consider in the analysis of these results. The dsRNA binding assay I used tested the activities of proteins expressed by transfection of cells. While this method is arguably more physiological in than using proteins produced by *in vitro* translation, it does not mirror the exact environment of a viral infection. The concentration of TRS1 achieved during transfection may differ from that expressed during infection, and the timing of expression may differ as well. Now that I have generated HCMV[TRS1-Mut 1], I will be able to test whether TRS1-Mut 1 can bind dsRNA, to UL44 and to Beclin 1 during infection of permissive cells.

Comparing the relative strength of dsRNA binding between TRS1 constructs is also difficult, as this assay is qualitative, rather than quantitative. Although our results suggested that TRS1-Mut 1 may not be able to dsRNA well, recent work from our lab shows that mutants of TRS1 with reduced abilities to bind dsRNA are still able to rescue VVΔE3L replication to levels achieved with wild-type TRS1 (Bierle et al., 2013). To clarify these results, I can estimate, after taking into account the relative input level of expression, the differences between the percent of input bound per construct. However, this method was not very reproducible. In the future, I plan to design a competition-based assay to determine the relative dsRNA binding affinities between TRS1 constructs. I can use the baculovirus system to express TRS1 and use an electrophoretic mobility

shift assay (EMSA) as described previously (Bierle et al., 2013) as a way of quantifying the capacity of each construct to bind dsRNA.

In summary, my data suggest that protein misfolding is a likely cause for the failure of TRS1[R697A] to bind PKR, to bind dsRNA, and to dimerize. I succeeded in finding mutants that appeared to be more selective in their deficiency in PKR binding but even these seemed to have a reduced ability to bind to dsRNA. PKR binding by TRS1 is required for antagonism of PKR during both vaccinia and HCMV infection. My study of HCMV[TRS1-Mut 1] suggests that PKR antagonism is at least one critical requirement in rescue of HCMV[ΔI/ΔT].

Role of dsRNA binding by TRS1

In the previously described experiments, I assessed the ability of CCTA mutants to antagonize PKR through rescue of VVΔE3L. To test whether this capability translated to the HCMV system to rescue HCMV[ΔI/ΔT], I first created a series of cell lines expressing each CCTA mutant. These cell lines expressed TRS1 with an N-terminal GFP fusion, which did not alter the behavior of these mutants. For example, TRS1-Mut 1 and Triple Mut remained unable to rescue VVΔE3L replication.

While I determined that cells expressing TRS1-Mut 1 were unable to support HCMV[ΔI/ΔT] replication, Triple Mut-expressing cells rescued HCMV[ΔI/ΔT]. Because Triple Mut did not rescue VVΔE3L, it remained possible

that this construct met the requirements for blocking PKR in the HCMV system, but not in the vaccinia system. However, we were unable to determine whether Triple Mut expression could block phospho-PKR accumulation through studying the cell line alone. In considering an alternative hypothesis, the N-terminal GFP fusion to Triple Mut may stabilize the protein by dimerizing and restoring dsRNA binding to the mutant. I used fresh lysates of TRS1- and Triple Mut-expressing cells via a dsRNA-binding assay to determine whether dsRNA binding was restored; however, with each passage of these cell lines, TRS1 expression decreased, possibly due to a shutoff in transgene expression. Thus, the difficulty experienced in manipulation of these cell lines led us to create recombinant viruses.

The generation of viruses HCMV[TRS1-S2H] and HCMV[TRS1-Mut 1] enabled me to answer the remaining questions about PKR antagonism during infection. Notably, we did create a virus expressing Triple Mut (HCMV[Triple Mut]) that failed to replicate in HF, corroborating the hypothesis that the fusion of GFP altered the function of Triple Mut when expressed *in trans* during HCMV infection. In summary, I determined that HCMV[Triple Mut] could not replicate in HF, indicating that dsRNA binding by TRS1 is required during HCMV replication. I plan to prepare adequate stocks of this virus for further testing, such as determining whether HCMV[Triple Mut] infection of HF results in the increase in phospho-PKR, as we expect of a TRS1 mutant that cannot block PKR.

Knockdown and knockout of PKR

Both knockdown and knockout of PKR in HF restored replication of HCMV[ΔI/ΔT], confirming that TRS1 is required solely to block PKR. I created two different PKR knockdown cell lines. While primary HF with PKR knocked down restored HCMV[ΔI/ΔT] replication, HF Tert cells did not. Although both expressed a similarly low level of PKR and both permitted VVΔE3L to replicate, the difference likely stemmed from differences in the parental cell lines prior to PKR knockdown. This phenotypic difference is also likely to be independent of Tert (telomerase reverse transcriptase) used to immortalize HF Tert cells, as I also used Tert-immortalized cells to knock out PKR that permit HCMV[ΔI/ΔT] to replicate. More likely is that HF Tert cells have been propagated in the lab for many generations and changes have accumulated that block HCMV[ΔI/ΔT] viral replication even with the depletion of PKR. Alternatively, the HF Tert PKR-kd cells were a clonal population, and while I selected a clone with the highest VVΔE3L replication, I may have inadvertently selected a clone with other mutations affecting HCMV replication. In retrospect, I could have selected and tested more HF Tert PKR-kd clones.

While PKR knockout cells also restored HCMV[ΔI/ΔT] replication, I noted that repeated passage of these cells resulted in a reduction in wild-type HCMV replication. This occurred in both HF Tert-based control and knockout cell lines. It would be interesting to examine the changes that have occurred in each of these cells by using RNASeq to study differences between later-passage HF Tert and earlier, lower-passage stocks (Cloonan and Grimmond, 2008). This may enable

our lab to learn more about mutations that limit viral replication in human fibroblasts.

In Chapter 3, I determined that HCMV[ΔI/ΔT] replication is restored to levels achieved by HCMV[TRS1-S2H] in two different PKR knockout cell lines, indicating that PKR antagonism is the only essential function of TRS1. Furthermore, in these cells, both HCMV[ΔI/ΔT] and HCMV[TRS1-Mut 1] replicated as well as HCMV[TRS1-S2H], suggesting that TRS1-Mut 1 did not have an ancillary role in HCMV replication in these cells.

Why does HCMV encode both TRS1 and IRS1?

Several viruses encode more than one PKR antagonist. For example, vaccinia virus encodes E3L and K3L, while HSV-1 encodes two copies of γ34.5 and one of US11 (Gil and Esteban, 2004; Mohr, 2004). In these cases, the antagonists target different steps of the PKR pathway, are expressed at different times during infection, or are more important in certain cell types than others. HMCV also has two PKR antagonists, but in this case the two genes are nearly identical in sequence and function. They are partially encoded within the repeats surrounding the unique short region of the genome and thus their N-terminal two-thirds are identical. In addition, their C-termini are ~50% similar. Thus, it is not surprising that they share several reported activities, including inhibition of autophagy and of the PKR and RNase L pathways (Chaumorcel et al., 2012; Child et al., 2004; Mouna et al., 2015). Both TRS1 and IRS1 are able to bind to

dsRNA, PKR, and Beclin-1 (Chaumorcel et al., 2012; Hakki and Geballe, 2005; Hakki et al., 2006; Mouna et al., 2015).

Why HCMV contains two genes with seemingly redundant roles is currently unknown. Because of the pressure for a compact genome, it is common for one viral gene to encode more than one function, rather than for one function to be encoded in two genes. Furthermore, only expression of TRS1 or IRS1 is required for HCMV to replicate, and I have shown here that the only essential function of TRS1 during HCMV replication is to block PKR. If encoding just TRS1 or IRS1 was important, I would expect to see a collapse to just one gene over time. In fact, while chimpanzee CMV encodes both TRS1 and IRS1, several other related CMVs, such as those specific to rhesus macaques and African green monkeys, encode just TRS1.

My studies reveal insights into why HCMV might have retained two seemingly redundant PKR antagonists. Although other reports have indicated that deletion of just IRS1 does not impact replication, I have observed a mild reduction in the replication of two different viruses that lack IRS1, HCMV[TRS1-HA] and HCMV[TRS1-S2H] (Blankenship and Shenk, 2002; Dunn et al., 2003; Jones and Muzithras, 1992; Marshall et al., 2009). Moreover, infection with these viruses results in an increased level of phospho-PKR compared to cells infected with wild-type HCMV (Chapter 2, Fig. 7b). Although it is possible that the epitope tags weaken TRS1's ability to block PKR in these viruses, my data suggest the possibility that TRS1 alone is not as efficient at blocking PKR as are TRS1 and IRS1 in combination. In studies involving experimental evolution of vaccinia virus

recombinants, we found that gene amplification of a weak PKR antagonist led to improved viral replication (Brennan et al., 2014; Elde et al., 2012). In the same way, it is possible that deletion of TRS1 or IRS1 leads to a reduction in the total level of PKR antagonism during HCMV replication. Some data suggest that wild-type HCMV expresses more TRS1 than IRS1 (Bierle et al., 2013; Mouna et al., 2015), which may explain the replication defect observed in single deletion mutants of TRS1 but not IRS1 (Blankenship and Shenk, 2002; Dunn et al., 2003; Jones and Muzithras, 1992). It is possible that simultaneous expression of both proteins might be necessary in order to generate an optimal concentration of TRS1 and IRS1 to block PKR.

The combined expression of TRS1 and IRS1 may be important for other roles attributed to these proteins, or in other cell types. Although blocking autophagy is not a critical role of TRS1 during HCMV replication in fibroblasts, a recent publication suggests that expression of both TRS1 and IRS1 maximizes this function (Mouna et al., 2015). Because at least two functions of TRS1 seem to benefit from the inclusion of IRS1 expression as well, we plan to take an experimental evolutionary approach to better understand the benefit of both TRS1 and IRS1 expression. HCMV expressing only TRS1 (or a nonhuman primate homolog of TRS1, such as rhesus TRS1) will be serially passaged in cell culture and monitored for gene duplication in the face of increased pressure—the overexpression of PKR. These cells are currently being engineered in our lab.

The relationship between autophagy, TRS1, and HCMV

Early in infection, HCMV stimulates autophagy; however, as viral protein expression increases, autophagy is downregulated (Cavignac and Esclatine, 2010). This phenomenon has been attributed to the function of TRS1 or IRS1 in blocking autophagy via direct interaction with the essential host autophagy protein, Beclin 1, to block the formation of autophagosomes (Chaumorcel et al., 2012; Mouna et al., 2015). Notably, this function of TRS1 or IRS1 is independent from the ability of each protein to antagonize PKR. Truncation of the N-terminal 44 codons of TRS1 or IRS1 prevents Beclin 1 binding and the resulting block in autophagy without affecting the ability of either protein to block PKR. Likewise, a C-terminal truncation, TRS1[1-679], which we have previously shown lacks the ability to bind or antagonize PKR, retains Beclin 1 binding properties and blocks autophagy. A virus lacking IRS1 and expressing a Beclin 1-nonbinding TRS1 replicates to wild-type levels in primary fibroblasts, consistent with our argument that blocking autophagy is a nonessential function of TRS1 in HCMV replication of HF.

The authors who characterized this function of TRS1 also found that the expression of both TRS1 and IRS1 during viral infection resulted in the maximum reduction of autophagy (Mouna et al., 2015). Surprisingly, in this study, the inhibition of autophagy reduced HCMV replication, while induction of autophagy significantly enhanced replication, suggesting that autophagy has a pro-viral role in HCMV replication. In contrast, recent findings from another lab suggest that activation of autophagy via a different pathway restricts viral replication in several different cell types (Belzile et al., 2015). It remains unclear as to whether this

modulation of autophagy by HCMV is proviral; manipulation of the autophagy process may differ at various points in the progression of infection.

It would be interesting to examine whether autophagic flux occurs during HCMV[ΔI/ΔT] infection in the absence of PKR and if autophagy is proviral in this instance. To test this, HCMV or HCMV[ΔI/ΔT] infection of PKR knockout cells would be monitored at several timepoints for evidence of autophagy. If TRS1 or IRS1 is necessary for the downregulation of autophagy during HCMV infection, I would expect to see autophagy maintained during HCMV[ΔI/ΔT] infection in the absence of PKR (where replication is permitted). Likewise, stimulation of autophagy via starvation during HCMV[ΔI/ΔT] replication in PKR knockdown cells may enhance replication. It would be of considerable interest if the impact of TRS1 and IRS1 were anti-viral in cells in which PKR antagonism was unnecessary. This result would indicate susceptibility that the anti-autophagy effects of TRS1 may be a response to host defenses.

Roles of other TRS1 functions

My finding that PKR antagonism is the only essential function of TRS1 and IRS1 raises questions about the importance of the other activities that have been attributed to these factors. Some of those functions may be indirect consequences of their ability to block PKR activation. For example, the described activation of reporter gene expression might simply be due to maintenance of protein synthesis as a result of TRS1 or IRS1 blocking PKR. Consistent with this

interpretation, I found that TRS1 did not activate reporter gene expression in PKR-deficient cells (Bierle et al., 2012). Ziehr et al found that most but not all of the increase in reporter gene expression by TRS1 was eliminated by PKR depletion (Ziehr et al., 2015). Their study implicated a PKR-independent effect of TRS1 on translation that might be mediated by the ability of TRS1 to bind to the mRNA cap. Likewise, TRS1's reported role in virion formation might be a secondary consequence of the effects of TRS1 on translation of factors needed for assembly (Adamo et al., 2004). We previously reported that TRS1 and IRS1 are able to prevent activation of the OAS/RNase L pathway in the setting of VVΔE3L infection (Child et al., 2004). However, infection with HCMV[ΔI/ΔT] did not activate this pathway (Marshall et al., 2009), possibly because PKR activation represses viral replication prior to accumulation of sufficiently high levels of dsRNA to activate OAS/RNase L. Regardless, the finding that HCMV[ΔI/ΔT] replicates in PKR-deficient cells demonstrates that antagonism of the RNase L pathway is not an essential function of TRS1 or IRS1.

While I studied the replication of recombinant viruses in both primary HF and HF Tert-based PKR knockdown and knockout cells, our knowledge about the roles of TRS1 functions in other cell types remains limited. We propose using CRISPR/Cas9 to knock out PKR in other HCMV-permissive cell types—such as endothelial cells, glial cells, and hepatocytes (Plachter et al., 1996)—to determine if replication of a viruses expressing TRS1-Mut 1 or lacking TRS1 and IRS1 remain equivalent as in HF. However, I would first need to repair the AD169 genome with genes necessary for replication in cell types other than HF (UL128-

131) (Sinzger et al., 2008). If we find that TRS1-Mut 1 can enhance replication in some cell types, we can test whether replication levels correlate to a specific function performed by TRS1-Mut 1, such as blocking autophagy. Finally, we plan to determine if whether this phenomenon is strain-specific by generating HCMV[TRS1-Mut 1] recombinant viruses within different clinical strains of HCMV. Although our model system has several limitations, strong evidence in another system supports out data. MCMV lacking PKR antagonists m142/m143 replicates as well as wild-type MCMV during infection of PKR knockout mice, suggesting that PKR antagonism by TRS1 is the only necessary function *in vivo* (Ostermann et al., 2015).

Other uses for PKR knockout cells

Because HCMV[ΔI/ΔT] cannot replicate in human fibroblasts, it could be an attractive candidate for a vaccine strain. In fact, in the guinea pig system, guinea pig CMV (GPCMV) lacking its PKR antagonist, gp145, replicates poorly in guinea pig cells. In a recent study of pregnant guinea pigs vaccinated with GPCMVΔgp145 and challenged with GPCMV thereafter, pup mortality was reduced by up to 77%, and congenital infections were reduced by 32% (Schleiss et al., 2015). This positive data suggests that further study of HCMV[ΔI/ΔT] infection of humans may be beneficial in determining whether it may stimulate a broad immune response worthy of a vaccine.

However, propagation of HCMV[ΔI/ΔT] to prepare stocks has historically been done in TRS1-expressing complementing cells. This presents a substantial risk for recombination of TRS1 back into the virus. Having found that HCMV[ΔI/ΔT] can replicate in PKR knockout cells, I can now prepare stocks without risk of recombination with the TRS1 transgene. These cells could be a useful tool in generating HCMV[ΔI/ΔT]; in fact, our lab has begun using these cells to prepare stocks of other viruses that can only replicate in complementing cells, such as HCMV[TRS1-Mut 1] and HCMV[Triple Mut].

Conclusion

My results reveal that inhibition of PKR is the single essential function of TRS1 or IRS1. Indeed, the observation that HCMV[ΔI/ΔT] replicates as well as wild-type virus in PKR-null cells suggests that TRS1's other activities contribute little to productive HCMV replication in cell culture. Although it remains possible that other functions of TRS1 and IRS1 might have an important or even essential role in natural HCMV infection, these studies highlight the importance of PKR as a strong viral restriction factor that has compelled many viruses to evolve effective evasion strategies. My speculation as to why HCMV encodes both TRS1 and IRS1 opens up more questions about gene duplication and the roles of seemingly "redundant" genes. These findings can be applied in a broader sense to other viruses encoding multiple PKR antagonists, especially those that also interact directly with both dsRNA and with PKR.

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CHAPTER 5

MATERIALS AND METHODS

Cells. All cells were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% NuSerum. Primary human foreskin fibroblasts (HF) and telomerase-immortalized HF (HF-Tert) were provided by Denise Galloway (Fred Hutchinson Cancer Center). HF transduced with a TRS1-expressing retrovirus (HF-TRS1) were reported previously (Marshall et al., 2009).

HF Tert cells expressing TRS1 constructs with N-terminal fusions were created by transduction of lentiviral vectors expressing these constructs (see Plasmids section). Transduction of cells was followed by selection in puromycin (1 μg/ml) and monitoring for GFP expression using microscopy.

PKR knockdown and control knockdown HF were created by transduction with lentiviral vectors expressing either an shRNA targeting PKR or a control shRNA (Open Biosystems, catalog numbers RHS4430-98844125 and RHS4346, respectively) followed by selection in puromycin (1 μg/ml).

Cells expressing transgenic forms of TRS1 with GFP fused to the N-terminus were generated by transduction of HF Tert cells with lentiviruses expressing TRS1, which were engineered using the vector pLVX-AcGFP1-N1 (a gift from the Lagunoff Lab). Cells underwent selection with puromycin (1 μg/ml).

PKR knockout A (PKR KO A) cells were constructed by transducing HF-Tert cells with a lentiviral vector that expresses Cas9 (Eckard et al., 2014) and a

guide RNA (gRNA) with the genomic target sequence 5'-TCTCTTCCATTGT**AG**GATA-3' (kindly provided by Elizabeth Gray and Dan Stetson, University of Washington). The predicted Cas9 cleavage site (between the two underlined nucleotides) is one bp downstream of the conserved AG (bold) of the exon 3 splice-acceptor site and 15 bps upstream of the PKR initiation codon. Following puromycin selection, single-cell clones were isolated and analyzed by PCR-amplifying this region of genomic DNA using primers #2001 and #2030 (Table 1), followed by TOPO-cloning and sequencing of multiple inserts from each of several cell clones. In every case, we detected mutations reflecting nonhomologous end joining resulting from cleavage at the expected site. In some cases, the resulting alleles retained the splice site and did not contain any large deletions. These clones expressed PKR as determined by immunoblot assays and were nonpermissive for VVΔE3L replication (data not shown). Other clones had alleles with mutations that removed the splice acceptor site and/or the PKR initiation codon. Analyses of PKR KO A revealed that this clone had one PKR allele with a 3 bp deletion that inactivated the splice acceptor site and a second allele with both a 66bp deletion and a 7bp duplication that deleted the PKR initiation codon. A control clone (Cas9 Ctrl A) was constructed by transducing HF-Tert with a similar vector that did not contain a gRNA. Sequence analyses of the PKR locus in this clone revealed the wild-type genomic sequence.

We constructed another PKR KO cell line (PKR KO B) by transducing HF with a construct containing doxycycline-inducible Cas9 and a gRNA targeting the

first dsRNA-binding domain of PKR (pEQ1510; target sequence 5'-TTCAGGACCTCCACATGAT-3'; Cas9 is predicted to cut between the two underlined nucleotides). Following puromycin selection (1 μg/ml), Cas9 expression was induced by addition of 1 μg/ml doxycycline for a minimum of two weeks prior to analysis. Screening of this cell line as described above (using primers #2049 and #2050 for PCR amplification) showed only a single bp insertion at the predicted cut site.

Plasmids. For initial experiments in the yeast two-hybrid assay, we mutated five TRS1 C-terminal residues to alanine by annealing oligos primers #950 and #951. This product was digested with BseRI and cloned into the pEQ1386 backbone. The resulting plasmid expressing TRS1[5-Ala] is pEQ1393. We made a series of C-terminal truncations in TRS1 for use in yeast two-hybrid assays. We first introduced a silent mutation to create a BstEII site within TRS1 by PCR-amplifying pEQ1284 (Child et al., 2012) with primers #783 and #841 (Table 1). The amplicon was digested with BglII and PstI and inserted into those same sites in pEQ1284, resulting in pEQ1304. We then annealed primers #876 and #877 and ligated the product into the BstEII and PstI sites of pEQ1304, generating pEQ1331, which added EcoRI and NotI sites and a 6XHis tag to the C-terminus of TRS1[1-667]. For each additional 3' truncation, we PCR-amplified pEQ1284 with forward primer #783 and the following reverse primers, digested the product with BglII and NotI, and inserted it into the BglII and NotI sites of pEQ1331. Reverse primer #917 was used for pEQ1360 (TRS1[1-679]); primer #918 for

pEQ1361 (TRS1[1-690]); primer #919 for pEQ1362 (TRS1[1-700]; Primer #878 for pEQ1340 (TRS1[1-710]); primer #879 for pEQ1341 (TRS1[1-720]); and primer #881 for pEQ1342 (full length TRS1[1-795]).

To make plasmids for use in transfection assays, individual alanine point mutant R696A (pEQ1298) was made using stitch PCR amplification of pEQ 1180 with primer pairs #808/#463 and #356/#809 and was TOPO-cloned into pcDNA3.1/v5 His. R697A (pEQ1432) was made using stitch PCR amplification of pEQ 1180 with primer pairs #622/#1015 and #463/#1014, then cutting with PpuMI and BsrGI to subclone into the pEQ1180 backbone. Primers #1052 and #1053, containing sequences encoding a TwinStrep tag, were annealed and ligated into pEQ1180 (Child et al., 2012) that had been digested with XhoI and XbaI to generate pEQ1435, a plasmid that expresses full length TRS1 with TwinStrep and 6XHis tags. The same primers inserted into an EGFP-6XHis expression plasmid derived from pEQ1100 (Child et al., 2006) to generate pEQ1436. TRS1 was excised from pEQ1427, which expresses a triple mutant of TRS1 that does not bind to dsRNA (Bierle et al., 2013) and from pEQ978 (Hakki and Geballe, 2005), which contains a C-terminal truncation (TRS1[1-648]) using HindIII and XhoI and the fragments were cloned into the same sites in pEQ1435 to generate pEQ1442 and pEQ1445, respectively. A plasmid expressing kinase-dead PKR with a biotinylation signal and 6XHis tag (pEQ1232) was constructed by transferring PKR from pEQ1198 (Child et al., 2012) into the pSV2 vector backbone and then cloning in the biotinylation signal from pEQ1068 (Child and Geballe, 2009). To design charged-cluster-to-alanine mutants of TRS1, we

aligned TRS1 and IRS1 sequences from HCMV Towne (Accession # FJ616285) and Chimpanzee CMV strain Heberling (Accession # NC_003521) using ClustalX 2.1. To introduce these mutations, TRS1 was amplified in two separate reactions, the first using a common upstream primer (#622) along with a mutant reverse primer (Table 1) and the second using a mutant forward primer and a common reverse primer (BGH). Subsequently, stitch PCR was used to join each set of products using the #622 and BGH reverse primers. The final PCR products were digested with PpuMI and XhoI and cloned into the same sites in pEQ1445. The specific mutant forward and reverse primer pairs were as follows: pEQ1468 (Mut 1) used #1096 and #1097, pEQ1469 (Mut 2) used #1098 and #1099, pEQ1470 (Mut 3) used #1100 and #1101, pEQ1471 (Mut 4) used #1102 and #1103, pEQ1472 (Mut 5) used #1104 and #1105, pEQ1473 (Mut 6) used #1106 and #1107, and pEQ1474 (Mut 7) used #1108 and #1109. The amino acids that were mutated to alanine in each of these constructs are indicated in Fig. 2a.

To construct plasmids for creating cell lines expressing each CCTA mutant, primer pair #1093 and #1094 were used to amplify TRS1 from pEQ1435 or other S2H-tagged mutants; these PCR products were digested with EcoRI and XmaI and were cloned into the similarly digested vector pLVX-AcGFP1-N1 (Clontech) to create expression vectors with GFP fused to the N-terminus of TRS1. This created pEQ1462 (wild-type TRS1), pEQ1463 (Triple Mut), pEQ1480 (Mut 1), pEQ1481 (Mut 3), and pEQ1492 (Mut 2).

To construct pEQ1510, the 2kb filler sequences in lentiCRISPR ((Shalem et al., 2014) a gift from Feng Zhang, Addgene plasmid # 49535) were removed

by BsmBI digestion, followed by the introduction of a pair of annealed primers that contain an AgeI restriction site (#1185 and #1186) using Gibson Assembly (New England Biolabs). A NotI to XhoI fragment was then excised and cloned into pCW-Cas9 ((Wang et al., 2014), a gift from Eric Lander and David Sabatini, Addgene plasmid # 50661) cut with the same enzymes. This yielded a lentiviral vector expressing tet-inducible Cas9 and containing the U6 promoter, an AgeI site into which different gRNAs can be introduced and the gRNA scaffold. The resulting vector, pEQ1508, was then digested with AgeI, and paired, annealed primers designed to target the first dsRBD of PKR (#1187 and #1188) were introduced by Gibson Assembly.

To construct plasmids for recombination into bacterial artificial chromosomes (BACs), a FRT-Kan-FRT cassette, PCR amplified from pSLFRTkn (Atalay et al., 2002) using primers #1170 and #1171, was cloned into the PmeI site of pcDNA3.1-V5-His-TOPO vectors containing TwinStrep-tagged wild-type TRS1 (pEQ1435) or TRS1-Mut 1 (pEQ1468) by Gibson assembly. The plasmids were digested with BamHI, then paired, annealed oligonucleotides containing the upstream 50 bp homology arm (#1197 and #1198) were introduced by Gibson Assembly. The resulting vectors were then digested with EcoR1 and BstEII, and paired, annealed primers #1199 and #2000 containing the downstream 50 bp homology arm were inserted by Gibson Assembly to yield pEQ1521 (HCMV[TRS1-S2H]) and pEQ1524 (HCMV[TRS1-Mut 1]).

Yeast two-hybrid assay. Yeast two-hybrid assays were conducted as previously described using a yeast codon-optimized variant of TRS1 fused to the GAL4 DNA binding domain and kinase-dead PKR fused to the GAL4 transcriptional activation domain in yeast expression vectors (Child et al., 2012).

Pulldown assays. For the PKR pulldown assay depicted in Chapter 2, Figure 2c, COS7 cells were transfected with 6XHis-tagged TRS1 constructs, a Biotin and 6XHis-tagged, kinase-dead form of human PKR (PKR-BH, pEQ1232), and biotinylase (BirA) using Lipofectamine 2000 (Invitrogen). At 24 hours post-transfection, biotin was added to the cells such that the final concentration was 25 μM. At 48 hours post-transfection, the cells were trypsinized and pelleted at 2000 x g for 5 minutes at 4°C and washed with cold PBS. The cell pellet was resuspended in RIPA low buffer (150mM NaCl, 0.1% Sodium Deoxycholate, 0.05% SDS, 50mM Tris pH 7.5, and 1% Triton X-100 with added NaVO4, Homoarginine, and Benzamidine, each at 1 μM final concentration, and added PMSF at 100 μM concentration) and incubated on ice for 20 minutes. Nuclei were removed by centrifugation at 10,000 x g for 20 minutes at 4°C. 11 μl per sample was saved on ice as the input and the remaining 99 μl was used for the pulldown. Avidin beads were washed twice in RIPA low buffer and resuspended in 130 μl RIPA low buffer. The lysates were added to the beads and incubated on a rotator at 4°C for 2 hours. The beads were pelleted and washed three times with RIPA low buffer. Proteins were eluted from the beads by heating to 95°C in loading buffer and separated by SDS-PAGE.

For the other PKR pulldown assay, COS7 cells were transfected with TwinStrep and 6XHis-tagged (-S2H) TRS1 constructs and a Biotin and 6XHis-tagged, kinase-dead form of human PKR (PKR-BH, pEQ1232), using Lipofectamine 2000 (Invitrogen). At 48 hours post-transfection, the cells were pelleted at 2000 x g for 5 minutes at 4°C and washed with cold PBS. The cell pellet was resuspended in 150 μl of lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, with freshly added 1 mM each of NaVO4, homoarginine, and benzamidine and 100uM PMSF) at 4°C and incubated on ice for 20 minutes, with periodic vortexing. Nuclei were removed by centrifugation at 10,000 x g for 10 minutes at 4°C. 5 μl per sample was saved on ice as the input and the remaining 145 μl was used for the pulldown. A 1:6.5 mixture of Streptactin beads (IBA Lifesciences; Schmidt 2013) and Sepharose CL-B6 beads (Sigma) was washed twice with lysis buffer and resuspended in 600ul lysis buffer. The lysates were added to the bead mixture and incubated on a rotator at 4°C for 2 hours. The beads were pelleted and washed three times with lysis buffer and twice with wash buffer (lysis buffer without the protease inhibitors and NP-40). Proteins were eluted from the beads by heating to 95°C in loading buffer and separated by SDS-PAGE.

For the dsRNA pulldowns, 293T cells were transfected with TRS1-expressing plasmids using Lipofectamine 2000 (Invitrogen) and lysates were harvested at 48 hours post-transfection. The dsRNA-binding assay was performed as previously described (Bierle et al., 2013).

VVΔE3L rescue assays. HeLa cells were transfected in triplicate with expression plasmids using Lipofectamine 2000 (Invitrogen). After 48 hours, the cells were infected (MOI = 0.1) with VVΔE3L, a vaccinia virus mutant in which E3L has been replaced with a LacZ cassette (provided by Bertram Jacobs). At 48 hours post-infection (hpi), VVΔE3L replication was monitored by quantification of β-galactosidase activity by a fluorometric substrate cleavage assay essentially as has been described (Hakki and Geballe, 2005). VVΔE3L replication in PKR knockdown and knockout cell lines was assessed at 48 hpi (MOI = 0.1).

BAC recombineering. After digestion of pEQ1521 (wild-type TRS1) and pEQ1524 (TRS1-Mut 1) with Asp718 and BstEII, the TRS1-FRT-kan-FRT fragments were transformed into *Escherichia coli* EL250 containing an HCMV BAC lacking both IRS1 and TRS1 (HCMV[ΔI/ΔT] (Marshall et al., 2009)). Following selection of kanamycin-resistant colonies, the kanR gene was removed by arabinose induction of FLP recombinase followed by selection for kan-sensitive colonies. BAC DNAs were analyzed by digestion with EcoRI, BamHI, or HindIII followed by electrophoresis on 0.7% agarose gels and ethidium bromide staining.

To reconstitute viruses, BAC DNA was purified from *E. coli* using the NucleoBond BAC 100 kit (Clontech) and transfected along with the cre-expression plasmid pPBRepcre into HF-TRS1 using Lipofectamine LTX reagent (Life Technologies). Mutant viruses were propagated and titered in HF-TRS1 or in PKR KO A cells. Viral DNA was harvested from infected PKR KO cells by lysis

with 2% SDS, proteinase K digestion, phenol:chloroform extraction and ethanol precipitation. The resulting DNAs were analyzed by PCR with primers located upstream (#2183) and downstream (#2180) of TRS1 and outside of the homologous recombination arms. The PCR products were sequenced with multiple forward and reverse primers, which yielded sequence covering the flanking regions and a minimum of 800 bp within both the 5' and 3' ends of TRS1. These results confirmed the identities of the wild-type and mutant TRS1 genes as well as their proper localization in the viral genome.

Immunoblot analyses. With the exception of pulldown assays (above), lysates for immunoblot assays were prepared by washing the monolayer with phosphate-buffered saline and lysing the cells with 2% SDS. After sonication to disrupt the cellular DNA, proteins were separated by 10% SDS-PAGE, transferred to a PVDF membrane (Millipore), and probed with the indicated antibodies using the Western Star chemiluminescent detection system (Applied Biosystems).

Antibodies used in these experiments include eIF2α L57A5 (#2103), phospho-eIF2α Ser51 (#3597), and PKR D7F7 (#12297), all from Cell Signaling Technology, PKR B-10 (sc6282, Santa Cruz Biotechnology), Penta-His (Qiagen), phospho-PKR T446 (ab32026, AbCam), and Actin (A2066, Sigma). Rabbit antiserum that recognizes the TRS1/IRS1 dsRNA binding domain (anti-p999) has been described previously (Marshall et al., 2009).

Metabolic radiolabeling. At 72hpi, infected cells were exposed to 50 uCi/ml of ^{35}S-Met for 1 hour at 37°C in methionine and cysteine-free media. Cells were then washed with PBS and lysed with 2% SDS. After sonication to disrupt the cellular DNA, proteins were separated by 10% SDS-PAGE. The gel was dried on a gel dryer and exposed to film for at least 24 hours, after which it was exposed to create an autoradiogram.

Primer Table:

Primer #	5' to 3' sequence
356	GCCTCGACGTCGGATCCGTCCGGCGGCCATGGCC
463	CACTACCATTACAATGCTCAA
622	CTCGAGACCATGGGCGTGGGCACCCCGCGC
783	GCCTCTAACATTGAGACAGCATA
808	CCGTAAAAACGACGTGTATCAAGCACGTTGGAAGAAAACCG
809	CGGTTTTCTTCCAACGTGCTTGATACACGTCGTTTTTACGG
841	GGTTCTGCAGCTTCACCTGGTAACCTTGGTGGTTTCCAATCTTC
876	GTTACCGAATTCGCGGCCGCCCATCATCACCATCACCATTAACTGCA
877	GTTAATGGTGATGGTGATGATGGGCGGCCGCGAATTCG
878	GCGCGGCCGCATCAATTTCCAAACCACATCTC
879	GCGCGGCCGCACAACCTTTTGGAACAGTTGG
881	GCGCGGCCGCTTGAGCGTTGTAATGGTAATG
917	GCGCGGCCGCATCCAAATCATACCAAGAATCTTCACC
918	GCGCGGCCGCCTTTCTATTAGAACCCAAAACCCAAAAAG
919	GCGCGGCCGCTTTCTTCCATCTTCTTTGATAAACATCG
950	AACGCTGTTTATGCTGCTGCTTGGAAGGCTAC
951	AGCCTTCCAAGCAGCAGCATAAACAGCGTTCT
1052	TCGAGCTGGAGCCACCCCCAGTTCGAGAAGGGCGGCGGCAGCGGCGGCGG CAGCGGCGGCGGCAGCTGGAGCCACCCCCAGTTCGAGAAGGGT
1053	CTAGACCCTTCTCGAACTGGGGGTGGCTCCAGCTGCCGCCGCCGCTGCCGC CGCCGCTGCCGCCGCCCTTCTCGAACTGGGGGTGGCTCCAGC
1093	TATAGAATTCGCCATGGCCCAGCGCAAC
1094	TACCCGGGCATGGTGATGGTGATGATG
1096	ACGTGCTTTGGCCCGGGCCGCCGCCGATTGGAAACCGCCACGTCT
1097	GTTTCCAATCGGCGGCGGCCCGGGCCAAAGCACGTCCCAAACTGG
1098	CGACGGGACGCCGCCGATTGGGCCCCGCCACGTCTCCCTGGGGA
1099	GACGTGGCGGGGCCCAATCGGCGGCGTCCCGTCGCAAAGCACGT
1100	CTCCCTGGGGCCGCCTCCTGGTACGACTTGGACG
1101	GTACCAGGAGGCGGCCCCAGGGAGACGTGGCGGT
1102	TCCTGGTACGCCTTGGCCGCCACTTTCTGGGTTCTGGGGAG
1103	CCCAGAAAGTGGCGGCCAAGGCGTACCAGGAGTCTTCCCCA
1104	GGGGAGTAACGCCAAAAACGCCGTGTATCAACGACGTTGGA
1105	TTGATACACGGCGTTTTTGGCGTTACTCCCCAGAACCCAGA
1106	AACCGTGTTAGCCTGTGGTTTGGCCATTGATCGTCCCATGCCAAC

1107	GACGATCAATGGCCAAACCACAGGCTAACACGGTTTTCTTCCAAC
1108	TGTGGTTTGGCCATTGCCGCCCCCATGCCAACGGTCCCCAA
1109	TTGGCATGGGGGCGGCAATGGCCAAACCACAGCGTAACACG
1014	GTATCAACGAGCCTGGAAGAAAACCGTGTTACG
1015	GGTTTTCTTCCAGGCTCGTTGATACACGTCGTTTT
1170	CGAGGCTGATCAGCGGGTTTTACGAGGACAGGCTGGAGC
1171	TCACCATCACCATTGAGTTTGACGACGACGACAAGTAAGAAG
1185	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGTTTTAGAGCTAG
1186	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCGGTGTTTCGTCCT
1187	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTTCAGGACCTCCACATGAT
1188	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACATCATGTGGAGGTCCTGAAC
1197	TAGTTAAGCTTGGTACCGAGCTCGTGACGCGGGTTTGCTTCCTATATAGTGGACGTCGGA
1198	CTGGGCCATGGCCGCCGGACGGATCCGGGCGCCGGACACCTCCGACGTCCACTATATAGG
1199	CTAGAAAGTATAGGAACTTCGAATTCACTGGTTTTCTTTTGCAGCTGTCGTTATGTTTCG
2000	GCTGGAGCCATGGCTGGTGACCTTGTAAAACAAGTTTTCGAAACATAACGACAGCTGCAA
2001	ATATGTTCTGTGAGCATCACTC
2030	GACCTCCACATGATAGGAG
2049	GCTACCACTCCACTTCACTTATT
2050	AGGCAATCACTCACCTTCTTT
2180	ATATGAAGCGTCGCGAGTATTA
2183	GCACGTCGCTGCCTATAAA
BGH rev.	TAGAAGGCACAGTCGAGG

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OBJECTIVE

Design, maintain, and manage high-quality materials and programs for the public to improve advocacy for scientific research.

RESEARCH EXPERIENCE

UW Department of Microbiology/Fred Hutchinson Cancer Research Center
Geballe Laboratory
May 2010-December 2015
Supervisor: Dr. Adam Geballe
Project: Essential Role of PKR Antagonism by TRS1 in HCMV Replication

UW Department of Microbiology
Mullins Laboratory
June 2009-April 2010
Supervisor: Dr. James Mullins
Project: Linking Genotype to Phenotype: Expansion of Position Specific Scoring Matrices to Score CCR5 vs. CXCR4 Co-Receptor Usage in HIV-1

UW Department of Microbiology
Mullins Laboratory
April 2009-June 2009 (Graduate Rotation III)
Supervisors: Dr. Morgane Rolland and Dr. James Mullins
Project: Co-Variation and Structural Analysis of Resistant HIV-1 Integrase

UW Department of Microbiology/Fred Hutchinson Cancer Research Center
Emerman Laboratory
January 2009-March 2009 (Graduate Rotation II)
Supervisor: Dr. Michael Emerman
Project: Differential Expression of Two African Green Monkey APOBEC3H Genes

UW Department of Microbiology/Washington National Primate Research Center
Hu Laboratory
September 2008-December 2008 (Graduate Rotation I)
Supervisors: Dr. Yun Li and Dr. Shiu-Lok Hu
Project: Antigenic Properties of a Single Glycan Modification on HIV-1 Envelope Proteins
California National Primate Research Center
Abel Lab
October 2007-June 2008
Supervisors: Dr. Koen van Rompay and Dr. Kristina Abel

Project: SIV vaccine study in the rhesus macaque model of infection

Clinical Virology Intern, Gilead Sciences, Inc.
Clinical Virology research group
June 2007-September 2007
Supervisor: Dr. Damian McColl
Project: Introduction of Integrase Enzymology in Clinical Virology

Hugh Edmondson Summer Pathology Research Fellow, UC Davis Medical Center
Afify Laboratory
June 2006-August 2006
Supervisor: Dr. Alaa Afify
Project: CD44v6 in Normal, Benign, and Malignant Lesions of the Breast

UC Davis Department of Medical Microbiology & Immunology
Asmuth Laboratory
October 2005-June 2006
Supervisors: SRA II Bridget McLaughlin, Dr. David M. Asmuth
Project: Flow cytometric analysis of HIV-infected and non-infected PBMCs

BIBLIOGRAPHY OF PEER-REVIEWED PUBLICATIONS

Braggin JE, Child S, Geballe A. Essential role of protein kinase R antagonism by TRS1 in human cytomegalovirus replication. *Virology*, in press (2015).

Child S, Brennan G, **Braggin JE**, Geballe A. Species specificity of protein kinase R antagonism by cytomegalovirus TRS1 genes. *J Virol* 86(7), 3880-9 (2012).

Afify A, McNiel MA, **Braggin J**, Bailey H, Paulino AF. Expression of CD44s, CD44v6, and hyaluronan across the spectrum of normal-hyperplasia-carcinoma in breast. *Appl Immunohistochem Mol Morphol.* 16(2), 121-7 (2008).

PUBLIC OUTREACH EXPERIENCE

Engage: The Science Speaker Series and Seminar (www.engage-science.com)
Mission statement of Engage: “Science outside the ivory tower: training today's graduate students in cutting-edge communication skills in order to re-connect the public with science and bring about a more informed tomorrow!”

- Completed *Engage* course, CENV 500 (Winter 2014), University of Washington
- Public lecture at Seattle’s Town Hall entitled “Viruses Sneak to Survive,” May 2014 (video link: http://www.engage-science.com/video-viruses/)
- Board member, Future/Vision Committee (2014-present); aiming to expand the organization and provide small workshops for graduate students
    - Successfully co-taught April 2015 science communication workshop for UW graduate students

Pacific Science Center

- Science Communication Fellow (June 2013-December 2014); created an interactive exhibit, "Sneaky Viruses," debuted several times each year on a volunteer basis during Scientist Spotlight events
- Volunteer for Paws on Science, UW's Microbiology Department table (April 2014)
- Volunteer for Science Expo, UW's Microbiology Department table (Summer 2013)

Speakers Bureau, Northwest Association for Biomedical Research

- Science café lecture for Science on Tap, "Drifting and Shifting: How the Influenza Virus Keeps Us Guessing," The Pub at Third Place Books in Seattle (January 2014)
- HIV Research lecture for Mercer Island High School Research and Ethics class (November 2012)

Science Night at Echo Lake Middle School

- Two-time volunteer for virology research table (February 2014, February 2013)

Girl Scouts Discover STEM

- Volunteer for virology research table (May 2013)

Congressman Inslee's Scoops on Cool Careers

- Volunteer at a virology research table representing Fred Hutchinson Cancer Research Center (Fall 2011)

Student chapter of the American Society for Microbiology, UC Davis

- Public outreach to the community of Davis and visitors for the annual Picnic Day award-winning Microbiology Exhibit (2004-2008)
- Public outreach to Cesar Chavez Elementary School through a microbiology display (2006)

TEACHING EXPERIENCE

Courses Taught, University of Washington

- MICROM 402, General Microbiology Lab for Microbiology Majors (Spring 2009 and Fall 2009)

Lectures, University of Washington

- Introduction to Virology/Bacteriophages, MICROM 301, General Microbiology (October 2011)
- Southern Blotting, MICROM 431, Recombinant DNA Technology (February 2013)

Microbiology Department Peer Advisor, UC Davis

- Guiding students in how to obtain laboratory internships in academia and the industry; assisting students in course scheduling to obtain the major (2007-08)

EDUCATION

University of Washington- Seattle, WA
Doctor of Philosophy, Microbiology (2015)
Attended September 2008-December 2015

- Graduate Research under the direction of Dr. Adam Geballe (May 2010-Dec 2015)
- Dissertation Defense held on December 7, 2015

University of California, Davis- Davis, CA
Bachelor of Science, Microbiology (2008)
Attended September 2004-June 2008

HONORS AND DISTINCTIONS

University of Washington

- Cell and Molecular Biology Training Grant awardee (July 2011-June 2012)

UC Davis

- Chancellor's Award of Merit (2008)
- Hugh Edmondson Summer Pathology Research Fellow (2006)
- Newport Corporation Future Stars Scholarship (2005, 2006)
- Dean's Honors List (2005)

EXTRACURRICULAR ORGANIZATIONS

Husky Masters Swimming (2008-present)

- Seattle-based adult competitive swim team
    - Competitor, board member and active co-manager
    - Certified Level I and II US Masters Swimming coach
    - Designed and taught three Introduction to Masters Swimming courses

Prytanean Women's Honor Society, UC Davis (2005-2008)

- Women's academic group focused on community service
    - Service Vice President (2006-2007)

American Society for Microbiology, UC Davis student chapter (2004-2008)

- Secretary (2005-2006)
- Publicity Chair (2006-2007)
- President (2007-2008)

Davis Aquatic Masters Swim Team

- Competitor (2005-2008)

UC Davis Division I Women's Swim Team

- ICA Student-Athlete (2004-2005)