Evolution of Protein Kinase R Antagonism in Primate Cytomegaloviruses

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Abstract

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During millions of years of coevolution with their hosts, cytomegaloviruses (CMVs) have succeeded in adapting to overcome host-specific immune defenses, including the protein kinase R (PKR) pathway. Consequently, these adaptations may also contribute to the inability of CMVs to cross species barriers. Here, we provide evidence that the evolutionary arms race between the antiviral factor PKR and its CMV antagonist TRS1 has led to extensive differences in the species-specificity of primate CMV TRS1 proteins. Moreover, we identify a single residue in human PKR that when mutated to the amino acid present in Agm PKR (F489S) is sufficient to confer resistance to $HCMV_{TRS1}$. Notably, this precise molecular determinant of PKR resistance has evolved under strong positive selection among primate PKR alleles and is positioned within the αG helix, which mediates the direct interaction of PKR with its substrate eIF2α. Remarkably, this same residue also impacts sensitivity to K3L, an eIF2α mimic encoded by poxviruses. Unlike K3L, TRS1 has no homology to eIF2α, suggesting that unrelated viral genes have convergently evolved to target this critical region of PKR. Despite its functional importance, the αG helix exhibits extraordinary plasticity, enabling adaptations that allow PKR

to evade diverse viral antagonists while still maintaining its critical interaction with eIF2α.

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DEDICATION

This dissertation is dedicated to my family and most of all to my husband Sam.

You bring so much joy to my life and I am eternally grateful for your love and encouragement.

Chapter 1. Introduction

Herpesviruses

The family *Herpesviridae* is composed of viruses that contain large and relatively complex dsDNA genomes. These genomes are encased within an icosahedral capsid, which is surrounded by an amorphous tegument and an envelope containing glycoprotein spikes. Herpesviruses are ubiquitous in nature and most animal species are naturally infected by at least one herpesvirus [1]. Upon entering cells, herpesviruses traffic to the nucleus where genome amplification and viral packaging occurs, followed by nuclear egress, envelopment, maturation and release. In addition to productive lytic infections that result in the destruction of the infected cell, herpesviruses are able to undergo a latency program, during which infectious progeny are not produced but the viral genome is maintained and retains the potential to initiate lytic replication.

Members of the herpesvirus family are further classified into three subfamilies—alpha, beta and gamma—based historically on biologically properties and reinforced more recently through genomic analyses [1,2]. Members of the alphaherpesvirinae, which include the important human pathogens Herpes simplex 1 and 2 (HSV-1 and HSV-2) and Varicella Zoster Virus (VZV), tend to be neurotropic, have a more rapid reproductive cycle, and infect a more variable host range. In contrast, beta-herpesviruses, including human herpresviruses 5, 6, and 7 (HHV-5, HHV-6, HHV-7), have a much longer reproductive cycle and are highly restricted in their host range. Finally, gamma herpesviruses such as Kaposi Sarcoma Associated Herpesvirus (KSHV) and Epstein Barr Virus (EBV), infect lymphoblastoid cells and are associated with malignancies.

Human Cytomegalovirus

HHV-5, more commonly known as human cytomegalovirus (HCMV), is the prototypic member of the beta-herpesvirus subfamily. HCMV has a genome of 235kb and encodes at least 150 open reading frames (ORFs), although recent ribosomal profiling analyses suggest HCMV may encode up to 750 ORFs [3]. As such, HCMV is the largest virus known to infect humans [4]. HCMV is readily spread through contact with infectious bodily fluids and is widely disseminated globally. In the United States, 50% of adults are seropositive, with seroprevalence reaching as high as 90% in certain subpopulations [5]. CMV seroprevalence rates are often higher in developing countries; for example, seroprevalence in southern Brazil was found to be 96.4% [6].

Infection with HCMV is typically asymptomatic but it remains an important cause of morbidity and mortality in immunocompromised individuals. Prior to the advent of highly active antiretroviral drugs, CMV was a frequent comorbidity of AIDS patients resulting in a number of end-organ disease manifestations, most commonly retinitis [4]. In hematopoietic stem cell transplant recipients, CMV pneumonia remains a serious complication that results in a mortality rate higher than 50%. Antiviral drugs have reduced the morbidity and mortality associated with HCMV infection, but the side-effects from the drugs and the costs associated with them are not trivial [7,8]. HCMV also has serious implications for neonates as it is the most prevalent congenital viral infection in the United States, resulting in as many as 40,000 cases annually. Of those infected, approximately 0.5% will succumb to the disease and an additional 20% will develop permanent neurological deficits including sensorineural hearing loss, mental retardation, and microcephaly [9]. In addition to these well-established roles, HCMV also has potential links to a number of additional diseases, including inflammatory bowel disease, glioblastoma, and

immunosenescence [10-12]. No vaccine for HCMV is currently available.

Species-specificity of Cytomegaloviruses

While HCMV is readily found throughout the human population, it is unable to cross species barriers. The species-specific nature of CMVs has been well established since early in their discovery. In the 1950s, Dr. Margret Smith isolated infectious agents from human and mouse salivary tissues, but found these agents only produced cytopathic effects in cells from their homologous host [13]. This high degree of specificity is corroborated by evolutionary analyses that demonstrate CMVs have co-speciated with their hosts throughout evolutionary history [14,15]. Through this process, each CMV has specifically adapted to the host that it naturally infects and in doing so diverged from closely related CMV species.

While the restricted host range of CMVs has been well established for decades, the host factors that contribute to cross-species barriers to CMV infection remain largely unknown. Several studies have demonstrated that CMVs can enter cells from more distantly related hosts, suggesting the block to replication occurs at a post-entry step [16-18]. Investigation of mouse CMV (MCMV) replication in human cells identified apoptosis as a contributing factor to cross-species barriers to CMV infection [19]. While MCMV fails to replicate to detectable titers in human fibroblasts, inhibition of apoptosis allowed for sustained viral replication. However, the level of MCMV replication observed in human cells in the absence of apoptosis was delayed and notably lower than that observed in murine cells. These findings suggest that apoptosis is one of several or possibly many barriers to MCMV replication in human cells. Thus, the cross-species barriers to CMV infection are highly complex and likely involve multiple divergent host factors

that the virus must either co-opt or overcome in order to productively replicate.

Host-virus Interactions

As obligate intracellular parasites, viruses are reliant on their hosts to productively replicate. Virus-host interactions dictate multiple steps of the CMV life cycle, from binding cellular receptors to mediate entry to usurping host kinases to mediate egress and release[20]. Moreover, viruses often co-opt host factors for their benefit. For example, components of the host DNA repair machinery are recruited to viral replication centers during HCMV infection and have been shown to facilitate preferential repair of damaged viral DNA relative to damaged cellular DNA [21]. Thus, divergence of host factors in different species may disrupt critical virus-host interactions and impede viral replication.

Beyond mediating critical steps of the virus life cycle, host-virus interactions are also essential to overcome host antiviral responses. In vertebrates, these immune responses can be divided into two main branches: innate and adaptive immunity. Also known as acquired immunity, the adaptive immune response can accurately target the pathogen by recognizing antigens in a highly specific manner and can generate immunological memory to protect from future exposures. However, achieving this specificity takes time and because of this, the adaptive response is slow to develop after a primary exposure. Therefore the innate and intrinsic immune responses provide an essential first line of defense to invading pathogens.

Distinguishing self from non-self: the innate immune response to dsRNA

The ability to distinguish self from non-self is a highly conserved antiviral defense and allows for a broad and rapid response to invading pathogens. In vertebrates, multiple pathogen recognition receptors (PRRs) are expressed throughout the cell and serve to detect invading pathogens by recognizing pathogen-associated molecular patterns (PAMPs). Upon stimulation by a PAMP, PRRs initiate signaling cascades that ultimately drive the production of interferon and proinflamatory cytokines. Interferon then acts in an autocrine and paracrine fashion to signal through the Jak-STAT signaling pathway and induce transcription of hundreds on interferon-stimulated genes (ISGs), thus establishing an antiviral state.

Produced as a byproduct of almost all viral lifecycles, dsRNA is arguably one of the most important viral PAMPs. For RNA viruses, dsRNA is a necessary intermediate in the replicative life cycle or in the case of dsRNA viruses, the genome itself. For DNA viruses, the source of the dsRNA is not as clear. The compact nature of the genomes of DNA viruses often results in genes being encoded on both strands of the genome and bidirectional convergent transcription is hypothesized as a potential source of dsRNA during DNA virus infections [22]. In support of this, ribosomal profiling data has shown that convergent transcripts with the potential to anneal are formed during HCMV infection [3]. However, it is also possible that viral infection stimulates production of structured host RNAs that activate antiviral pathways. Regardless of the source, dsRNA has been detected during infection with a number of viruses, including vesicular stomatitis virus, measles virus, influenza A virus, and HCMV [23,24]. Given its ubiquity during viral infections, dsRNA is sensed by multiple PRRs in different cellular compartments, including Toll-like receptors (TLRs), and RIG-I like receptors (RLRs) [22].

Toll-like receptor 3 (TLR3) is a member of the TLR family, which is composed of a number of membrane bound receptors that recognize and respond to different PAMPs. In the absence of infection, TLR3 localizes to the endoplasmic reticulum, and in some cells types resides at the cell surface [25,26]. Upon viral infection, TLR3 is recruited to the endosome where it is poised to detect invading viruses [27,28]. Upon recognition of dsRNA, TLR3 recruits the adaptor protein TRIF to initiate its signaling cascade, driving transcription of type I interferon and proinflammatory molecules.

Critical for detecting dsRNA in the cytosol is the RLR family, which contains RIG-I, and MDA5. As many RNA viruses replicate in the cytoplasm, detection of RNA by RIG-I and MDA5 is an important component of the innate immune response to viral infection. Complimentary in function, RIG-I and MDA5 recognize slightly different forms of viral RNA ligands. RIG-I recognizes short dsRNA and ssRNA molecules (<300bp) that contain a 5'-triphosphate end [29-31]. In contrast, MDA5 preferentially recognizes long (<1000bp) dsRNA with no sequence or end specificities [31]. Recognition of their respective RNA molecules allow RIG-I and MDA5 to associate with and activate the adaptor protein MAVS. Activation of MAVS results in a signaling cascade that ultimately leads to the activation of NF-κB and IRF3, which translocate to the nucleus to induce expression of type I interferon and pro-inflammatory molecules [32,33].

Collectively, the detection of PAMPs by these and other PRRs leads to the establishment of an antiviral state. Contributing to this antiviral state is the expression of hundreds of interferon-stimulated genes (ISGs). These ISGs serve as the effectors of the antiviral response and act directly to inhibit viral replication. Activation of these effector proteins often depends on

detection of viral PAMPs, including dsRNA. While there are multiple ISGs that respond to dsRNA, this thesis will focus on the antiviral factor Protein Kinase R (PKR).

Protein Kinase R

PKR is one of four cellular kinases that phosphorylate the alpha subunit of the eukaryotic initiation factor 2 (eIF2α), ultimately leading to a global shutoff in translation initiation. eIF2α is an essential component of the translation initiation machinery as it shuttles the initiating methionine tRNA (Met-tRNAi) to the 40S ribosomal subunit [34]. eIF2α binds the Met-tRNAi in a GTP dependent manner, and after delivery of the Met-tRNAi to the 40S ribosomal subunit, pairing between Met-tRNAi anticodon and the AUG initiation codon triggers release of eIF2α through GTP hydrolysis. Inactive eIF2α-GDP is recycled to eIF2α-GTP through the GTP exchange factor eIF2B. Upon phosphorylation of eIF2α, eIF2B binds P-eIF2α with a 100 fold higher affinity, thus competitively inhibiting eIF2B interactions with non-phosphorylated eIF2α [35]. As eIF2B is limiting in the cell, sequestration of eIF2B by P-eIF2α prevents recharging of eIF2α and thus delivery of Met-tRNA to the 40S ribosomal subunit [36].

Unlike the other eIF2α kinases, PKR is IFN induced, highlighting its role in the antiviral response. PKR is composed of two canonical N-terminal dsRNA binding domains (dsRBDs) and a C-terminal kinase domain. In the absence of viral infection, PKR exists in an inactive, monomeric state as the dsRNA binding domains are thought to occlude the kinase domain and inhibit its activity [36]. The dsRBDs can recognize and bind to dsRNAs greater than 30bp in length in a sequence independent manner [37]. Upon dsRNA binding, a conformation change in PKR is induced that results in dimerization, auto-phosphorylation and activation of PKR. In this active state, PKR can bind its downstream substrate, eIF2α. Structural data has revealed that the

β-barrel of eIF2a engages with the C lobe of the kinase domain of PKR, centered on the N-terminal end of the αG helix of PKR [38]. This interaction allows for phosphorylation of S51 within eIF2α, ultimately resulting in a block to translation initiation. As viruses are reliant on host translation machinery, this global inhibition of translation effectively blocks viral replication.

Viral PKR Antagonists

To overcome the antiviral effects imposed by PKR, most viruses have evolved strategies to prevent PKR activation. The strategies employed by viruses are incredibly diverse and examples have been found of inhibitors acting at virtually every step of the pathway [39].

RNA Inhibitors

Some viruses have evolved mechanisms to generate inhibitory RNA structures that directly bind to PKR in a manner that does not stimulate PKR activation. For example, adenovirus encodes two small RNAs called virus-associated RNAs I and II (VAI and VAII) that interact with the dsRBDs of PKR and competitively inhibit dsRNA binding to prevent PKR activation [40,41]. Likewise, Epstein-Barr virus encodes EBER-1 and EBER-2 which contain significant secondary structure, allowing EBER-1 and EBER2 to bind PKR in a manner that fails to activate PKR [42,43].

Protein Antagonists

Given the importance of dsRNA as a PAMP, it is not surprising that many viruses encode dsRNA-binding proteins to sequester dsRNA. One prime example is the Vaccinia Virus (VacV)

antagonist E3L. E3L contains one canonical dsRBD in its C-terminus, and this domain has been shown to be essential for E3L activity and sufficient for sustained replication of the virus in a wide range of host cells[44-46]. Similarly, the NSP3 protein of rotaviruses contains a conserved dsRBD to sequester dsRNA and prevent PKR pathway activation [47]. In addition to viruses expressing proteins with canonical dsRBDs, there are also examples of viral antagonists that can bind dsRNA in an atypical manner, including the reovirus antagonist σ3 and the influenza antagonist NS1 [48,49].

As an alternative or complementary strategy, viruses also encode proteins that directly interact with PKR to inhibit its activity. NS5A from hepatitis C has been shown to directly bind to PKR, and this interaction is mediated by amino acids 244-296 of PKR [50,51]. As this region encompasses the dimerization domain of PKR [52], NS5A binding inhibits PKR dimerization and thus activation. The VacV encoded antagonist K3L shares significant structural homology to eIF2α [53,54]. In fact, it is thought that VacV acquired eIF2α from the host and modified it to function as a competitive inhibitor of eIF2α, resulting in the antagonist K3L. Although less well characterized, the Kaposi Sarcoma Associated Herpesvirus (KSHV) protein vIRF2 was shown to bind PKR in vitro and this interaction was sufficient to inhibit PKR autophosphorylation upon stimulation with dsRNA, suggesting it may function as a PKR antagonist [55]. Moreover, poliovirus and Rift Valley fever virus encode proteins that induce PKR degradation, representing yet another viral solution to PKR antiviral activity [56,57].

There are also examples of viral antagonists that act downstream of PKR activation. HSV-1 encodes γ34.5, which is essential for inhibition of the PKR pathway during early infection. γ34.5 shares homology with the host factor GADD34, which regulates the host protein phosphatase 1α (PP1α) by targeting it to eIF2α when necessary to replenish pools of non-

phosphorylated eIF2α [58]. Analogously, γ34.5 recruits PP1α to eIF2α during infection to dephosphorylate eIF2α and maintain translation of viral proteins [59]. Similarly, the E6 protein of Human papillomavirus (HPV) binds to both GADD34 and PP1α to promote dephosphorylation of eIF2α during infection [60]. Thus, throughout the course of evolution, viruses have found diverse mechanisms to overcome this critical antiviral response.

PKR Antagonism by HCMV

HCMV was first shown to inhibit the PKR pathway through its ability to complement a VacV deleted of its PKR antagonist, E3L (VacVΔE3L) [61]. In contrast, UV irradiated CMV failed to complement VacVΔE3L, suggesting that *de-novo* CMV gene synthesis was necessary to rescue VacVΔE3L. To identify the CMV gene(s) responsible, a library of recombinant VacVΔE3L viruses expressing fragments from the CMV genome were generated and evaluated for their ability to replicate in human fibroblasts (HFs) [62]. A single recombinant virus was able to replicate in the non-permissive HFs, and contained a fragment of the HCMV genome encoding TRS1 and part of the adjacent J1S gene. To determine which gene was responsible for overcoming PKR, additional recombinant viruses expressing TRS1, the fragment of J1S, or a frameshifted mutant of TRS1 were generated and only the virus expressing TRS1 in frame was capable of replicating. Moreover, VacVΔE3L+TRS1 maintained viral protein synthesis and inhibited PKR autophosphorylation, demonstrating that TRS1 is a PKR antagonist.

Because TRS1 is encoded partially within a repeat region of the CMV genome, it is highly similar to IRS1. The N-terminal two-thirds of each protein are 100% identical, while the remaining third is 50% identical. Therefore, a recombinant VacVΔE3L expressing IRS1 was

generated and found to replicate in HFs, demonstrating that IRS1 is also capable of inhibiting PKR pathway activation [62].

To evaluate the role of these genes in the context of the HCMV lifecycle, a series of TRS1 and IRS1 mutant viruses were evaluated. While deletion of TRS1 resulted in a modest replication defect, deletion of IRS1 had no impact on viral replication [63]. In contrast, a virus deleted of both IRS1 and TRS1 (HCMVΔI/ΔT) was unable to replicate to detectable levels [24]. Moreover, protein synthesis was inhibited following HCMVΔI/ΔT infection and levels of phosphorylated eIF2α were markedly increased, demonstrating that the PKR pathway is efficiently activated by HCMV in the absence of TRS1 or IRS1. HCMVΔI/ΔT could be complemented by the VacV PKR antagonist E3L, suggesting PKR activation was the primary block to viral replication [24]. While other activities have been attributed to TRS1, HCMVΔI/ΔT is able to replicate as well as WT HCMV in HFs deficient in PKR, suggesting that PKR antagonism is the only essential function of TRS1 or IRS1 during HCMV replication in vitro [64]. Collectively, these finding demonstrate that TRS1 and IRS1 are functionally redundant in their abilities to antagonize PKR and that either TRS1 or IRS1 is essential for HCMV replication.

Mapping the Functional Domains of TRS1

Given the number of viral antagonists that bind dsRNA, TRS1 was initially evaluated for its ability to bind dsRNA. TRS1 generated both in vitro and during infection in mammalian cells was found to bind to poly(I:C) agarose beads [65]. This interaction could be competitively inhibited by pre-incubating TRS1 with dsRNA but not dsDNA, demonstrating TRS1 specifically binds dsRNA. Analysis of the amino acid sequence of TRS1 did not yield any homology to

know dsRBDs. Therefore, serial truncations of TRS1 were evaluated for their ability to bind dsRNA in order to more precisely map the dsRBD, and residues 74-248 were found to be sufficient for dsRNA binding [65]. However, recombinant VacVΔE3L viruses expressing either a mutant of TRS1 lacking the dsRBD or the dsRBD of TRS1 alone were unable to replicate in HFs, suggesting the dsRBD of TRS1 is necessary but not sufficient for PKR antagonism [65].

In addition to dsRNA binding, PKR was also found to directly interact with PKR and cause relocalization to the nucleus [66]. However, whether or not the altered localization of PKR during infection is important for HCMV PKR antagonism or replication remains unknown. Truncational analysis of TRS1 and IRS1 demonstrated that the C-terminal region of each protein was necessary for PKR binding, as deletion of amino acids 679-795 of TRS1 or amino acids 668-847 of IRS1 disrupted PKR binding [66]. Moreover, PKR binding by TRS1 mutants directly correlated with the level of rescue of VacVΔE3L, suggesting that PKR binding is an important component of the mechanism of antagonism used by TRS1 [64].

TRS1: Mechanism of PKR Antagonism

While these truncational analyses suggest that both dsRNA binding and PKR binding by TRS1 are necessary for PKR antagonism, our understanding of the mechanism by which TRS1 inhibits PKR remains incomplete. The ability of TRS1 to bind dsRNA suggests it could sequester dsRNA away from PKR, similar to VacVΔE3L. However, TRS1 binds dsRNA with a weaker affinity than does PKR, but is expressed to a higher level during infection [67]. Therefore, it is difficult to discern whether the relatively weak dsRNA binding activity but increased expression of TRS1 would be sufficient to sequester dsRNA away from PKR [67]. Interestingly, TRS1 mutants that bind dsRNA with a weaker affinity than WT TRS1 retained the

ability to rescue VacVΔE3L, suggesting that efficient dsRNA binding may not be necessary for PKR antagonism [67]. Therefore, it is unlikely that TRS1 antagonizes PKR by sequestering dsRNA.

Another possibility is that dsRNA binding contributes to PKR binding, and disrupting dsRNA binding inhibits the ability of TRS1 to bind to and antagonize PKR. Introduction of three alanine mutations into the dsRNA binding domain of TRS1 was sufficient to disrupt any detectable dsRNA binding by TRS1 and this mutant was unable to rescue VacVΔE3L replication [67]. However, this mutant was still able to efficiently bind PKR, demonstrating that dRNA binding and PKR binding are genetically separable. Introduction of the triple mutant of TRS1 into HCMVΔI/ΔT was not sufficient to rescue replication in HFs (unpublished data), again validating that dsRNA binding by TRS1 is necessary for PKR antagonism.

In addition to dsRNA binding, PKR binding is also essential for TRS1 to overcome the PKR pathway. To rule out the possibility that truncation of the C-terminus of TRS1 altered some other function of TRS1, point mutations were introduced into the C-terminal region of TRS1 to generate TRS1-Mut-1, which is deficient in PKR binding [64]. TRS1-Mut 1 was unable to rescue either VacVΔE3L or HCMVΔI/ΔT, and robust phosphorylation of PKR and eIF2a was observed following infection of HFs with HCMVΔI/ΔT+TRS1-Mut 1 [64]. Thus, even in the context of HCMV replication, the ability of TRS1 to bind to PKR is essential for its activity.

While we know that TRS1 must directly bind to PKR to inhibit its activity, we do not know which domain of PKR is targeted by TRS1, and identifying the TRS1:PKR interface may further elucidate the mechanism of action used by TRS1. For example, NS5A was shown to bind to the dimerization domain of PKR and disrupt this critical step in the PKR pathway [50]. In

contrast, VacV K3L is known to bind at the aG helix of PKR, and thus disrupt interactions between PKR and its downstream substrate.

The selective pressure imposed by multiple viral antagonists has driven rapid evolution of PKR. This divergence of PKR can be leveraged to dissect host-virus interactions, as detailed in Chapter 2, and will be used in this thesis to improve our understanding of how the rapid evolution of PKR has impacted the evolution of the primate CMV TRS1 proteins.

Chapter 2. An evolutionary view of the arms race between protein kinase R and large DNA viruses.

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Summary

To establish productive infections, viruses must counteract numerous cellular defenses that are poised to recognize viruses as non-self and to activate anti-viral pathways. The opposing goals of host and viral factors lead to evolutionary arms races that can be illuminated by evolutionary and computational methods and tested in experimental models. Here we illustrate how this perspective has been contributing to our understanding of the interactions of the protein kinase R pathway with large DNA viruses.

Introduction

Recent studies have identified numerous host cell restriction factors that constitute the first line of defense against viruses. These factors, which include many IFN-induced proteins, can act in a generalized manner by recognizing common molecular byproducts of viral replication, or more exclusively by targeting specific viral proteins. In the former case, viral antagonists that counteract the restriction factor put the host gene "on defense" as it must evolve to evade viral antagonists [68]. In contrast, restriction factors with more specificity evolve "on offense" to maintain their ability to recognize viral targets. In both cases, the resulting arms race

drives rapid evolution of the host and viral genes as they continually adapt to evade or restore recognition (Fig. 2.1A). These evolutionary signatures of conflict can be detected and quantified by analyses of the rates of nonsynonymous (dN) and synonymous (dS) substitutions among orthologous genes, with a dN/dS ratio greater than one indicating positive selection. Various algorithms enable estimation of the extent to which a gene has been evolving under positive selection, which lineages within a phylogenetic tree have experienced the strongest selection, and even which codons have been under recurrent pressure to evolve. These "hot spots" of evolution often pinpoint critical interacting surfaces between host and viral proteins [68]. While this evolutionary framework has been applied to assess a variety of host restriction factors (reviewed in [68] and [69]), here we focus on the evolutionary approaches that are uncovering insights into the molecular conflicts between protein kinase R (PKR) and its viral antagonists in large DNA viruses.

Protein Kinase R: A dsRNA Sensor with Broad Antiviral Activity

PKR is a member of a family of kinases that respond to various cellular stresses by phosphorylating the eukaryotic initiation factor 2α (eIF2α), resulting in arrest of translation initiation. Unlike the other eIF2α kinases, PKR is interferon inducible, highlighting its role in the innate immune response. PKR has broad antiviral activity as a result of its activation by double-stranded RNA (dsRNA), a nearly ubiquitous byproduct of viral infections. While dsRNA is known to be a necessary intermediate in the lifecycle of most RNA viruses, immunological methods have also identified dsRNA in cells infected with DNA viruses [70,71]. Whether this dsRNA arises from viral or cellular transcripts is unclear. RNAseq data in several systems have identified overlapping complementary viral transcripts that have the potential to form dsRNA

[3,72-74], suggesting that the pressure to maintain compact genomes may be an Achilles heel for these DNA viruses. However, there are also data suggesting that DNA viruses induce structured host transcripts that can regulate PKR activity [75-77]. Regardless, dsRNA is an important pathogen associated molecular pattern (PAMP) that induces dimerization, autophosphorylation and activation of PKR.

Role of PKR Antagonists in Large DNA Viruses

The importance of the PKR antiviral response is substantiated by the presence of antagonists in many virus families. Furthermore, these antagonists are often essential for viral replication. For example, deletion of the human CMV (HCMV) PKR antagonists IRS1 and TRS1 results in a virus (HCMV[ΔI/ΔT]) that fails to replicate in cell culture, but can be rescued by introduction of the vaccinia virus (VACV) PKR antagonist E3L [24]. Moreover, HCMV[ΔI/ΔT] replicates efficiently in PKR-deficient cells, supporting the conclusion that the replication defect in wild type cells is due to PKR activity [64,78]. Similarly, deletion of the PKR antagonists from mouse CMV eliminates replication in wild-type cells and mice, but has no effect in PKR-null cells and mice [79].

Even in systems where PKR antagonists appear not to be absolutely essential, they still often contribute to replication and pathogenicity. Guinea pig CMV depleted of its PKR antagonist, gp145, replicates poorly in cell culture, has reduced virulence in animals, and appears to be a safe and potentially effective vaccine for preventing congenital transmission in guinea pigs [80]. The fact that it replicates at all suggests that the guinea pig PKR defense system may be less effective than that in humans or mice, or that guinea pig CMV has a second, as yet unidentified PKR antagonist. Herpes simplex-1 (HSV-1) with a deletion of γ34.5, one of its two

known PKR antagonists, is avirulent in wild type mice, even when directly inoculated into the CNS [81]; however, it does replicate in some cell types and, notably, is fully virulent in PKR-null mice [82]. VACV lacking its PKR antagonist, E3L (VACVΔE3L), replicates poorly in many cell types and in mice, but this defect is ameliorated at least partially in PKR deficient cells and animals [83-85]. Like HSV-1, VACV encodes at least one other PKR antagonist (K3L), which may explain why VACVΔE3L is able to replicate at all. Although some PKR antagonists have additional activities that might account for the presence of more than one in a single virus, it is also possible that the combination increases total potency of the PKR antagonism or that each one is effective in alternative settings, such as different cell types. The fact that several large DNA viruses encode more than one antagonist underscores how critical overcoming the PKR pathway has been during viral evolution.

PKR vs Viral Antagonists: Evolutionary Signatures of Conflict

The broad antiviral activity of PKR and the diversity of its viral antagonists predict that PKR has engaged in multiple evolutionary arms races. Consistent with this hypothesis, two groups reported that PKR has a strong signature of positive selection [86,87]. Similar findings of positive selection have been found for retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) [88], revealing that multiple dsRNA-sensors have been engaged in arms races with viruses. In the case of PKR, rapidly evolving codons were identified in both the N-terminal half of PKR (though not in the dsRNA-binding residues), and throughout the C-terminal kinase domain, consistent with multiple regions of PKR being targets of viral antagonists [86,87]. These papers identified surprisingly distinct sets of rapidly evolving codons, likely due to differences in the number and evolutionary breadth of the sequences used

for the analyses and in the algorithms employed [89]. Nevertheless, both papers conclude that the broad antiviral activity of PKR has made it a target of diverse viral antagonists, resulting in rapid evolution of PKR.

Given the broad diversity of PKR across species, it is somewhat surprising that human PKR has so few polymorphisms. In fact, the frequency of missense mutations at any codon in over 100,000 human PKR alleles is less than 0.02%; this is in contrast to missense mutations existing at frequencies from 1 to 15% in PKR for other great ape species and frequencies of up to 22% and 50% in human RIG-I and MDA5, respectively (unpublished observation, data from [90] and the Exome Aggregation Consortium [ExAC], Cambridge, MA, November 2015 [http://exac.broadinstitute.org]). The low variation in human PKR may be a consequence of rapid population expansion following a bottleneck event or of a selective sweep occurring sometime after humans diverged from other hominoids.

Consistent with the arms race paradigm, rapid evolution of PKR appears to have driven the evolution of its viral antagonists. For instance, analysis of the PKR antagonist K3L from variola major and VACV revealed a dN/dS ratio of 2.8, while fewer than 10% of other orthologs in these viruses show signatures of positive selection [86]. Similarly, recent comparisons of more than 100 isolates of HCMV revealed that TRS1 is evolving under positive selection [91], which is surprising considering that PKR is so invariant among humans. However, TRS1 has been reported to have multiple other functions in addition to PKR antagonism, so it is possible that one or more of these activities or the host adaptive immune response is driving TRS1 evolution 4. Regardless, these evolutionary analyses indicate that the host viral arms race has significantly impacted the evolution of PKR and its viral antagonists.

Consequences of the PKR-Antagonist Arms Races.

The rapid evolution and divergence of PKR and its antagonists has consequences for cross species viral transmission events (Fig. 2.1B). For instance, HCMV TRS1 has coevolved with human PKR and can readily antagonize it, but fails to antagonize Old World monkey PKRs, likely contributing to the inability of HCMV to replicate in monkey cells [83]. In contrast, Rhesus CMV (RhCMV) TRS1 antagonizes some Old World monkey PKR alleles, but has limited activity against human PKR. In addition, RhCMV TRS1 and HCMV TRS1 block PKR pathway activation at different steps. While HCMV TRS1 blocks prior to PKR phosphorylation, RhCMV TRS1 allows PKR phosphorylation but prevents phosphorylation of eIF2α, the downstream substrate of PKR [83]. Thus, the evolutionary adaptations in both PKR and TRS1 have led to qualitatively different mechanisms of antagonism, likely reflecting alterations in the protein-protein interfaces.

Despite these clear differences between HCMV and RhCMV TRS1, the species-specificity of PKR antagonism is surprisingly complex. For example, even though humans and rodents are much more distantly related than humans and Old World monkeys, HCMV TRS1 functions in mouse cells and the PKR antagonists from mouse and guinea pig CMV function in human cells [92-94]. In addition, although RhCMV TRS1 does not inhibit human PKR in VACVΔE3L rescue and reporter gene expression assays ([83] and unpublished data), RhCMV replicates in human fibroblasts (HFs), suggesting that RhTRS1's weak activity against human PKR may be sufficient for RhCMV replication in HF. The explanations for these puzzling observations will require further investigation.

The species-specific differences driven by the host-virus arms race can guide experimental dissection of these interactions and mechanisms. For example, primate PKR alleles

vary in their resistance to K3L [86], the VACV eIF2α mimic that competitively inhibits PKR activity [95]. Analyses of chimeric PKR alleles revealed that critical differences in K3L resistance mapped to residues under positive selection within the αG helix [86]. In fact, a single amino acid present in human PKR was sufficient to confer resistance to the otherwise susceptible gibbon PKR allele. However, other residues, including one not identified as under positive selection, also contribute to K3L resistance, reflecting the intricacies arising from PKR having adapted to numerous extant and extinct viruses. Nonetheless, the utility of an evolutionary-guided approach for identifying critical interacting surfaces of restriction factors and their viral antagonists in other systems suggests that this approach will be useful for elucidating the mechanisms of antagonism used by other PKR antagonists, including TRS1.

Conclusions

Evolutionary-guided analyses coupled to functional studies have led to many significant insights into the conflicts between host restriction factors and their viral antagonists. This approach has applications in many viral systems to identify participating factors and mechanisms underlying host-virus interactions. Moreover, these types of analysis may be useful for evaluating the strength and limitations of animal models. Given the power of this approach, this thesis aims to improve our understanding of the function of CMV TRS1 proteins by applying an evolutionary perspective.

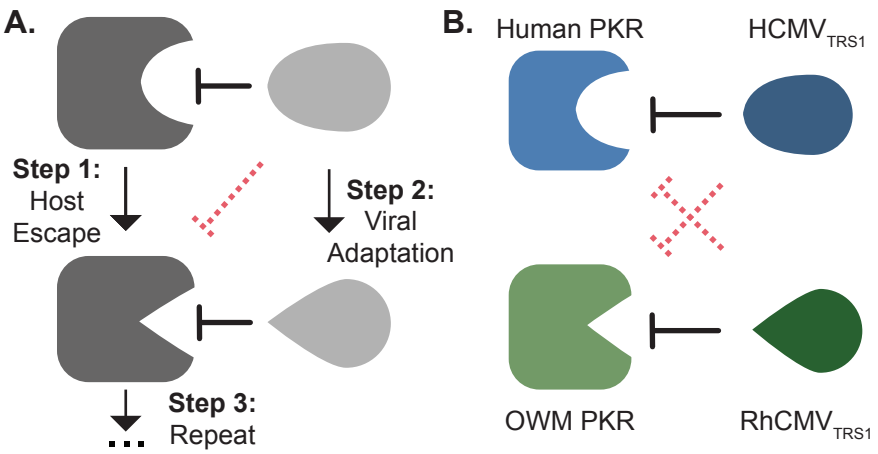

Figure 2.1 Host-virus arms race and functional consequences.
(A) Schematic representation of the evolutionary steps taken as a host gene evolves "on defense" to evade a viral antagonist. (B) Rapid evolution has led to the divergence of PKR in primates and contributes to cross-species barriers to infection. While HCMV$_{TRS1}$ can readily antagonize human PKR, it has limited activity against Old World monkey (OWM) PKRs. In contrast, RhCMV$_{TRS1}$ can antagonize some Old World monkey PKRs but not human PKR.

Chapter 3. Species-specific differences in Protein Kinase R Antagonism by Primate Cytomegaloviruses

Portions of this chapter are adapted from an article originally published in PLoS Pathogens:

Carpentier KS, Esparo NM, Child SJ, Geballe AP. 2016
A Single Amino Acid Dictates Protein Kinase R Susceptibility to Unrelated Viral Antagonists.
PLoS Pathogens. 12(10): e1005966. doi:10.1371/journal.ppat.1005966

Summary

Cytomegaloviruses are highly specie-specific. However, the host factors that contribute to cross-species barriers to CMV infection are not well characterized. The rapid evolution of cell intrinsic antiviral factors has led to their divergence in different primate species, making them attractive candidates to be factors that contribute to barriers to cross species-transmission events. Here, we show that the rapid evolution of the antiviral factor Protein Kinase R has significantly impacted the evolution of the primate CMV antagonist TRS1. While Human CMV TRS1 ($HCMV_{TRS1}$) is able to readily antagonize human PKR, it has limited activity against the distantly related African green monkey PKR (AgmPKR). Conversely, TRS1 proteins from two old world monkey viruses, African green monkey CMV (AgmCMV) and Rhesus CMV (RhCMV), exhibit strong activity against AgmPKR but are unable to overcome human PKR. Surprisingly, TRS1 encoded by the new world monkey virus Squirrel monkey CMV (SmCMV) is able to antagonize both human PKR and AgmPKR, suggesting it is more broadly acting. In addition to the species-specific differences in activity, there are also differences in the mechanism by which different TRS1 proteins antagonize PKR. While $HCMV_{TRS1}$ blocks PKR autophosphorylation, $RhCMV_{TRS1}$ and $SmCMV_{TRS1}$ allow this step to proceed but inhibit phosphorylation of the downstream substrate

eIf2α. These findings suggest that the rapid evolution of PKR and the co-adaptation of primate CMV TRS1 proteins has led to significant divergence of the PKR:TRS1 interface, leading to qualitatively different mechanisms of antagonism. Moreover, these species-specific differences in TRS1 activities suggest that PKR is likely a contributing factor in cross-species barriers to CMV replication.

Introduction

HCMV is a ubiquitous virus that persists for the lifespan of the infected host, highlighting its ability to evade host defenses [96]. While most infections are asymptomatic, HCMV causes life-threatening diseases in immunocompromised patients and is the most frequent congenital viral infection in developed countries, leading to permanent neurological deficits in thousands of newborns each year [9]. Despite its success in spreading throughout the human population, HCMV is unable to cross species barriers. Genomic analyses have demonstrated that CMVs have been co-speciating with their hosts for ~80 million years [14,15]. Through this process, each CMV has specifically adapted to its cognate host and in doing so, diverged from closely related CMV species. Among the many factors that may contribute to cross-species barriers to infection, cell-intrinsic immune factors likely play a central role because the selective pressure imposed by viral antagonists has driven their rapid evolution. Support for this arms race paradigm comes from computational and functional studies that demonstrate ongoing reciprocal innovation by host and viral factors at host:virus interfaces [97,98]. The millions of years of shared evolutionary history between CMVs and their hosts provide an invaluable model for investigating the consequences of host-virus arms races.

Protein Kinase R (PKR) is a broadly acting restriction factor that phosphorylates the translation initiation factor eIF2α in response to cytoplasmic double-stranded RNA (dsRNA),

resulting in a block to translation initiation and viral replication [34]. The importance of PKR in the host cell's anti-viral arsenal is highlighted by the presence of PKR antagonists in many virus families [99,100]. Furthermore, deletion of PKR antagonists renders many viruses replication deficient [24,80,81,92,101,102], demonstrating that PKR poses a strong molecular barrier to viral replication. To overcome the onslaught of diverse viral antagonists, PKR has had to continually adapt while still being constrained by the need to maintain its critical interactions with dsRNA and eIF2α. Consistent with this perspective, evolutionary analyses have identified dramatic episodes of positive selection in PKR during primate evolution [86,87]. Thus, we leveraged the long co-evolutionary history of CMVs and their hosts to investigate how the rapid evolution of PKR has impacted the evolution of the CMV-encoded PKR antagonist TRS1.

Results

Primate Cytomegaloviruses have evolved species-specific differences in PKR antagonism

To determine whether the evolutionary divergence of PKR in primates has affected the ability of CMVs to antagonize PKR, we used a heterologous system to readily test TRS1 alleles from several primate CMV species. This system takes advantage of the fact that wild type VacV (WT VacV) replicates well in a broad range of primate cells, including human (HeLa) and Agm (BSC40) cells, while deletion of the PKR antagonist E3L (VacVΔE3L) markedly reduces replication [101] (Fig 3.1A). PKR activity is responsible for the replication blockade in HeLa cells, as knocking out PKR by CRISPR/Cas9 gene editing (HeLa PKR KO) completely rescued VacVΔE3L replication. To evaluate whether the CMV TRS1 proteins can antagonize human or Agm PKR, we recombined four CMV *TRS1* genes from species that infect hominoids, Old World monkeys, and New World monkeys (HCMV, African green monkey CMV (AgmCMV),

Rhesus CMV (RhCMV)), and Squirrel monkey CMV (SmCMV)) into VacVΔE3L and evaluated their ability to rescue replication in human and Agm cells. All viruses replicated well in the HeLa-PKR KO cells (Fig 3.1A) and expressed comparable levels of the TRS1 proteins (Fig 3.1B). However, we observed species-specific differences in viral replication in the human and Agm cell lines. Consistent with previous findings, VacVΔE3L+$HCMV_{TRS1}$ replicated well in human but not Agm cells [83]. Conversely, $AgmCMV_{TRS1}$ and $RhCMV_{TRS1}$ rescued VacVΔE3L in Agm but not human cells. Surprisingly, $SmCMV_{TRS1}$ rescued replication in both human and Agm cells, indicating that it is more broadly acting. These results suggest that evolutionary pressures have had a substantial impact on the CMV *TRS1* genes, resulting in species-specific differences in their ability to block host defenses necessary to rescue VacVΔE3L replication.

CMV TRS1 genes antagonize PKR-mediated inhibition of reporter gene expression in a species-specific manner

We hypothesized that the differences in the ability of *TRS1* genes to rescue VacVΔE3L in human and Agm cells are due to differing abilities to antagonize human vs. Agm PKR. To test this, we utilized an assay in which expression of PKR inhibits translation of a co-transfected secreted embryonic alkaline phosphatase (SEAP) reporter gene [87,94] (Fig 3.2A). Co-transfection of a functional PKR antagonist reverses, at least in part, the inhibitory effect of PKR, leading to an increase in reporter gene expression. We co-transfected HeLa PKR KO cells with plasmids expressing SEAP along with HuPKR or with a control plasmid and a panel of TRS1 antagonists or a vector control. In the absence of any antagonist, transfection of the HuPKR expression plasmid reduced SEAP expression (Fig 3.2B, bars 1 vs 6). Co-transfection of $HCMV_{TRS1}$ or $SmCMV_{TRS1}$ each lessened the inhibitory effect of HuPKR (bars 7 and 10), while

co-transfection of AgmCMV$_{TRS1}$ or RhCMV$_{TRS1}$ had little effect (bars 8 and 9). In contrast, in cells transfected with Agm PKR, AgmCMV$_{TRS1}$ rescued reporter activity relative to the vector control while HCMV$_{TRS1}$ did not (Fig 3.2C), consistent with what was observed with VacVΔE3L rescue in Agm cells (Fig 3.1A). These results substantiate the co-evolutionary history of CMVs with their hosts, during which specific adaptations to their cognate PKR limited hominoid and Old World monkey CMVs ability to restrict more distant PKR alleles.

RhCMV$_{TRS1}$ fails to complement HCMV deleted of its PKR antagonists

Our findings from the recombinant VacV and SEAP assays demonstrate that RhCMV$_{TRS1}$ and AgmCMV$_{TRS1}$ have weak activity against human PKR. However, VacV replication kinetics are much more rapid than that of CMV, and the amount of dsRNA produced during infection by CMV and VacV may differ. Therefore, it is possible that a weak PKR antagonist would be sufficient to overcome PKR in the context of CMV infection. To determine if a weak PKR antagonist could support CMV replication, we generated a recombinant HCMV-eGFP virus that lacks the PKR antagonists HCMV$_{TRS1}$ and HCMV$_{IRS1}$, but expresses the orthologous RhCMV$_{TRS1}$ (HCMVΔI/ΔT-eGFP+RhCMV$_{TRS1}$). While this virus replicated well in human fibroblasts lacking PKR (HF-PKR KO), no growth was observed in HFs. The presence of RhCMV$_{TRS1}$ in the virus was confirmed using PCR, but future work is needed to verify that RhCMV$_{TRS1}$ is expressed during infection. These results suggest that even in the context of HCMV infection, a weak antagonist is unable to prevent PKR pathway activation to support viral replication. Thus, these findings support the hypothesis that evolutionary adaptations in PKR constitute one of the barriers to cross-species transmission of CMVs.

Primate CMV TRS1 proteins utilize different mechanisms to prevent PKR pathway activation

In addition to the observed differences in the species-specific activity of orthologous TRS1 proteins, we have also observed apparent differences in the mechanism by which TRS1 proteins block pathway activation. Previous work in our lab demonstrated that while $HCMV_{TRS1}$ blocks phosphorylation and thus activation of PKR, $RhCMV_{TRS1}$ allows this step to proceed but prevents phosphorylation of the downstream substrate, eIF2α. Given these differences, we expanded these analyses to evaluate which step of the PKR pathway is blocked by $AgmCMV_{TRS1}$ and $SmCMV_{TRS1}$. These analyses were first conducted in HFs as these cells support at least moderate replication of all four of these CMV viruses. Moreover, all of these viruses block activation of the PKR pathway in HFs as evidenced by continued protein synthesis during infection (Fig 3.4B). Similar to previous findings, we observed that HCMV blocked phosphorylation of PKR, while RhCMV allowed this step to occur (Fig 3.4A). Interestingly, despite being more closely related to RhCMV, AgmCMV functioned like HCMV and prevented phosphorylation of PKR. In contrast, robust phosphorylation of PKR was observed following infection with SmCMV. However, protein synthesis was also slightly decreased during infection with SmCMV, suggesting the PKR pathway may have been partially activated. Regardless, these findings clearly indicate that primate CMVs utilize distinct mechanisms to prevent PKR pathway activation.

To determine if the observed differences in the status of PKR phosphorylation during CMV infection were due to differences in TRS1 activities, we returned to our recombinant VacV assay. We infected HFs and BSC40s with WT VacV, VacVΔE3L, and recombinant VacVΔE3L viruses expressing $HCMV_{TRS1}$, $AgmCMV_{TRS1}$, or $RhCMV_{TRS1}$. At the time these experiments were conducted our recombinant $SmCMV_{TRS1}$ virus had not yet been generated and additional

experiments will be needed to further evaluate its mechanism of action. In the HF cells, only WT VacV and VacVΔE3L+HCMV$_{TRS1}$ were able to overcome the PKR pathway and allow viral replication to proceed (Fig 3.5A). Moreover, PKR phosphorylation levels were similar to mock in cells infected with WT VacV and VacVΔE3L+HCMV$_{TRS1}$, demonstrating that HCMV$_{TRS1}$ and E3L inhibit the PKR pathway prior to PKR phosphorylation. In contrast, PKR phosphorylation increased following infection with VacVΔE3L and the recombinant viruses expressing AgmCMV$_{TRS1}$ and RhCMV$_{TRS1}$ (Fig 3.5B), consistent with activation of the PKR pathway resulting in an inability of these viruses to replicate in HFs (Fig. 2.5A).

In the BSC40 cell line, AgmCMV$_{TRS1}$ and RhCMV$_{TRS1}$ complemented VacVΔE3L and restored replication to levels similar to that observed with WT VacV (Fig 3.5A). However, in contrast to WT VacV, both AgmCMV$_{TRS1}$ and RhCMV$_{TRS1}$ allowed PKR phosphorylation to occur (Fig 3.5B). As viral replication was maintained, these results suggest that AgmCMV$_{TRS1}$ and RhCMV$_{TRS1}$ block the PKR pathway at a step downstream of PKR phosphorylation during recombinant VacV infection of BSC40 cells.

While these results are consistent with what was observed in the context of RhCMV infection in HFs (Fig 3.4 and [83]), they diverge from what was observed during AgmCMV infection of HFs (Fig 3.4). AgmCMV infection of HFs resulted in a block to PKR phosphorylation, while VacVΔE3L+AgmCMV$_{TRS1}$ infection of BSC40s allowed PKR phosphorylation but prevented full pathway activation. The observed differences may be due to the fact that different viruses were used in these experiments (CMV vs VacV). Alternatively, it is possible that the mechanism of antagonism used by AgmCMV$_{TRS1}$ is different in human cells relative to AGM cells. To distinguish between these alternative hypotheses, I evaluated the status of PKR phosphorylation in an AMG cell line after infection with primate CMVs. I infected

AGM FBs with HCMV, RhCMV, AgmCMV, and WT VacV and VacVΔE3L as controls and evaluated the levels of P-PKR. I found elevated levels of phosphorylated PKR in Agm FBs infected with HCMV, consistent with the inability of this virus to overcome the PKR pathway and replicate in AGM cells. Despite the fact that both AgmCMV and RhCMV replicate well in this cell line, I also observed increased levels of PKR phosphorylation, suggesting these viruses block the PKR pathway at a downstream step. However, additional analyses such as evaluation of the status of eIF2α phosphorylation or metabolic labeling to evaluate total protein synthesis would be necessary to test whether the PKR pathway is inhibited by AgmCMV and RhCMV during infection of AGM FBs. Collectively, these results suggest that the mechanism of antagonism used by $AgmCMV_{TRS1}$ is dependent on the PKR variant it is pitted against.

Discussion

Cytomegaloviruses have coevolved with their respective hosts for millions of years, allowing them to specifically adapt to their cognate host. However, this specific adaptation may also contribute to the inability of CMVs to cross species barriers. While there are likely multiple host factors that contribute to cross species barriers to CMV infection, cell intrinsic antiviral factors like PKR are attractive candidates. This is because selective pressures imposed by viral antagonists has driven rapid evolution of host antiviral factors, thus leading to significant divergence of these antiviral factors in primate species. PKR has evolved under positive selection throughout primate evolution, and rapidly evolving codons were identified throughout the protein, consistent with its broad antiviral activity.

The rapid evolution of PKR has in turn impacted the evolution of viral PKR antagonists. For example, primate PKRs exhibit differential sensitivity to the VacV antagonist K3L, which

functions as a PKR pseudosubstrate and structurally mimics eIF2α. Similarly, previous work from our lab demonstrates that $HCMV_{TRS1}$ can readily antagonize human but not AGM PKR, with the reverse being true for $RhCMV_{TRS1}$. Given these species-specific differences in TRS1 activity, we sought to expand these analyses to gain a more comprehensive view of how the rapid evolution of PKR has impacted the evolution of CMV PKR antagonism. Our findings indicate that some CMV TRS1 proteins have specifically adapted to changes in the PKR variant from their cognate host. For example, both $HCMV_{TRS1}$ and $AgmCMV_{TRS1}$ can readily antagonize the PKR variants from their respective hosts. However, this specific adaptation comes at a cost, as $HCMV_{TRS1}$ exhibits weak activity against AGM PKR and similarly $AgmCMV_{TRS1}$ has little activity against human PKR. These findings suggest that the species-specific differences in TRS1 activities likely contribute to cross-species barriers to CMV transmission as overcoming PKR is essential for CMV replication[24].

Looking beyond $HCMV_{TRS1}$ and $AgmCMV_{TRS1}$ we find the story of PKR antagonism in CMVs to be more complex. For example, $RhCMV_{TRS1}$ has strong activity against AgmPKR in VacVΔE3L rescue assays despite the fact that AGM is not its cognate host. This finding is not too surprising considering the relatively close evolutionary relationship between AGMs and Rhesus macaques. Moreover, evolutionary analyses of PKR have shown that a significant burst of evolution occurred in a branch leading to a common ancestor of AGMs and Rhesus macaques, where 22 nonsynonymous and zero synonymous mutations were observed [86]. While additional selection occurred following the divergence of AGM and Rhesus PKR, these changes do not seem to have had a significant impact on $RhCMV_{TRS1}$ activity and thus were likely driven by other viral antagonists. Surprisingly, we found that $SmCMV_{TRS1}$ appears to have broad activity, as it is able to antagonize both HuPKR and AgmPKR in VacVΔE3L rescue assays. These results

suggest that $SmCMV_{TRS1}$ may have evolved to target a region of PKR that is more conserved. Alternatively, $SmCMV_{TRS1}$ may be more flexible and thus more tolerant of different amino acids at its binding site. Future work is necessary to determine what accounts for the broad activity of $SmCMV_{TRS1}$.

While the VacVΔE3L rescue assay provides a valuable tool to evaluate the activity of different TRS1 proteins in a common viral background, it may not accurately model PKR activation during CMV infection. In fact, both RhCMV and AgmCMV are capable of replicating in human cells, suggesting that weak antagonism by TRS1 genes in these viruses may be sufficient to inhibit PKR during CMV infection. Therefore, to test this we generated an HCMV virus that expressed $RhCMV_{TRS1}$ as its only PKR antagonist. While this virus exhibited robust replication in HFs lacking PKR, it failed to replicate in WT HFs (Fig 3.3). Thus, even in the context of HCMV replication the weak activity of $RhCMV_{TRS1}$ against human PKR is not sufficient to inhibit pathway activation. Given these findings, it is surprising that RhCMV and AgmCMV are able to replicate at all in HFs. Both AgmCMV and RhCMV replicate more rapidly than HCMV, and thus it is possible that the weak activity of their antagonists is sufficient in this context. Alternatively, it is possible that these viruses contain additional PKR antagonists that permit replication. However, in the case of RhCMV, we have shown that TRS1 is essential for viral replication, suggesting other RhCMV genes are at least not sufficient to antagonize PKR (unpublished data). Moreover, $RhCMV_{TRS1}$ and $AgmCMV_{TRS1}$ are each sufficient to antagonize AGM PKR in context of the VacVΔE3L rescue assay and thus don't require additional factors from CMV to function. Another possibility is that HCMV generates more dsRNA than RhCMV or AgmCMV and thus requires a more active PKR antagonist. The ability of RhCMV and AgmCMV to replicate in HFs is still not fully understood and will require further investigation.

Beyond the species-specific difference in the activities of primate CMV TRS1 proteins, we have also observed difference in the mechanisms of antagonism employed. Previous work demonstrated that $HCMV_{TRS1}$ functions to block phosphorylation and thus activation of PKR, while $RhCMV_{TRS1}$ allows this step to occur but prevents phosphorylation of the downstream substrate eIF2α [83]. Thus, we sought to expand these analyses to other primate CMV TRS1 proteins. Preliminary analyses suggest that $SmCMV_{TRS1}$ functions similar to $RhCMV_{TRS1}$ and acts downstream of PKR auto-phosphorylation as robust phosphorylation of PKR was observed during SmCMV infection of HFs. However, total protein synthesis was slightly decreased in SmCMV infected cells, suggesting the PKR pathway may have been partially activated. Additional experiments evaluating both P-PKR and P-eIF2α during SmCMV infection are necessary to more carefully characterize $SmCMV_{TRS1}$'s mechanism of action. The activity of $AgmCMV_{TRS1}$ appears to be more complex as the mechanism of action used by $AgmCMV_{TRS1}$ is dependent on the PKR variant it is antagonizing—$AgmCMV_{TRS1}$ blocks phosphorylation of human PKR but acts post auto-phosphorylation when antagonizing AgmPKR. This surprising finding suggests that the interactions of $AgmCMV_{TRS1}$ with human PKR and AgmPKR are qualitatively distinct.

Collectively, our findings provide evidence that the host-viral arms race between PKR and CMV TRS1 proteins has led to substantial divergence of the interface between PKR variants and CMV TRS1 proteins, as reflected by species-specific differences in CMV PKR antagonism. However, the molecular basis of these species-specific differences remains undefined and further work is necessary to identify the critical differences within primate PKR alleles that are responsible for the differential sensitivity to CMV TRS1 proteins.

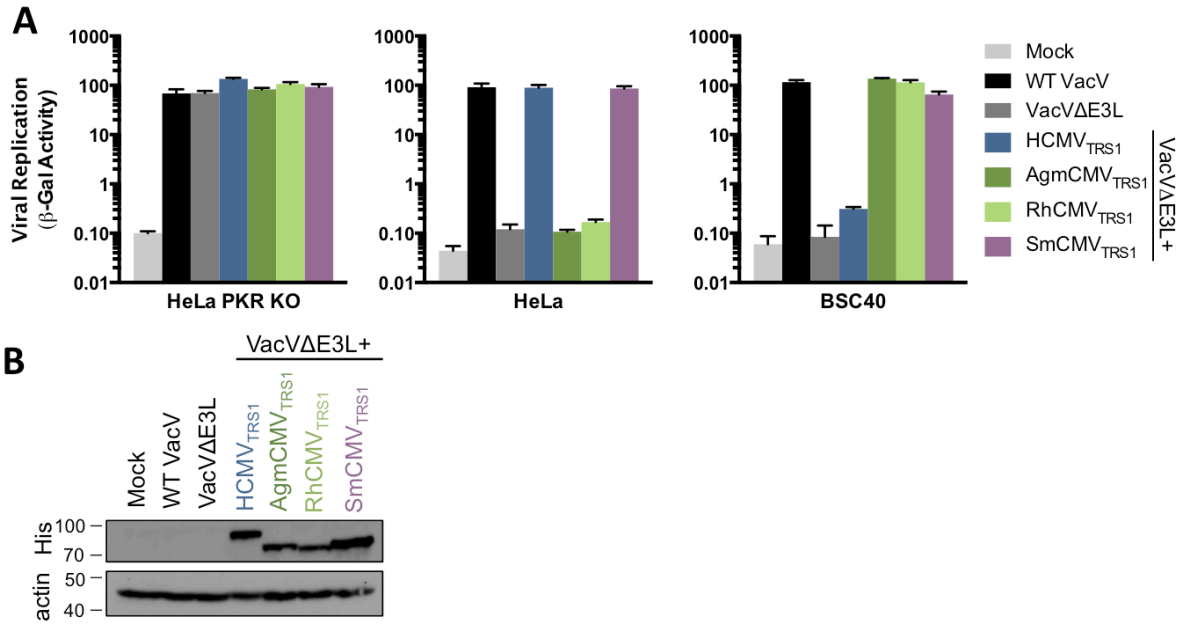

Figure 3.1 Species-specific differences in VacVE3L rescue by primate CMV TRS1 proteins. (A) Triplicate wells of HeLa PKR KO, HeLa, or BSC40 cells were mock infected or infected (MOI 0.1) with WT VacV, VacVΔE3L, or VacVΔE3L recombinants containing $HCMV_{TRS1}$, $AgmCMV_{TRS1}$, $RhCMV_{TRS1}$, or $SmCMV_{TRS1}$. At 48 hpi, viral replication was quantified by measuring β-gal activity (mean ± s.d.). (B) His-tagged TRS1 constructs were detected in lysates of the infected HeLa PKR KO cells from (A) by western blotting. TRS1 size variation is expected based on differences in coding length.

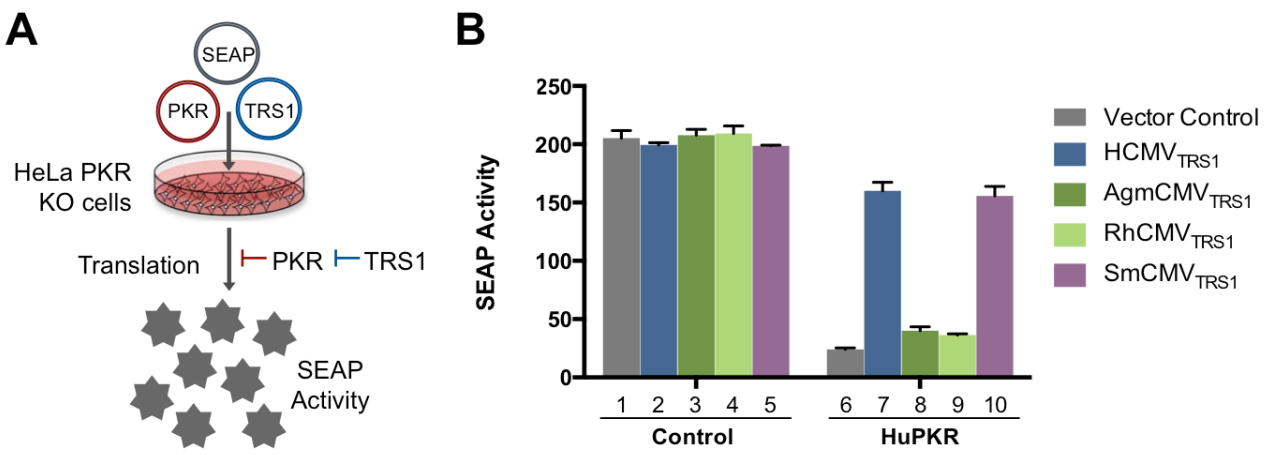

Figure 3.2 Primate CMV TRS1 proteins exhibit species-specific differences in their ability to antagonize PKR variants.
(A) Schematic representation of the SEAP assay. Transfection of PKR leads to decreased activity of a co-transfected reporter construct expressing SEAP. This PKR-driven repression can be counteracted by co-transfection of a functional TRS1 antagonist, resulting in a rescue of SEAP activity. (B) The SEAP assay recapitulates species-specific differences in HuPKR antagonism by TRS1 alleles. HeLa PKR KO cells were co-transfected with a SEAP reporter plasmid along with either a control vector or HuPKR and the indicated TRS1 alleles or a vector control. At 48 h post transfection, SEAP activity in the medium was measured (mean ± s.d.).

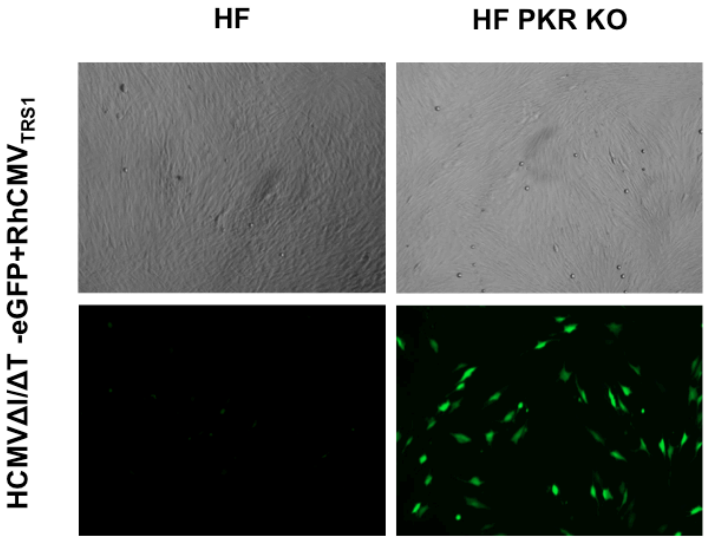

Figure 3.3 RhCMV$_{TRS1}$ fails to support HCMV replication in HFs.
Human Fibroblasts (HF) and HF PKR KO cells were infected with equal amounts of HCMVΔI/ΔT-eGFP+RhCMV$_{TRS1}$ and imaged at 5dpi.

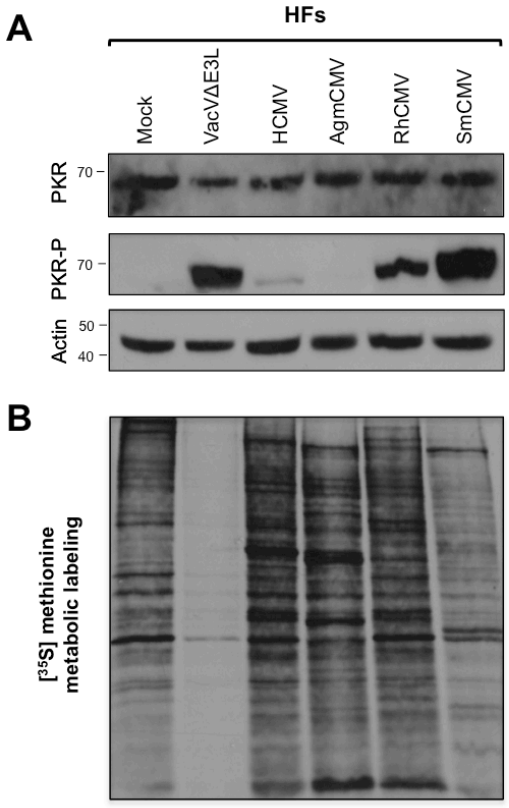

Figure 3.4 Primate CMVs block the PKR pathway at different steps.
(A) Evaluation of the status of PKR phosphorylation during infection. HFs were infected with the indicated viruses at an MOI of 3. At 48hpi, cells were lysed and levels of PKR-P, total PKR and actin were evaluated by Western blotting. (B) Analysis of total protein synthesis during infection by metabolic labeling. HFs were infected with the indicated viruses at an MOI of 3 and at 24hpi, cells were pulse- labeled with [^{35}S] methionine. Lysates were evaluated through SDS-PAGE and autoradiography.

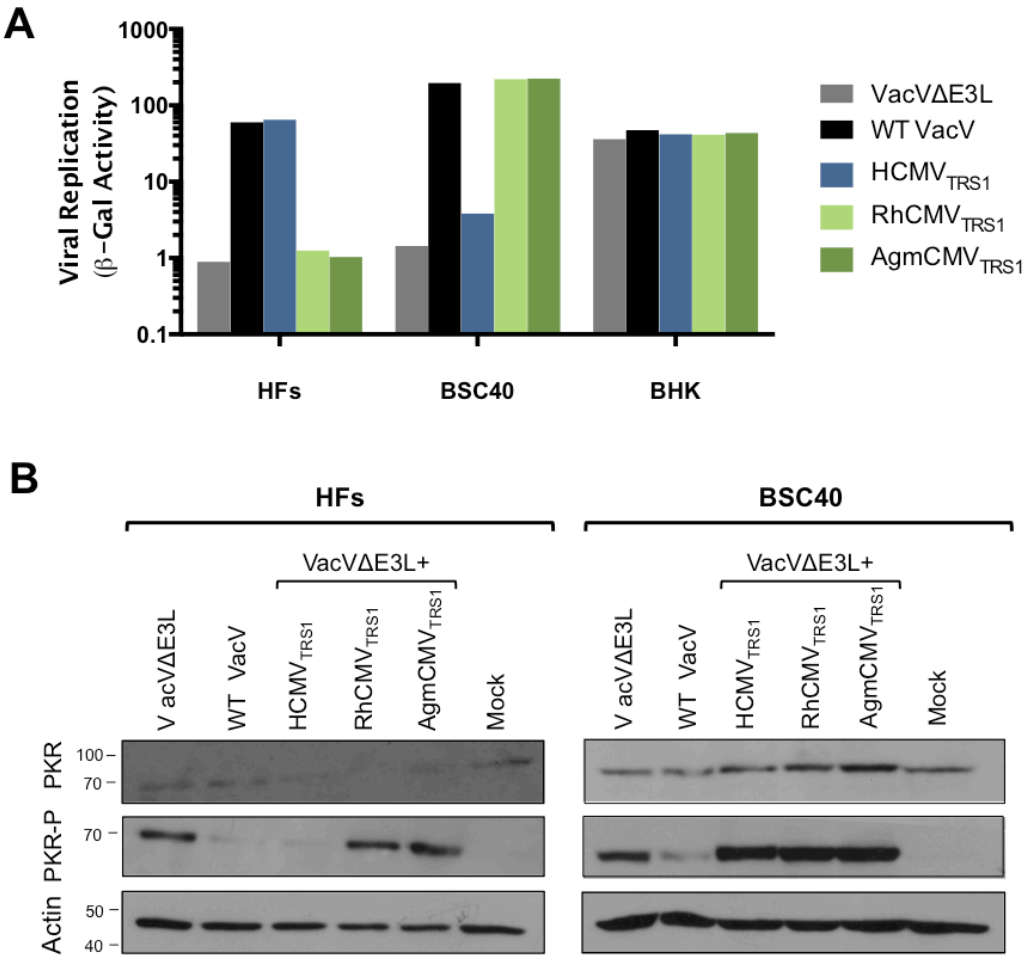

Figure 3.5 AgmCMVTRS1 allows PKR phosphorylation in BSC40 cells.
(A) Evaluation of viral replication in HF, BSC40 and BHK cells. Each cell type was infected at an MOI of 3 with the indicated viruses. At 24hpi, β-gal activity was measured to evaluate viral replication. (B). Evaluation of PKR phosphorylation in infected cells. Lysates were harvested from the same cells analyzed in panel (A) and levels of PKR-P, total PKR and actin were evaluated by Western blotting.

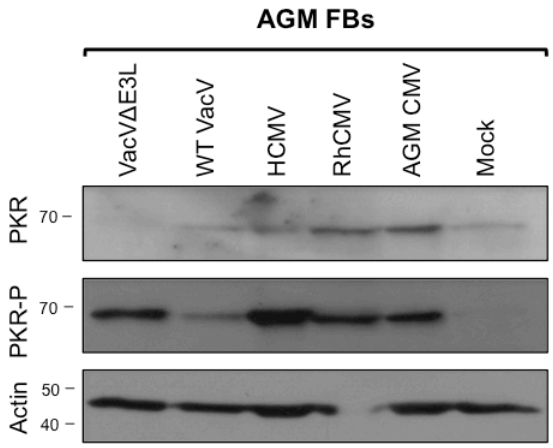

Figure 3.6 Structure of α-defensins. AgmCMV allows phosphorylation of PKR in AGM FBs.

AGM FBs were infected at an MOI of 3 with the indicated viruses. At 48hpi, lysates were harvested and levels of PKR-P, total PKR and actin were evaluated by Western blotting.

Chapter 4. A single amino acid dictates PKR susceptibility to unrelated viral antagonists

Portions of this chapter are adapted from an article originally published in PLoS Pathogens:

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A Single Amino Acid Dictates Protein Kinase R Susceptibility to Unrelated Viral Antagonists.
PLoS Pathogens. 12(10): e1005966. doi:10.1371/journal.ppat.1005966

Summary

The rapid evolution and divergence of PKR in primates has led to specific adaptations of primate CMVs to the PKR allele of their cognate host, ultimately resulting in species-specific differences in CMV PKR antagonism. For example, $HCMV_{TRS1}$ can readily antagonize human PKR, but not African green monkey PKR. By leveraging these differences, we identified a single amino acid at codon 489 in human PKR that dictates PKR susceptibility to the human CMV PKR antagonist, $HCMV_{TRS1}$. This amino acid is positioned within a helix that mediates the critical interaction between PKR and its downstream substrate eIF2α. Despite this seemingly important structural role, human PKR is highly tolerant of amino acid substitutions at position 489, allowing it the flexibility to adapt in order to evade viral antagonists without disrupting its antiviral activity. Remarkably, position 489 also dictates PKR sensitivity to the entirely unrelated poxvirus-encoded PKR antagonist, K3L. Thus, mutations driven by one virus can impact the host's sensitivity to unrelated viral antagonists, illustrating the multilateral nature of the host-viral "arms-races" between viruses and broadly acting antiviral host defenses.

Introduction

Host restriction factors provide a potent block to viral replication, making them an important component of the innate immune response. In order to establish productive infections, viruses have evolved mechanisms to evade these antiviral factors. This antagonism exerts evolutionary pressure on the host, triggering an arms race between the host and the virus that drives continual innovation of the conflicting proteins. This arms-race paradigm is exemplified by the rapid evolution of the antiviral factor Protein Kinase R (PKR) [86]. PKR is activated by double-stranded dsRNA, a common byproduct of viral replication. In the presence of dsRNA, PKR dimerizes around it, allowing PKR to autophosphorylate and become active. In this active state, PKR then phosphorylates its downstream substrata, eIF2α, ultimately leading to a global shutoff in translation and subsequent inhibition of viral replication [36]. Given this potent antiviral effect, many viruses encode PKR antagonists, including the cytomegaloviruses (CMVs).

Primate CMVs encode the PKR antagonist TRS1, which interacts directly with both dsRNA and PKR to prevent pathway activation [65,66]. Co-evolution of the CMVs with their hosts for millions of years has allowed them to become well adapted to their cognate PKR [14,15]. However, this specific adaptation has led to the divergence of CMV TRS1 proteins resulting in species-specific differences in TRS1 activity. Herein we leverage these differences to precisely map a molecular basis of differential sensitivity of human PKR and AgmPKR to $HCMV_{TRS1}$ to a single amino acid. This amino acid falls within the αG helix of PKR, which mediates the critical interaction between PKR and its downstream substrate, eIF2α. Despite this seemingly important structural role, we find this amino acid is highly tolerant of amino acid substitutions and is thus poised to evolve to evade viral antagonists. Moreover, we find that

altering this reside impacts not only $HCMV_{TRS1}$, but also an unrelated antagonist from poxviruses that also targets the αG helix. Thus, for broadly acting factors like PKR, the host-viral arms race is surprisingly complex and host adaptations to evade one virus have the potential to unintentionally impact the host's sensitivity to unrelated viruses.

Results

Mapping the species-specific difference in PKR susceptibility to a single amino acid

The rapid evolution of PKR in primates has led to significant divergence of the CMV *TRS1* genes, resulting in species-specific differences in their ability to antagonize primate PKR variants. For example, $HCMV_{TRS1}$ can readily antagonize human PKR, but has weak activity against the more distantly related African green monkey (Agm) PKR. These functional differences are likely explained by species-specific changes in the binding interface of PKR and the CMV TRS1 proteins. However, the location of this interface on PKR is unknown. As an alternative to blind approaches like alanine scanning and random mutagenesis, we leveraged the power of the species-specific differences in TRS1 activity to precisely map the PKR:TRS1 interface. We generated chimeras between the susceptible HuPKR allele and the resistant AgmPKR allele. The 98 amino acids that differ between HuPKR and AgmPKR are scattered throughout the protein, as are the sites that were previously found to be evolving under positive selection in primates [86], making it difficult to predict which region(s) is likely to be responsible for their differential sensitivity to $HCMV_{TRS1}$. Therefore, we first made chimeras with a break point within the linker region near the middle of PKR and found that the species-specificity of $HCMV_{TRS1}$ and $AgmCMV_{TRS1}$ mapped to the C-terminal half of PKR (Fig 4.1A). Further subdivision identified a small region (codons 475-520, designated region D2) within

Agm PKR which when introduced into HuPKR was sufficient to confer resistance to $HCMV_{TRS1}$ (Fig 4.1B). Six amino acids within this region differ between HuPKR and AgmPKR. To determine whether any of these differences alone is necessary for sensitivity to $HCMV_{TRS1}$, we generated point mutants at each of these sites by introducing the amino acid present in AgmPKR into HuPKR. Strikingly, mutating position 489 of HuPKR from phenylalanine to serine (F489S) was sufficient to confer resistance to $HCMV_{TRS1}$, while the other five point mutants had no effect (Fig 4.1C). Thus, this experimental system allowed us to rapidly identify position 489 as a critical species-specific determinant of sensitivity to $HCMV_{TRS1}$.

HuPKR F489S confers resistance to $HCMV_{TRS1}$ in the context of viral replication

We next wished to test the impact of this mutation in the context of a complete viral infection by challenging viruses with heterologous PKRs. Unfortunately, this approach has been difficult to establish because PKR overexpression inhibits cell growth. To circumvent this problem, we stably transduced PKR knockout cells with heterologous PKR genes (HuPKR, AgmPKR, HuPKR F489S, or the empty vector) under the control of a doxycycline-inducible promoter. We then used these cells to assess the replication profiles of our recombinant VacVs. All of the viruses replicated well in the control empty vector cell line regardless of doxycycline induction (Fig 4.2A), and TRS1 proteins were expressed to similar levels in these cells (Fig 4.2B). Upon induction, the HuPKR and HuPKR F489S cell lines expressed PKR to levels comparable to that observed in wild-type HeLa cells (Fig 4.2C). Although we could not assess expression of AgmPKR as it does not cross-react with the PKR antibody, the fact that the AgmPKR line restricted VacVΔE3L replication after induction of PKR by addition of doxycycline strongly suggests that AgmPKR was expressed in these cells. As expected, WT

VacV was able to overcome PKR antiviral activity in each of these cell lines, while VacVΔE3L did not replicate well in any (Fig 4.2A). In the HuPKR- and AgmPKR-expressing cell lines, the replication profiles of the panel of TRS1-expressing viruses mirrored those observed in human (HeLa) and Agm (BSC40) cell lines (Fig 3.1A and Fig 4.2A). Importantly, the cell line expressing HuPKR F489S restricted VacVΔE3L+$HCMV_{TRS1}$, demonstrating that this mutation renders HuPKR resistant to $HCMV_{TRS1}$ activity in the context of viral infection. $RhCMV_{TRS1}$ and $AgmCMV_{TRS1}$ were also unable to antagonize HuPKR F489S efficiently and rescue replication, suggesting that mutating position 489 of HuPKR to the AgmPKR variant is not sufficient for conferring sensitivity to these Old World monkey CMV TRS1 proteins (Fig 4.2A). Interestingly, $SmCMV_{TRS1}$ rescued VacVΔE3L replication even in cells expressing HuPKR F489S, consistent with its broad PKR inhibitory activity.

The inability of VacVΔE3L+$HCMV_{TRS1}$ to replicate in HuPKR F489S cells strongly suggests that $HCMV_{TRS1}$ is unable to prevent activation of HuPKR F489S. To test this, we evaluated the levels of phosphorylated PKR and eIF2α following infection. As expected, PKR phosphorylation was observed in response to VVΔE3L infection in both HuPKR and HuPKR F489S cells, demonstrating activation of the PKR pathway had been initiated (Fig 4.3). While we did not observe increased eIF2α-P levels in response to VVΔE3L infection in the HuPKR cell line in this experiment, we did observe increased eIF2α-P in the HuPKR F489S cells. Consistent with previous findings, VacVΔE3L+$HCMV_{TRS1}$ was able to block phosphorylation of PKR and eIF2α in HuPKR cells [83]. However, robust phosphorylation of both PKR and eIF2α was observed in HuPKR F489S cells in response to VacVΔE3L+$HCMV_{TRS1}$ infection (Fig 4.3, lanes 4 vs 8). Thus, consistent with the replication data (Fig 4.2A), $HCMV_{TRS1}$ prevents activation of the PKR pathway in HuPKR cells but not in HuPKR F489S cells.

Mutating position 489 disrupts $HCMV_{TRS1}$ binding to HuPKR

We next investigated how this single amino acid change made HuPKR resistant to $HCMV_{TRS1}$. Since prior studies suggested that $HCMV_{TRS1}$ must bind directly to PKR to effectively antagonize the PKR pathway [64,66,78], we tested the hypothesis that altering residue 489 of HuPKR from phenylalanine to serine interfered with $HCMV_{TRS1}$ binding to PKR. We transfected HeLa PKR KO cells with either WT HuPKR or HuPKR F489S along with a panel of His-tagged TRS1 constructs or His-tagged GFP as a negative control. Following cell lysis, we affinity purified the His-tagged proteins along with any bound PKR. We detected TRS1 and PKR in the lysate and bound fractions using anti-His and anti-PKR antibodies, respectively (Fig 4.4). As expected, $HCMV_{TRS1}$ bound to WT HuPKR; however, this interaction was completely disrupted by the F489S mutation. In contrast, $SmCMV_{TRS1}$ bound to both WT HuPKR and the F489S mutant equally well, consistent with its ability to antagonize both forms of HuPKR (Fig 4.2A), while $AgmCMV_{TRS1}$ did not bind to either PKR variant. These results indicate that position 489 is a critical residue mediating the interaction between $HCMV_{TRS1}$ and HuPKR and that disrupting this interaction renders $HCMV_{TRS1}$ ineffective.

Position 489 of human PKR is highly tolerant of amino acid substitutions, many of which confer resistance to antagonism by $HCMV_{TRS1}$.

Amino acid 489 falls within the αG-helix of PKR, which mediates the interaction between PKR and its downstream substrate, eIF2α [38,103]. In fact, structural data suggest that F489 directly interacts with eIF2α by projecting into a hydrophobic pocket [38] (Fig 4.5A). Despite this seemingly critical interaction, position 489 is rapidly evolving in primates, consistent with it being engaged in an ongoing arms race with viral antagonists [86] (Fig 4.5B).

To determine how tolerant HuPKR function is to changes at position 489, we generated HuPKR variants by introducing all possible amino acid substitutions at position 489. These variants were then evaluated for their ability to restrict SEAP reporter gene expression, which reflects the ability of PKR to bind to and phosphorylate eIF2α and thereby inhibit translation [34]. Surprisingly, with the exception of the weak activity of proline, all of the amino acid variants maintained the ability to restrict SEAP expression to levels comparable to that of wild-type HuPKR (Fig 4.5C, gray bars). In contrast, a point mutant of PKR that lacks kinase activity [104] (KD HuPKR) and cannot phosphorylate eIF2α had little effect on SEAP activity. Thus, position 489 is highly tolerant of amino acid changes and does not play an essential role in the interaction between HuPKR and eIF2α. Of note, the other three cellular eIF2α kinases, which do not appear to be engaged in arms races with viral factors, have completely conserved the site corresponding to position 489 of PKR [86]. Thus, these results demonstrate the robustness of PKR's interaction with eIF2α despite the pressure for continual innovation within the αG-helix in order to evade viral antagonists.

We next wanted to know whether $HCMV_{TRS1}$ exhibited similar flexibility it its ability to recognize different amino acids at position 489. We found that in addition to phenylalanine, $HCMV_{TRS1}$ could antagonize HuPKR expressing tryptophan, methionine, or tyrosine at position 489, and had moderate activity against leucine (Fig 4.5C). These amino acids each have hydrophobic side chains, suggesting that they may interact with a hydrophobic pocket in $HCMV_{TRS1}$. In contrast, $HCMV_{TRS1}$ had little activity against the other amino acid substitutions. Thus, our results clearly indicate that while HuPKR can tolerate a diverse range of amino acids at position 489 without losing its functional interaction with eIF2α, $HCMV_{TRS1}$ is active in counteracting only a small subset of these variants. The surprising plasticity at this site may

contribute to PKR's ability to remain competitive in the arms race with rapidly evolving viral genes.

HuPKR F489S also confers resistance to the unrelated poxvirus antagonist K3L (H47R)

In addition to position 489, two other codons (492 and 496, Fig 4.5B) within the αG-helix are rapidly evolving among primates [86], suggesting that this helix may be a hot spot for targeting by viral antagonists. Consistent with this hypothesis, poxviruses encode eIF2α mimics that competitively inhibit eIF2α docking by binding to the αG helix. In fact, previous work on the VacV eIF2α mimic K3L identified position 492 as a major species-specific determinant of PKR activity within hominoids [86]. Another neighboring codon in human PKR, A488, has been implicated in the differential sensitivity of human vs. mouse PKR to VacV K3L [87]. The proximity of the amino acids that affect K3L and $HCMV_{TRS1}$ activities indicate that interactions at the αG helix are essential for both antagonists. However, unlike K3L, $HCMV_{TRS1}$ shows no obvious homology to eIF2α, suggesting that two unrelated viruses convergently evolved to target this vulnerable region of PKR. Given the shared interaction at the αG-helix, we investigated whether altering position 489 of HuPKR would also impact the activity of K3L. WT K3L has very little activity against HuPKR, while an experimentally evolved form of K3L containing a single amino acid change (H47R) confers moderate activity against HuPKR [105,106]. Similar to previous findings [105], K3L H47R rescued VACVΔE3L replication relative to WT K3L in cells expressing HuPKR (Fig 4.6). Notably, the replication advantage conferred by K3L H47R was completely abrogated in cells expressing HuPKR F489S, demonstrating that this single amino acid change in PKR confers resistance to two unrelated viral antagonists. This result highlights the complexity of the host-viral arms race in broadly acting factors like PKR where multiple

viral antagonists may target a shared interface. In these scenarios, mutations driven by one virus have the potential to alter sensitivity or resistance to unrelated viral antagonists.

Discussion

Overall, our results provide evidence for a co-evolutionary history between PKR and the CMV TRS1 proteins, as reflected by specific adaptation of $HCMV_{TRS1}$ and $AgmCMV_{TRS1}$ to their cognate PKRs. These adaptations have led to divergence of the PKR:TRS1 interface, resulting in species-specific differences in TRS1 activity. For example, in contrast to the critical role of amino acid 489 of PKR for $HCMV_{TRS1}$ activity, this site does not appear to mediate sensitivity to the orthologous proteins from three other primate CMVs. $SmCMV_{TRS1}$ retains the ability to bind to and antagonize HuPKR F489S (Fig 4.2A and Fig 4.4), and in fact it appears to be active against a broad range of primate PKRs as well as multiple HuPKR 489 variants (Fig 3.1A and data not shown). Thus, compared to $HCMV_{TRS1,}$ $SqMCMV_{TRS1}$ likely has evolved to recognize and bind to a different site that may be more conserved among primate PKRs. On the other hand, the RhCMV and AgmCMV TRS1 alleles are unable to inhibit HuPKR even when codon 489 is the variant (489S) found in their Old World monkey hosts. Although we have not precisely mapped species-specific determinants in these cases, we observed that the Agm PKR kinase domain was sufficient to confer sensitivity to inhibition by $AgmCMV_{TRS1}$ (Fig 4.1A). Unlike $HCMV_{TRS1}$, which inhibits the PKR pathway prior to autophosphorylation, $RhCMV_{TRS1}$ allows PKR autophosphorylation but blocks eIF2α phosphorylation [83]. Thus, the evolution of PKR in primates appears to have led to quite divergent adaptions in the CMV TRS1 antagonists that changed their precise binding interactions and resulted in alternative species-specific mechanisms of PKR inhibition.

Even while PKR has been evolving to evade viral antagonists, it is constrained by the need to maintain recognition of dsRNA and eIF2α. Thus, residues that are critical for these interactions are more likely to be immutable, while those that are detected by the viral antagonists are more likely to change. In fact, codons evolving under positive selection in primates were reported to be widely dispersed across the PKR coding sequences, though none were found within the dsRNA-binding domains [86]. On the other hand, at least three codons within the αG helix are highly variable among primates [86], even though the PKR kinase domain-eIF2α co-crystal shows that this region, and residue 489 in particular, makes direct contact with eIF2α [38] and thus might be expected to be particularly intolerant of substitutions. Although variation at this site in other primate PKR genes might depend on epistatic mutations elsewhere in PKR that help maintain the interaction with eIF2α, we found that HuPKR retains its inhibitory activity when any of 19 different amino acid substitutions are introduced at residue 489 in human PKR. The only exception is proline, which may not be tolerated due to its helix-breaking properties. These findings support structural data showing that the helical insert between βstrands 3 and 4 of eIF2α is relatively flexible (high B-factor) [107,108] and thus able to adapt to maintain interactions with a rapidly evolving PKR interface. In contrast, VacV K3L, the structural mimic of eIF2α, has been proposed to be more rigid and thus less tolerant of mutations in the αG helix [109]. Prior studies have identified other mutations in the αG helix that preserve (codons 490 and 499) or eliminate (codons 487 and 495) eIF2α recognition by PKR [103], but none of these are variable among primate PKR alleles. Thus, HuPKR appears to retain its eIF2α kinase function despite mutations at a subset of positions within this otherwise critical structure. This mutational tolerance at codon 489 and nearby residues may facilitate acquisition of adaptive changes in PKR during its arms race with viral antagonists that target this site.

Because PKR senses dsRNA, which accumulates during replication of diverse viruses [23] it has broad anti-viral activity and has also been the target of antagonists encoded by many different virus families [99,100]. Even within the CMV subfamily, adaptations to diverging PKR alleles during co-speciation of CMVs with their hosts has altered the PKR:TRS1 binding interfaces. In contrast to this divergent evolution of closely related PKR antagonists, here we also identify convergent evolution by the unrelated antagonists $HCMV_{TRS1}$ and poxvirus K3L. Prior studies mapped species-specific determinants of VacV K3L sensitivity to PKR codons 488 [87] and 492 [86], both of which are within the αG helix and proximal to the site we found to be critical for sensitivity to $HCMV_{TRS1}$. Furthermore, we demonstrate that codon 489 also impacts K3L activity, as introducing a serine at position 489 of human PKR is sufficient to confer resistance to both $HCMV_{TRS1}$ and VacV K3L (H47R). Thus, the conflict between PKR and its viral antagonists should be viewed as a multilateral arms race, in which mutations driven by one virus can have collateral effects on other antagonists. This is true for the αG helix of PKR, which might be targeted by unrelated viral antagonists precisely because it is critical for PKR function. In response, during the evolution of host restriction factors like PKR, preservation of robustness may depend on residues, such as 489, embedded within critical functional domains that can serve as mutable decoys to enable rapid adaptation to multiple viruses without compromising the core functions of the protein.

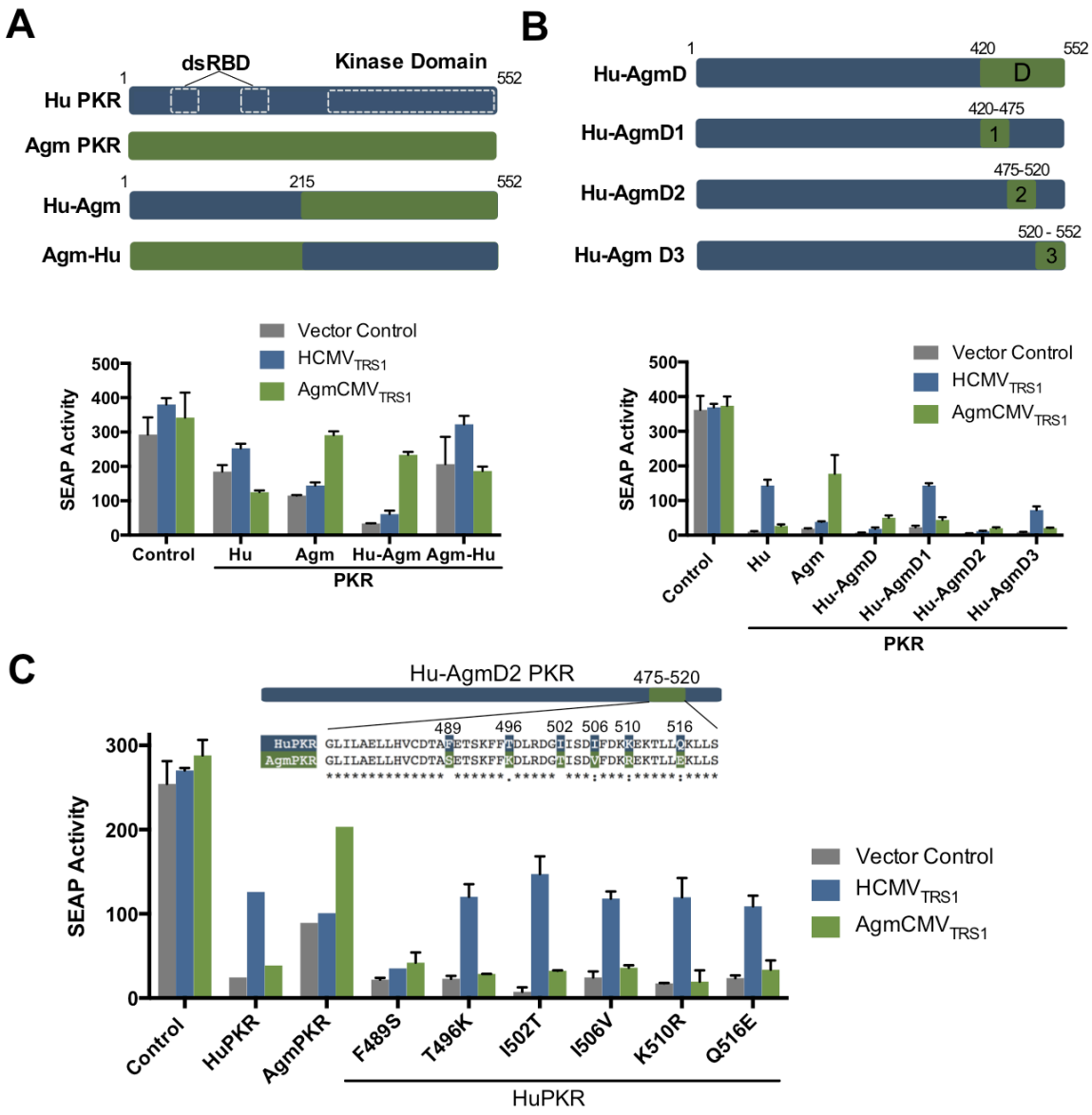

Figure 4.1 Mapping of the molecular determinant of sensitivity of HuPKR to HCMVTRS1. (A) PKR resistance maps to the kinase domain. Chimeras generated between human PKR and AgmPKR are shown. HeLa PKR KO cells were co-transfected with a SEAP reporter plasmid along with either a control vector or PKR variants and the indicated TRS1 alleles or a vector control. At 48 h post transfection, SEAP activity in the medium was measured (mean ± s.d.). In this experiment, all PKR constructs contained C-terminal epitope tags, which were not used in other experiments as we discovered that the tags attenuated the inhibitory effect of PKR. (B) Resistance to HCMV$_{TRS1}$ maps to the D2 region, codons 475-520, of Agm PKR. The kinase domain was subdivided to create additional chimeras, which were evaluated as described in (A) (mean ± s.d.). (C) A single amino acid change, F489S, confers resistance to HCMV$_{TRS1}$. Point mutants were generated in HuPKR to introduce the six AgmPKR-specific residues that differ between HuPKR and AgmPKR within the region spanning codons 475 to 520. The ability of the point mutants to antagonizing HuPKR were evaluated as described in (A).

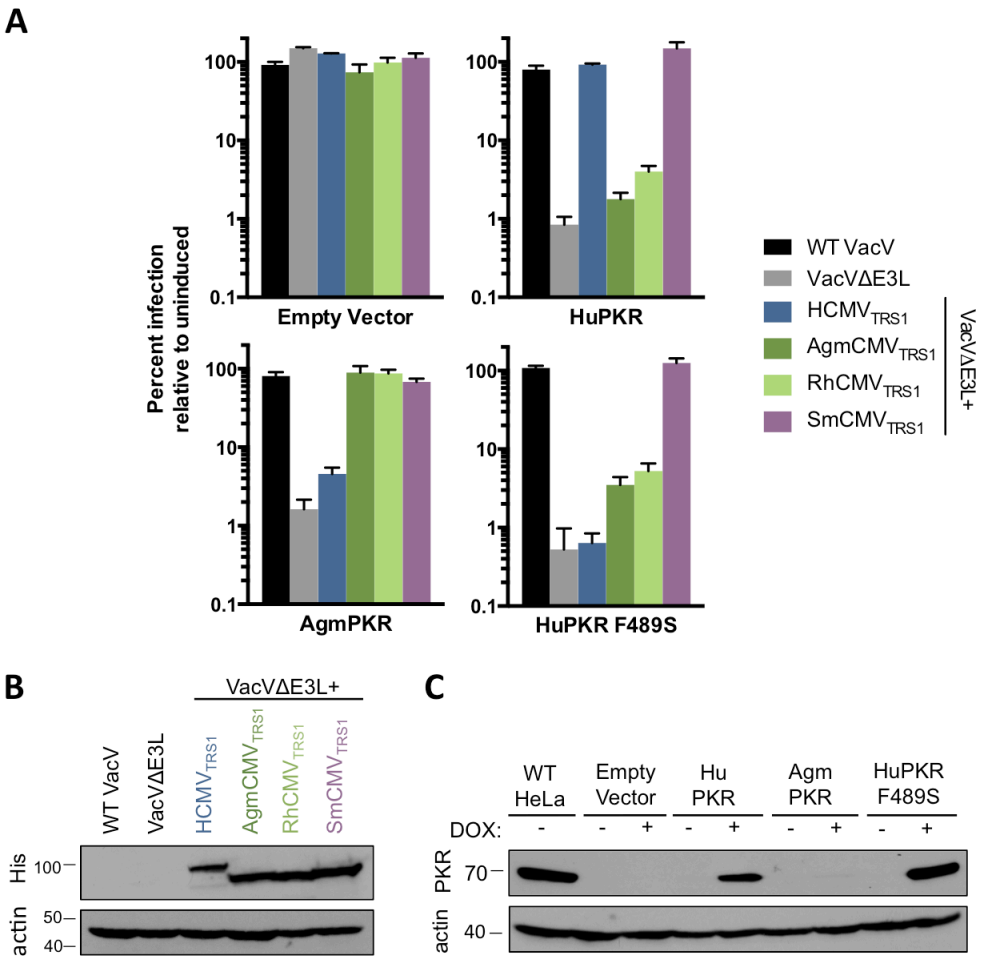

Figure 4.2 HuPKR F489S confers resistance in the context of viral replication.
(A) Triplicate wells of HeLa PKR KO cells inducibly expressing the indicated PKR variants were treated +/- doxycycline and infected (MOI 0.1) with a panel of VacVs. At 48hpi, viral replication was quantified by measuring β-gal activity and is reported as percent replication in doxycycline treated cells relative to replication in the same cells without induction of PKR expression (mean ± s.d.). (B) His-tagged TRS1 constructs were detected in lysates of the infected empty vector cells from (A) by western blotting. TRS1 size variation is expected based on differences in coding length. (C) PKR expression in lysates of mock-infected cells from (A) was evaluated by western blotting. *AgmPKR does not cross react with the antibody used.

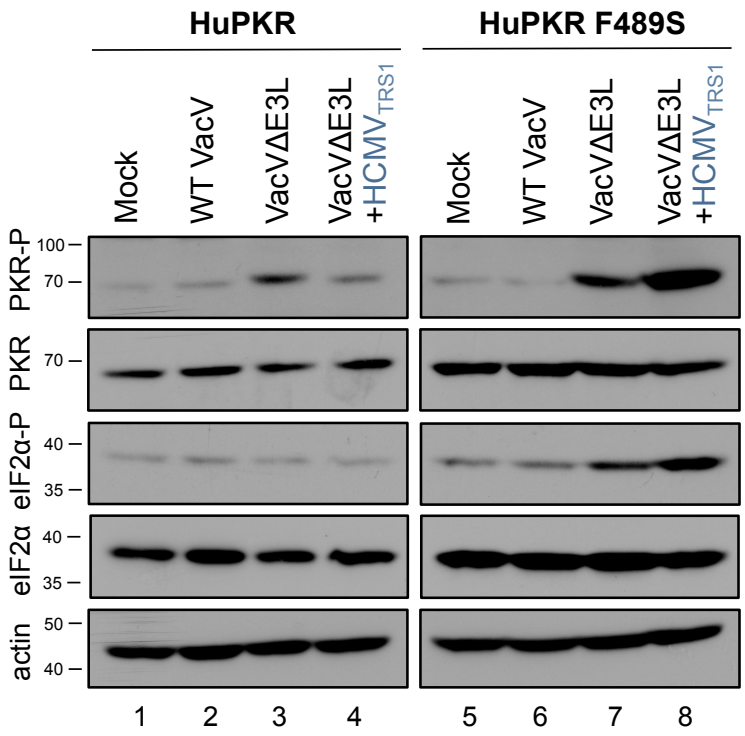

Figure 4.3 The PKR pathway is activated in HuPKR F489S cells after infection with VacVΔE3L+HCMVTRS1.

HeLa PKR KO cells with stably integrated HuPKR or HuPKR F489S were induced with doxycycline to express PKR and 24 hours later mock-infected or infected at an MOI of 3 with the indicated viruses. At 6 hpi, cells were lysed and levels of PKR-P, total PKR, eIF2α-P and actin were evaluated by Western blotting.

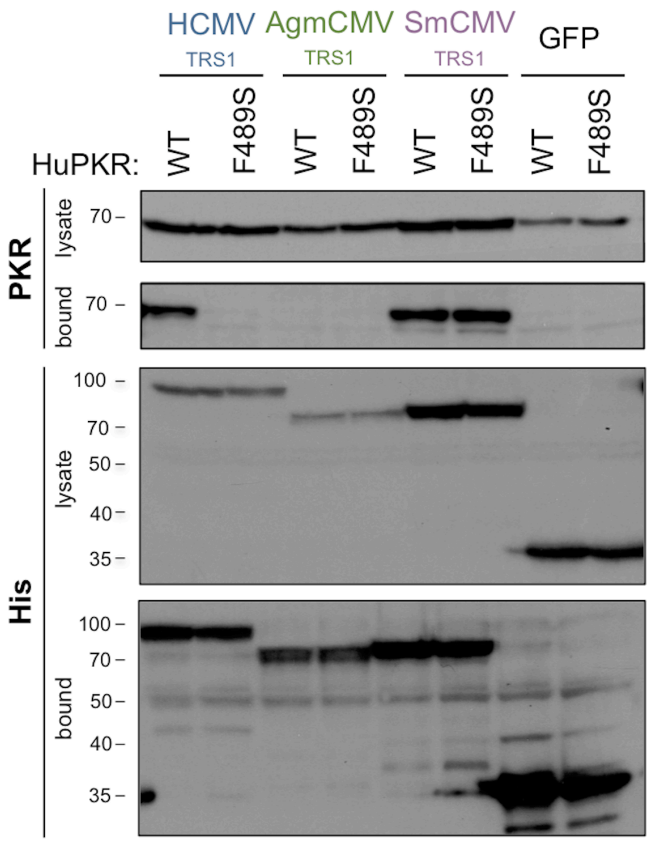

Figure 4.4 The F489S mutation eliminates HCMVTRS1 binding to PKR.
HeLa PKR KO cells were co-transfected with WT HuPKR or HuPKR F489S and either His-tagged $HCMV_{TRS1}$, $AgmCMV_{TRS1}$, $SmCMV_{TRS1}$ or EGFP. At 48h post transfection, lysates were prepared and incubated with nickel-agarose beads. Cell lysates and bound proteins were analyzed by Western blotting, probing for His-tagged TRS1 proteins and for PKR.

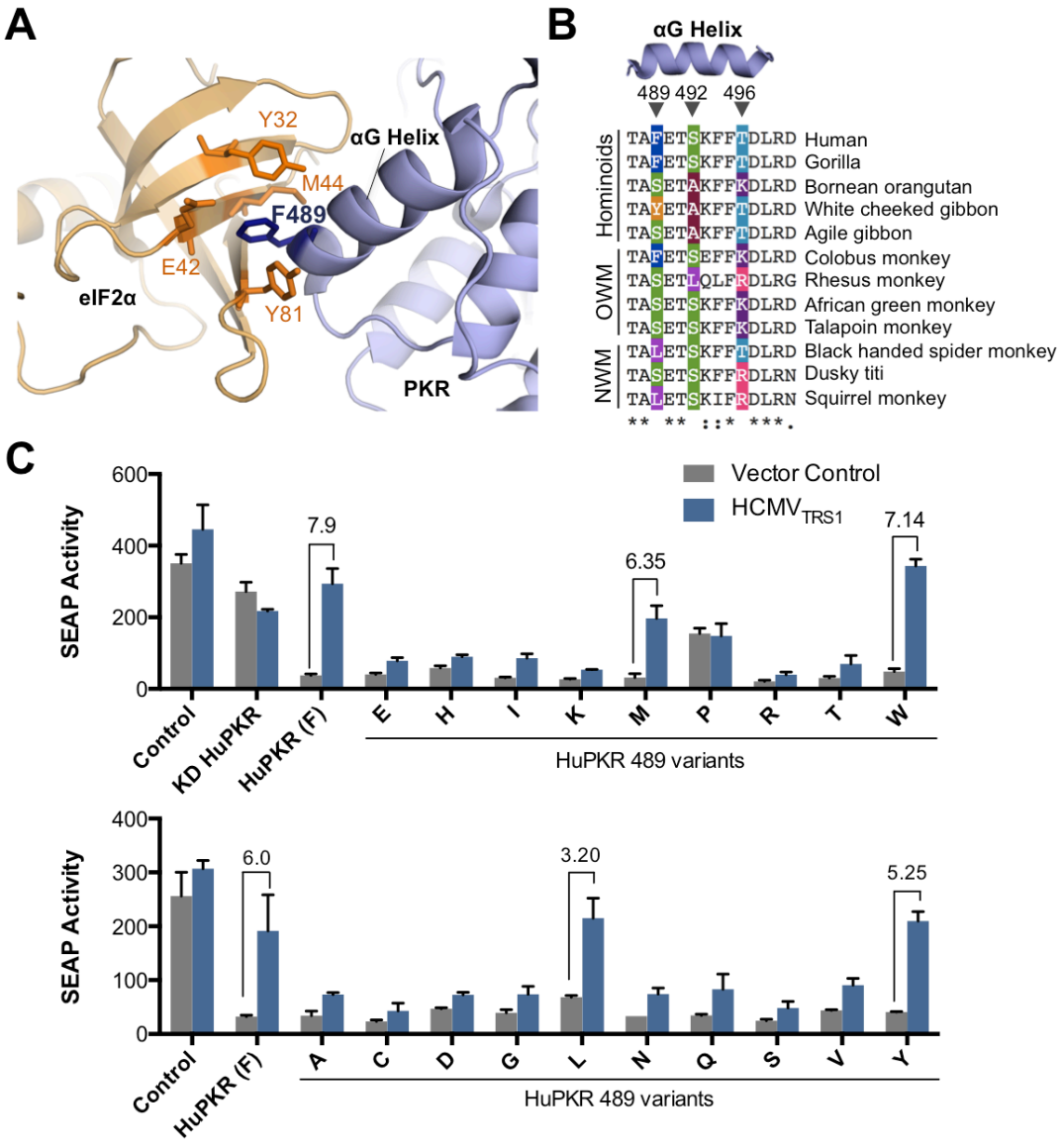

Figure 4.5 Position 489 of HuPKR is highly tolerant of amino acid substitutions.
(A) Position 489 (dark blue) falls within the αG helix of PKR (light blue) and projects into a hydrophobic pocket of eIF2α (pale orange) composed of the side chains of Y32, E42, M44 and Y81 (dark orange). (B) Position 489 is highly variable in primates. The protein sequence alignment of the αG helix of PKR from representative primates is shown. Amino acids previously found to be evolving under positive selection among primates [86] are indicated with arrowheads. (C) Most mutations at position 489 retain PKR activity but only a subset are inhibited by HCMVTRS1. HuPKR 489 variants were evaluated as described in Fig 3.2B (mean ± s.d., n=2). The fold change relative to a vector control is indicated for amino acids that are most sensitive to inhibition by HCMVTRS1. KD PKR is a mutant of PKR (K296R) that lacks kinase activity.

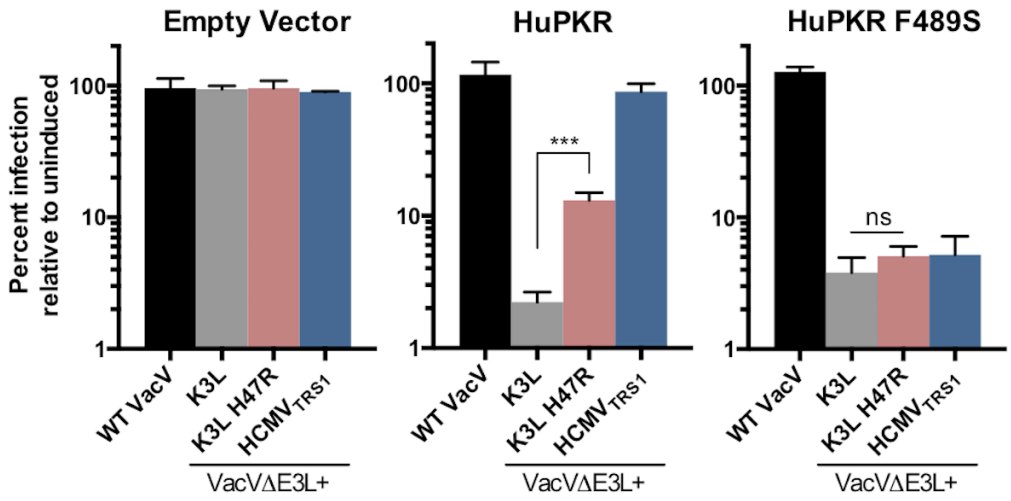

Figure 4.6 HuPKR F489S confers resistance to VacV K3L H47R.
Triplicate wells of HeLa PKR KO cells inducibly expressing the indicated PKR variants were infected and evaluated as described in Fig 4.2A. (mean ± s.d.).

Chapter 5. Materials and Methods

This chapter is adapted from an article originally published in PLoS Pathogens:

Carpentier KS, Esparo NM, Child SJ, Geballe AP. 2016
A Single Amino Acid Dictates Protein Kinase R Susceptibility to Unrelated Viral Antagonists.
PLoS Pathogens. 12(10): e1005966. doi:10.1371/journal.ppat.1005966

Primers.

All primers used to construct plasmids are listed in Table 5.1.

SEAP reporter assay and plasmids

The SEAP assay was carried out as described previously [94], except that 0.05 μg of SEAP reporter was transfected along with a given PKR and TRS1 construct at a ratio of 1:2, respectively.

The SEAP expression vector (pEQ886) has been described previously [110]. A plasmid expressing EGFP with a 6x-His tag (pEQ1100) was used as a the control and vector control in SEAP assays and has been described previously [93]. $HCMV_{TRS1}$ was expressed from pEQ1180 (formerly 981) [65]. $AgmCMV_{TRS1}$ was PCR amplified from AgmCMV DNA (ATTC VR-706) using primers 931 and 932 and TOPO cloned into pcDNA3.1V5-6xHis (ThermoFicher Scientific) to yield pEQ1377. $RhCMV_{TRS1}$ was digested from pEQ1215 [83] with HindIII and NotI and ligated into pEQ1180 that had been cut with the same enzymes to yield pEQ1261. Because we were unsuccessful in PCR ampligfying $SmCMV_{TRS1}$ from viral DNA, possibly due to its very high GC content, we synthesized a mammalian codon-optimized form of $SmCMV_{TRS1}$ flanked by the MCS from pcDNA3.1v5-His to facilitate later cloning (GenScript Inc.; GenBank accession number KX518569). This synthesized construct was inserted into pUC57 using EcoRI

and HindIII to produce pEQ1494. $SmCMV_{TRS1}$ was removed from pEQ1494 using Asp718 and NotI and ligated into pEQ1377 cut with the same enzymes, resulting in pEQ1495, which was used in SEAP experiments.

A knockdown resistant form of HuPKR (SR#329) generously provided by Stefan Rothenburg (Kansas State University) [87] was used for HuPKR expression in Fig 3.2B and Fig 4.5C. To generate a construct expressing active HuPKR with a 6xHis tag, pSB819+HuPKR [86] was digested with EcoRI to yield a PKR fragment that was then ligated into the same sites in pEQ1198 [83], resulting in pEQ1356. However, PKR expression from pEQ1356 was insufficient to repress SEAP expression in the reporter assay, so HuPKR with a 6xHis tag was then PCR amplified from pEQ1356 using primers 2058 and 2059, and cloned into SR#329 cut with KpnI and HindIII using Gibson Assembly (NEB), resulting in pEQ1563. Similarily, AgmPKR was isolated from pSB819+AgmPKR, which has been described previously [86], and introduced into a pcDNA3.1v5-His backbone that also contained a biotinylation signal between XhoI and XbaI, resulting in pEQ1357. AgmPKR was PCR amplified from pEQ1357 using primers 2058 and 2059 and introduced into SR#329 that had been digested with KpnI and HindIII using Gibson assembly (NEB) to produce pEQ1564. pEQ1563 and pEQ1564 were used in S1A Fig.

To generate chimeras between HuPKR and AgmPKR, the N-terminal half of HuPKR or AgmPKR was amplified from pEQ1356 and pEQ1357, respectively, using primers 2033 and 2036, while the C-terminal half of each PKR was amplified using primers 2034 and 2035. The N-terminal and C-terminal fragments were then cloned into pEQ1357 digested with BamHI and EcoRV using Gibson Assembly (NEB) to yield a Hu-Agm chimera (pEQ1555) and an Agm-Hu chimera (pEQ1556) in the pcDNA3.1v5-His backbone. The two his-tagged chimeras were then PCR-amplified from these constructs using primers 2058 and 2059 and inserted into SR#329

digested with KpnI and HindIII using Gibson Assembly (NEB), resulting in pEQ1571 (Hu-Agm PKR-6xHis) and pEQ1572 (Agm-Hu PKR-6xHis), which were used in S2A Fig. Constructs without tags were generated as follows: HuPKR or AgmPKR were PCR amplified from pEQ1356 and pEQ1357 using primers 2102 and 2104 or 2102 and 2103, respectively. The PCR products were then introduced into SR#329 cut with KpnI and HindIII by Gibson Assembly (NEB) to yield pEQ1602 (HuPKR) and pEQ1598 (AgmPKR), which were used in Fig 4.1B and Fig 4.1C.

To generate the Hu-AgmD chimera, the N-terminal portion of HuPKR was PCR amplified from pEQ1356 using primers 2102 and 2085, while the AgmD fragment was amplified from pEQ1357 using primers 2084 and 2103. These fragments were cloned into SR#329 using Gibson assembly, resulting in pEQ1600. The Hu-AgmD1, D2 and D3 chimeras were first constructed with 6xHis tags by PCR amplifying the various regions of HuPKR and AgmPKR as follows. Hu-AgmD1: pEQ1356 was PCR-amplified using primer pairs 2058, 2085 and 2098, 2059; pEQ1357 was PCR amplified using primers 2084, 2099. Hu-AgmD2: pEQ1356 was PCR-amplified using primer pairs 2058, 2099 and 2100, 2059; pEQ1357 was PCR amplified using primers 2098, 2101. Hu-AgmD3: pEQ1356 was PCR amplified using primers 2058, 2101; pEQ1357 was PCR amplified using primers 2100, 2059. The fragments were then cloned into SR#329 using Gibson Assembly, resulting in pEQ1595 (Hu-AgmD1-His), pEQ1596 (Hu-AgmD2-His), and pEQ1597 (Hu-AgmD3-His). To remove the 6xHis tags, the chimeras were PCR-amplified using the following primers: 2102, 2104 (pEQ1595 and pEQ1596) or 2102, 2103 (pEQ1597). The PCR products were cloned into SR#329 to generate pEQ1603 (Hu-AgmD1), pEQ1604 (Hu-AgmD2) and pEQ1599 (Hu-AgmD3), which were used in S1B Fig.

Point mutations in HuPKR were constructed by using complementary forward and reverse

primers harboring the desired mutation paired with primers at the beginning or end of PKR, as shown in Table 5.2. pEQ1356 was used as a template for PCR amplification and the resulting N and C terminal fragments containing the desired mutations were cloned into SR#329 cut with KpnI and HindIII of using Gibson Assembly.

VacV recombinant viruses and infections

WT VacV (VC2+lacZ), VacVΔE3L, VacVΔE3L+$HCMV_{TRS1}$ (VVeq1148), and VacVΔE3L+$RhCMV_{TRS1}$ (VVeq1233) have been described previously [83]. To generate VacVΔE3L+$AgmCMV_{TRS1}$, $AgmCMV_{TRS1}$ was first removed from pEQ1377 (see SEAP plasmids, above) using Asp718 and NotI and ligated into pEQ1233[83], that had been digested with the same enzymes, resulting in pEQ1453. pEQ1453 was then introduced into the thymidine kinase locus in VacVΔE3L through homologous recombination to generate VacVΔE3L+$AgmCMV_{TRS1}$ (VVeq1453). Similarly, $SmCMV_{TRS1}$ was cut from pEQ1495 (see SEAP plasmids) with Asp718 and NotI and inserted into the same sites of pEQ1453 to produce pEQ1497, which was recombined into VacVΔE3L to produce VacVΔE3L+$SmCMV_{TRS1}$ (VVeq1497). Recombinant VacVs were propagated and titered in BHK cells.

In all viral replication experiments, cells were infected at an MOI of 0.1 for 1 hour, after which the medium was replaced. Viral replication was evaluated at 48hpi by measuring β-Galactosidase (β-Gal) activity via a fluorometric substrate cleavage assay, as described previously (Child et al, 2006). With the stable cell lines expressing PKR in an inducible manner (Fig 4.2A and Fig 4.6), cells were treated with medium containing 1 μg/ml of doxycycline 24

hours prior to infection, infected in the absence of doxycycline, and refed with medium containing 1ug/ml of doxycycline after the hour-long infection period.

To evaluate the status of PKR phosphorylation during infection, cells were treated with medium containing 1 μg/ml of doxycycline for 24 hours and then infected at an MOI of 3 for 1 hour, after which the cells were re-fed with medium containing 1 μg/ml of doxycycline. Cell lysates were harvested in 2% SDS at 6hpi.

Cell Lines

HeLa, BSC40, and HeLa PKR KO cell lines were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% NuSerum (BD Biosciences). HeLa cells were provided by Bertram Jacobs (Arizona State University) and BSC40 cells were provided by Stanley Riddell (Fred Hutchinson Cancer Research Center, Seattle, WA).

HeLa PKR KO cell lines were generated by transfecting HeLa cells with plasmid vectors that express Cas9 (HCas9, a gift from George Church, Addgene #41815 (REF)), two guide RNAs (pEQ 1451 and pEQ1452) that target genomic sequences upstream (5'-TCTCTTCCATTGTAGGATA-3') and downstream (5'-CTTTTCTTCCACACAGTCA-3') of PKR, and a homologous recombination vector containing mCherry and puromycin (pEQ1489). After puromycin selection, single-cell clones were evaluated by sequencing and immunoblotting to identify clean knockouts; Clone #6 was used in these studies.

To generate stable cell lines expressing different PKR constructs in the HeLa PKR KO cell line, we first cloned each PKR into the pSLIK-Hygro lentiviral vector (a gift from Iain Fraser,

Addgene plasmid # 25737 [111]). HuPKR was PCR amplified from pEQ1356 using primers 2105 and 2106 and inserted into pEN_TmiRc3 Entry Vector (a gift from Iain Fraser, Addgene plasmid # 25748 [111]) at sties SpeI and XbaI using Gibson Assembly (NEB) to produce pEQ1606. HuPKR was then moved from pEQ1606 into the pSLIK-Hygro destination vector using Gateway Cloning (Thermo Fisher Scientific), resulting in pEQ1607. To move AgmPKR into this vector, AgmPKR was PCR amplified from pEQ1357 using primers 2175 and 2176, and inserted into pEQ1607 that had been digested with BstEII using Gibson Cloning (NEB), yielding pEQ1641. Similarly, HuPKR F489S was PCR-amplified from pEQ1624 using primers 2173 and 2174 and introduced into pEQ1607 that had been digested with BstEII using Gibson Assembly, resulting in pEQ1642. HeLa PKR KO cells were transduced with lentiviral vectors encoding pEQ1607, pEQ1641, pEQ1642, and pSLIK-Hygro (empty vector). After hygromycin selection, single cell clones were evaluated for their ability to restrict VacVΔE3L and for PKR expression levels, with the following clones used in experiments: HeLa PKR KO+1607#9, 1641#3 and 1642#2.

Immunoblot Assays

Cell lysates were harvested in 2% sodium dodecyl sulfate (SDS) and equal amounts of lysates were separated on a 10% SDS-polyacrylamide gel and transferred to polyvinylide difluoride (PVDF) membranes. Proteins were detected using the Western-Star™ chemiluminescent detection system (Applied Biosystems) with the following primary antibodies: PKR D7F7 (Cell Signaling Technology), P-PKR E120 (ab32036, abcam), Penta-His (34660, Qiagen), and Actin (A2066, Sigma).

Pulldown assays.

HeLa PKR KO cells were transfected using Lipofectamine 2000 (Invitrogen) with 0.15 µg of HuPKR (pEQ1602) or HuPKR F489S (pEQ1624) and 2.35µg of $HCMV_{TRS1}$ (pEQ1180), $AgmCMV_{TRS1}$ (pEQ1377), $SmCMV_{TRS1}$ (pEQ1495) or EGFP (pEQ1100). At 48 hours post-transfection, cells were washed with PBS and harvested in 250µl of cold NiNTA Lysis Buffer (50mM NaH2PO4, 300mM NaCl, 10mM imidazole, 0.75% Tween 20, 1µM benzamidine and 100 µM PMSF). Lysates were incubated on ice for 20 minutes with occasional vortexing and centrifuged at 16,000xg for 10 min at 4°C to remove cell debris. 20 µl of each lysate was reserved, while 200 µl of the remaining lysate was added to 30 µl of PerfectPro NiNTA Superflow (5prime) agarose and incubated at 4°C on a rotating mixer for 2 hours. After binding, the beads were pelleted and washed 3x with 500µl of NiNTA Wash buffer (50mM NaH2PO4, 300mM NaCl, 20mM imidazole, 0.75% Tween 20). The lysate and bound samples were denatured in SDS-PAGE sample buffer at 95°C (5 minutes) and separated on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane and probed with PKR D7F7 (Cell Signaling Technology) and Penta-His (Qiagen) antibodies.

Structure observations and protein alignments.

The structure of PKR in complex with eIF2α was visualized using data from the protein databank (http://www.pdb.org; ID 2A1A) and MacPyMol. For sequence comparisons of the αG helix among primate PKRs, the following sequences were obtained from NCBI and aligned using Clustal Omega: Homo sapiens (human; BC093676), Gorilla gorilla (Gorilla; EU733258), Pongo pygmaeus pygmaeus (Bornean orangutan; EU733259), Nomascus leucogenys (northern white-cheeked gibbon; EU733257), Hylobates agilis albibarbis (Agile gibbon; EU733270), Colobus

guereza (Colobus monkey; EU733267), Macaca mulatta (Rhesus monkey; EU733261), Cercopithecus aethiops (African green monkey; EU733254), Miopithecus talapoin talapoin (Talapoin monkey; EU733269), Ateles geoffroyi (Black-handed spider monkey; EU733263), Callicebus moloch (Dusky titi; EU733265), Saimiri boliviensis boliviensis (Squirrel monkey, XM_003926814).

Nucleotide sequence accession numbers

The sequence of the mammalian codon-optimized $SmCMV_{TRS1}$ has been deposited in GenBank under the following accession number: KX518569.

Table 5.1. List of primers used in materials and methods.

Primer number	Sequence (5' to 3')
931	ACCACCATGACCGGCCGCAGACCTCCGCGC
932	ACAGGGAATGACGAGCACCCCGTCGCTGCT
2033	GTT AAG CTT GGT ACC GAG CTC G
2034	GGC CGC CAC TGT GCT GGA T
2035	GCA GAT ACA TCA GAG ATA AAT TCT AAC AG
2036	CTG TTA GAA TTT ATC TCT GAT GTA TCT GC
2058	AAT TCG AGC TCG GTA CGC TAG TTA AGC TTG GTA CC
2059	TAA TAA GAT CTC TCG AGA GGG TTT AAA CTC AAT GGT G
2084	CAT AGA GAT CTT AAG CCA AGT AAT ATA TTC
2085	GAA TAT ATT ACT TGG CTT AAG ATC TCT ATG
2098	GAA GTG GAC CTC TAC GCT TTG G
2099	CCA AAG CGT AGA GGT CCA CTT C
2100	AGA AAT TAC TCT CAA AGA AAC CTG AGG
2101	CCT CAG GTT TCT TTG AGA GTA ATT TCT
2102	AAT TCG AGC TCG GTA CCA TGG CTG GTG ATC TT
2103	TAA TAA GAT CTC TCG AGA AGC TTC TAA CAT GTA TGT CGT TCC TTT TT
2104	TAA TAA GAT CTC TCG AGA AGC TTC TAA CAT GTG TGT CGT TCA TT
2105	CCT AGG CGT CTG ATC ACT AGT GGT AAC CAT GGC TGG TGA TCT TTC AG
2106	GTA CAA GAA AGC TGG GTC TAG AGG TGA CCC TAA CAT GTG TGT CGT TCA TT
2128	ATG TGA CAC TGC TTC CGA AAC ATC AAA GTT
2129	AAC TTT GAT GTT TCG GAA GCA GTG TCA CAT
2130	ATC AAA GTT TTT CAA AGA CCT ACG GGA TG
2131	CAT CCC GTA GGT CTT TGA AAA ACT TTG AT
2132	TAC GGG ATG GCA CCA TCT CAG ATA TAT
2133	ATA TAT CTG AGA TGG TGC CAT CCC GTA
2134	GCA TCA TCT CAG ATG TGT TTG ATA AAA AAG A
2135	TCT TTT TTA TCA AAC ACA TCT GAG ATG ATG C
2136	TAT ATT TGA TAA AAG AGA AAA AAC TCT TC
2137	GAA GAG TTT TTT CTC TTT TAT CAA ATA TA
2138	GAA AAA ACT CTT CTA GAG AAA TTA CTC TCA
2139	TGA GAG TAA TTT CTC TAG AAG AGT TTT TTC

2167	ATG TGA CAC TGC TTA TGA AAC ATC AAA GTT
2168	AAC TTT GAT GTT TCA TAA GCA GTG TCA CAT
2169	ATG TGA CAC TGC TTT GGA AAC ATC AAA GTT
2170	AAC TTT GAT GTT TCC AAA GCA GTG TCA CAT
2173	CGT CTG ATC ACT AGT GGT AAC CAT GGC TGG TGA TCT TTC AG
2174	AAA GCT GGG TCT AGA GGT CAC CCT AAC ATG TGT GTC GTT C
2175	CGT CTG ATC ACT AGT GGT AAC CAT GGC TGG TGA TCT TGC AC
2176	AAA GCT GGG TCT AGA GGT CAC CCT AAC ATG TAT GTC GTT C
2198	ATG TGA CAC TGC TAT CGA AAC ATC AAA GTT
2199	AAC TTT GAT GTT TCG ATA GCA GTG TCA CAT
2200	AGA CTT ACG TTA TTA GAC GTG TTA AAT ATA A
2201	TTA TAT TTA ACA CGT CTA ATA ACG TAA GTC T
2206	GTA TGT GAC ACT GCT TGG GAA ACA TCA AAG TTT
2207	AAA CTT TGA TGT TTC CCA AGC AGT GTC ACA TAC
2208	GTA TGT GAC ACT GCT GAG GAA ACA TCA AAG TTT
2209	AAA CTT TGA TGT TTC CTC AGC AGT GTC ACA TAC
2210	GTA TGT GAC ACT GCT CAC GAA ACA TCA AAG TTT
2211	AAA CTT TGA TGT TTC GTG AGC AGT GTC ACA TAC
2212	GTA TGT GAC ACT GCT ATG GAA ACA TCA AAG TTT
2213	AAA CTT TGA TGT TTC CAT AGC AGT GTC ACA TAC
2214	GTA TGT GAC ACT GCT CCC GAA ACA TCA AAG TTT
2215	AAA CTT TGA TGT TTC GGG AGC AGT GTC ACA TAC
2216	GTA TGT GAC ACT GCT CGG GAA ACA TCA AAG TTT
2217	AAA CTT TGA TGT TTC CCG AGC AGT GTC ACA TAC
2218	GTA TGT GAC ACT GCT AAG GAA ACA TCA AAG TTT
2219	AAA CTT TGA TGT TTC CTT AGC AGT GTC ACA TAC
2220	GTA TGT GAC ACT GCT GAC GAA ACA TCA AAG TTT
2221	AAA CTT TGA TGT TTC GTC AGC AGT GTC ACA TAC
2222	GTA TGT GAC ACT GCT AAC GAA ACA TCA AAG TTT
2223	AAA CTT TGA TGT TTC GTT AGC AGT GTC ACA TAC
2224	GTA TGT GAC ACT GCT CAG GAA ACA TCA AAG TTT
2225	AAA CTT TGA TGT TTC CTG AGC AGT GTC ACA TAC
2226	GTA TGT GAC ACT GCT TGC GAA ACA TCA AAG TTT
2227	AAA CTT TGA TGT TTC GCA AGC AGT GTC ACA TAC

2228	GTA TGT GAC ACT GCT GGC GAA ACA TCA AAG TTT
2229	AAA CTT TGA TGT TTC GCC AGC AGT GTC ACA TAC
2230	GTA TGT GAC ACT GCT GCC GAA ACA TCA AAG TTT
2231	AAA CTT TGA TGT TTC GGC AGC AGT GTC ACA TAC
2232	GTA TGT GAC ACT GCT GTG GAA ACA TCA AAG TTT
2233	AAA CTT TGA TGT TTC CAC AGC AGT GTC ACA TAC
2234	GTA TGT GAC ACT GCT ACC GAA ACA TCA AAG TTT
2235	AAA CTT TGA TGT TTC GGT AGC AGT GTC ACA TAC

Table 5.2. Sets of primers used to construct point mutants.

HuPKR Mutation	pEQ number	N-terminal fragment primers	C-terminal fragment primers
K296R	1645	2102, 2201	2104, 2200
F489S	1624	2102, 2129	2104, 2128
T496K	1625	2102, 2131	2104, 2130
I502T	1626	2102, 2133	2104, 2132
I506V	1627	2102, 2135	2104, 2134
K510R	1628	2102, 2137	2104, 2136
Q516E	1629	2102, 2139	2104, 2138
F489Y	1633	2102, 2168	2104, 2167
F489L	1631	2102, 2170	2104, 2169
F489I	1646	2102, 2199	2104, 2198
F489E	1647	2102, 2209	2104, 2208
F489W	1648	2102, 2207	2104, 2206
F489H	1649	2102, 2211	2104, 2210
F489M	1650	2102, 2213	2104, 2212
F489P	1651	2102, 2215	2104, 2214
F489T	1652	2102, 2235	2104, 2234
F489R	1655	2102, 2217	2104, 2216
F489K	1656	2102, 2219	2104, 2218
F489D	1657	2102, 2221	2104, 2220
F489Q	1658	2102, 2225	2104, 2224
F489A	1659	2102, 2231	2104, 2230
F489G	1662	2102, 2229	2104, 2228
F489V	1663	2102, 2233	2104, 2232
F489N	1666	2102, 2223	2104, 2222

Chapter 6. Conclusions and Future Directions

A portion of this chapter is adapted from an article originally published in the Journal of Virology:

Carpentier KS, **Geballe AP**. 2016.
An Evolutionary View of the Arms Race between Protein Kinase R and Large DNA Viruses. *Journal of Virology* **90**:3280–3283.

Conclusions

In order to productively replicate, viruses encode antagonists to overcome intrinsic host antiviral defenses. The selective pressure imposed by viral antagonists initiates ongoing arms races that drive reciprocal innovation within the conflicting proteins. Over evolutionary time, this ultimately leads to the divergence of host restriction factors in different species, as exemplified by the antiviral factor PKR. The broad antiviral activity of PKR has made it vulnerable to multiple viral antagonists, and consistent with this, rapidly evolving codons are scattered throughout the protein [86,87]. The divergence of PKR in primates has established differential susceptibilities to viral antagonists[83,86,87].

In this dissertation, we leveraged the species-specificity of CMVs to evaluate how the rapid evolution of PKR has impacted the evolution of the CMV PKR antagonist, TRS1. In Chapter 3, we provide evidence for a co-evolutionary history between PKR and TRS1, as both $HCMV_{TRS1}$ and $AgmCMV_{TRS1}$ have specifically adapted to overcome the PKR variant of the host they naturally infect. However, this specific adaptation may also contribute to cross species barriers to CMV transmission as neither of these antagonists function against more distantly related PKR variants. Moreover, we find that the CMV TRS1 proteins have evolved different mechanisms of PKR antagonism, as some function upstream of PKR phosphorylation while

others act at a downstream step. Thus, host-viral arms race has had a significant impact on the evolution of CMV TRS1 proteins.

In Chapter 4, we evaluated the molecular basis for the differential sensitivity of HuPKR and AgmPKR to antagonism by $HCMV_{TRS1}$. By leveraging the differential susceptibilities of these PKR variants, we were able to readily and precisely map a molecular determinant of PKR susceptibility to $HCMV_{TRS1,}$ as mutating position 489 of HuPKR to the residue present in AgmPKR (F489S) was sufficient to confer resistance. This single amino acid change disrupted the interaction between PKR and $HCMV_{TRS1}$, thus preventing $HCMV_{TRS1}$ from antagonizing PKR. Notably, this amino acid falls within the αG helix of PKR, which mediates the interaction between PKR and its downstream substrate, eIF2α. Despite the critical role of this helix, we find that position 489 is highly tolerant of amino acid substitutions, consistent with it being engaged in a host-viral arms race. In fact, position 489 dictates the susceptibility of PKR to not only $HCMV_{TRS1}$, but also to the unrelated poxvirus antagonist K3L. These results highlight the complexity of the host viral arms race in broadly acting factors like PKR, as selective pressure imposed by one virus has the potential to drive changes in the host that may impact host susceptibility to an unrelated viral antagonist.

Future Directions

While our work has advanced our understanding of how the rapid evolution of PKR has impacted the evolution of CMV TRS1 proteins, it has also exposed additional questions.

Does HuPKR F489S restrict HCMV replication?

In Chapter 4, we identified HuPKR F489S as a PKR variant resistant to $HCMV_{TRS1}$ activity. In our reporter based SEAP assay, $HCMV_{TRS1}$ was unable to rescue PKR mediated translational repression in the presence of HuPKR F489S. Furthermore, VacVΔE3L+$HCMV_{TRS1}$ was unable to replicate in HuPKR F489S HeLa cells upon induction of PKR expression. This inability to replicate correlated with robust activation of the PKR pathway, demonstrating that even in the context of VacV infection, $HCMV_{TRS1}$ is unable to antagonize HuPKR F489S. Finally, we showed that mutating position 489 of HuPKR from phenylalanine to serine disrupted the interaction between $HCMV_{TRS1}$ and PKR. As previous work has shown that PKR binding is essential for $HCMV_{TRS1}$ to antagonize PKR [64,66], this disrupted interaction likely explains why $HCMV_{TRS1}$ is ineffective against HuPKR F489S in the above described assays.

Because PKR antagonism is essential for HCMV replication [24,64], our findings suggest that HCMV would be unable to replicate in cells expressing HuPKR F489S. However, we have not formally tested this prediction. As HCMV does not replicate in HeLa cells, an HCMV permissive cell line expressing HuPKR F489S is necessary to test this. Therefore, I stably introduced HuPKR, AgmPKR, or HuPKR F489S into HF PKR KO cells [64]. Upon doxycycline induction, both the HuPKR and HuPKR F489S cell lines expressed PKR, although the expression of HuPKR F489S was noticeably lower (Fig 6.1). As a preliminary experiment, I then evaluated the replication of HCMV[TRS1-HA] in these cells in the presence or absence of PKR induction. HCMV[TRS1-HA] replicated to similar levels regardless of PKR induction in the HuPKR cell line, but exhibited a 7-fold decrease in replication in cells expressing HuPKR F489S (Fig 6.2). While these results were promising, they did not reproduce in one additional experiment, possibly due to silencing of of the PKR transgene in the bulk population. To

circumvent this problem, I began isolating clonal cells from the bulk populations in an effort to generate a cell line that expressed high levels of PKR, as I had done with the HeLa cells. However, because HFs are primary cells, they are not as amenable to cloning and generating clonal lines has proven difficult. Additional work is underway to generate clonal HFs expressing HuPKR F489S to evaluate whether HCMV replication is inhibited in these cells.

How do other TRS1 proteins target PKR?

Residue 489 of HuPKR is an important species-specific determinant of the activity of $HCMV_{TRS1}$. However, rapid evolution of PKR in primates has led to divergence of CMV TRS1 proteins, likely resulting in species-specific differences in the interactions between primate CMV TRS1 proteins and their cognate host PKR variants. In support of this, mutating position 489 of HuPKR has no effect on the ability of $SmCMV_{TRS1}$ to bind and antagonize PKR (Fig 4.2A and Fig 4.4). Moreover, despite the fact that AgmPKR encodes a serine at position 489, introducing this residue into HuPKR (F489S) is not sufficient for $AgmCMV_{TRS1}$ or $RhCMV_{TRS1}$ to recognize and antagonize PKR (Fig 4.2A). This result suggests that either position 489 does not mediate the interaction between AgmPKR and the OWM PKR antagonists, or that additional points of contact are additionally required.

To test whether or not position 489 was necessary for PKR antagonism by $AgmCMV_{TRS1}$ and $RhCMV_{TRS1}$, I generated a stable HeLa PKR KO cell line expressing AgmPKR S489F (the reciprocal mutant relative to HuPKR F489S). As expected, WT VacV replicated well in this cell line while VacVΔE3L was restricted, demonstrating that functional PKR was expressed (Fig 6.3). Introducing the phenylalanine from HuPKR at position 489 of AgmPKR was not sufficient to rescue replication of VacVΔE3L+$HCMV_{TRS1}$, suggesting that additional points of contact are

necessary for $HCMV_{TRS1}$ to bind HuPKR. Interestingly, mutating position 489 had only a modest impact on the replication of viruses expressing $AgmCMV_{TRS1}$ or $RhCMV_{TRS1}$, suggesting that this residue is not as critical for their activity as it is for $HCMV_{TRS1}$. This finding suggests that the PKR:TRS1 interface has diverged in different species.

Surprisingly, $SmCMV_{TRS1}$ was unable to rescue replication of VacVΔE3L in these cells expressing AgmPKR S489F (Fig 6.3). This result was quite unexpected as $SmCMV_{TRS1}$ seems to have more broad activity and functions against both HuPKR and AgmPKR. One potential explanation is that in HuPKR, position 489 is not essential for $SmCMV_{TRS1}$ as it can successfully bind a second point of contact. However, in AgmPKR, this second point of contact is missing, thus making position 489 necessary for $SmCMV_{TRS1}$ to interact with AgmPKR (Fig 6.3). Within the αG helix, there is an additional codon evolving under positive selection that differs between HuPKR and AgmPKR, position 496. It would be interesting to introduce the αG helix from HuPKR into AgmPKR to evaluate whether or not position 496 might mediate the interaction between HuPKR and $SmCMV_{TRS1}$. If position 496 is not sufficient for this interaction, chimeras could be generated between HuPKR and AgmPKR S489F to more precisely map the molecular basis of HuPKR's sensitive to $SmCMV_{TRS1}$ regardless of what is present at position 489. These lines of investigation could improve our understanding of how diverged the PKR:TRS1 interface is among primate CMV TRS1 proteins, further illuminate the role of the PKR αG helix as a site of interaction with diverse viral antagonists, and provide insight into the consequences of host-virus arms races.

Can HCMV TRS1 adapt to overcome HuPKR F489S

The host-virus arms race has driven continual innovation in restriction factors and viral antagonists alike, as reflected by species-specific differences in PKR antagonism. The species-specific functional differences between antagonists can be leveraged to evaluate how viruses might adapt to overcome restriction factors. For example, the VACV PKR antagonist K3L has limited activity against human PKR [86]. To investigate how VACV could adapt to this resistant PKR variant, a VACV expressing K3L as the only PKR antagonist was serially passaged through human cells. Deep sequencing and functional assays of progeny viruses revealed that improved viral fitness resulted from amplification of the K3L locus [105]. This amplification not only augmented K3L expression, but also increased the genomic space available for sampling mutations. In fact, a mutant K3L with improved activity against human PKR emerged, which enabled collapse of the amplified locus. These experiments suggested a "gene-accordion" model to explain how large DNA viruses may rapidly adapt despite their low mutation rates [105]. A similar cycle of gene amplification, mutation, and collapse was observed upon serial passage of a VACV expressing $RhCMV_{TRS1}$ as its sole PKR antagonist in cells containing a relatively resistant PKR allele [112]. In this case, the $RhCMV_{TRS1}$ locus amplified, but subsequent collapse of the locus appeared to be enabled by mutations in other loci affecting genes not previously implicated in PKR pathway antagonism [113].

The strategy of using experimental evolution of a virus in a setting in which it is poorly adapted to overcome a host restriction factor is potentially broadly applicable to studies of other host-virus conflicts. In Chapter four, we identified a single amino acid change that renders HuPKR resistant to $HCMV_{TRS1}$ activity. This finding now provides the opportunity to simulate the host-virus arms race in vitro and evaluate whether $HCMV_{TRS1}$ can adapt to overcome HuPKR

F489S. Serial passage of VacVΔE3L+HCMV$_{TRS1}$ in HeLa cells expressing HuPKR F489S would apply selective pressure to the virus and may drive adaptive changes in HCMV$_{TRS1}$. While adaptation is not guaranteed, in this system HCMV$_{TRS1}$ would only need to adapt to a single amino acid change in PKR and thus may be more feasible. However, it is also possible that the virus will not adapt or that adaptive mutations arise external to TRS1, as was observed in experimental evolution of VacVΔE3L+RhCMV$_{TRS1}$. To circumvent this, an alternative approach would be to generate a library of mutant HCMV$_{TRS1}$ constructs and evaluate their ability to rescue VacVΔE3L in HeLa cells expressing HuPKR F489S. Regardless of the method used, identifying adaptive mutations within HCMV$_{TRS1}$ would be informative as to which residues mediate the interaction with PKR and improve our understanding of how HCMV$_{TRS1}$ functions.

Experimental evolution of VacVΔE3L in human cells uncovered the role of gene amplification as a first step in the "gene-accordion" model. Whether or not other large DNA viruses similarly use amplification as a mechanism of adaptation has not yet been tested. The presence of gene families in CMV suggests it has the capacity to amplify its genome, although in these cases the amplified genes appear to have neofunctionalized, which is an alternative solution relative to adaptive mutations facilitating collapse of the amplified locus. Our identification of a PKR variant resistant to HCMV$_{TRS1}$ may provide a model to evaluate whether HCMV can adapt by gene amplification to overcome host restriction factors. If HF cells expressing HuPKR F489S are restrictive to HCMV replication, HCMV could be serially passaged in these cells and adapted viruses could be evaluated using deep sequencing to identify gene amplifications and adaptive point mutations, which could shed light on whether the accordion model applies to CMV evolution.

What is the mechanism of PKR antagonism used by TRS1?

Many PKR antagonists directly bind to PKR to inhibit its antiviral activity, but the mechanisms used by these antagonists are diverse and range from inhibiting dimerization to impeding eIF2α docking. In this thesis, we identified position 489 within the αG helix of PKR as a critical mediator of the interaction between PKR and $HCMV_{TRS1}$. The identification of a PKR:TRS1 interface allows us to speculate about the mechanism of action of $HCMV_{TRS1}$. Similar to $HCMV_{TRS1}$, the VacV antagonist K3L is known to interact αG helix of PKR. K3L has been extensively studied and is known to both block the auto-phosphorylation of PKR [114] and act as a competitive inhibitor to eIF2α docking [95]. Similarly, $HCMV_{TRS1}$ prevents PKR auto-phosphorylation [83]. These data suggest that interactions at the αG-helix might affect PKR kinase activity, which is not too surprising given the proximity of the αG-helix to the catalytic loop of PKR. Moreover, while $HCMV_{TRS1}$ largely prevents PKR auto-phosphorylation during infection, TRS1 is also capable of binding active PKR, and this interaction might serve as a contingency strategy to inhibit pathway activation by occluding eIF2α docking. Evaluating whether or not pre-incubation of PKR with TRS1 inhibits eIF2α binding would be useful for determining whether $HCMV_{TRS1}$ binding does in fact prevent eIF2α docking, as was observed with K3L.

In addition to PKR binding, $HCMV_{TRS1}$ must also interact with dsRNA to inhibit PKR activation but why this interaction is essential is not fully understood. It is possible that TRS1 functions both by binding PKR and by sequestering dsRNA, and that this combinatorial approach is necessary to fully inhibit pathway activation. However, TRS1 binds dsRNA with a weaker affinity than PKR, and mutants of TRS1 that bound dsRNA less well than WT TRS1 were not compromised in their ability to inhibit PKR and rescue VacVΔE3L replication [67].

Thus, it seems unlikely that the dsRBD of TRS1 functions to sequester dsRNA. Instead, we hypothesize that that the dsRBD of PKR is necessary to correctly orient TRS1 such that its binding inhibits PKR activation. Solving the crystal structure of TRS1 would greatly enhance our ability to predict how TRS1 interacts with PKR and thus provide insight into the mechanism of action used by $HCMV_{TRS1.}$

The broad presence of PKR antagonists across viral families highlights the critical role of this antiviral factor [39]. Moreover, viral PKR antagonists are often essential for replication or pathogenicity [24,64,101,115]. Thus, understanding how viral PKR antagonists function provides insight into mechanisms of pathogenicity and has the potential to inform the development of therapeutics and vaccine strategies. CMV remains the most prevalent neonatal viral infection, establishing neurological deficits in thousands of neonates annually in the United States [9]. Moreover, CMV is a common comorbidity in immunocompromised patients [4]. Thus, it is critical to understand how CMV is able to evade host antiviral responses to establish infections. While there is still more work to be done to fully understand how $HCMV_{TRS1}$ functions, our findings demonstrate the utility of leveraging the host-viral arms race to gain insight into how viruses have evolved in response to changing host defenses and to better identify the diverse mechanisms used by viruses to overcome host restriction factors.

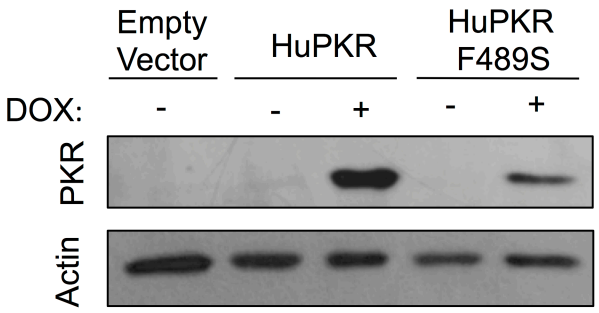

Figure 6.1 PKR expression in HF-PKR KO cells stably transduced with PKR variants.
HF PKR-KO Cells stably expressing an empty vector, HuPKR, or HuPKR F489S were treated +/- doxycycline (1ug/ml) for 72 hours. Lysates were collected and evaluated for PKR expression by western blotting.

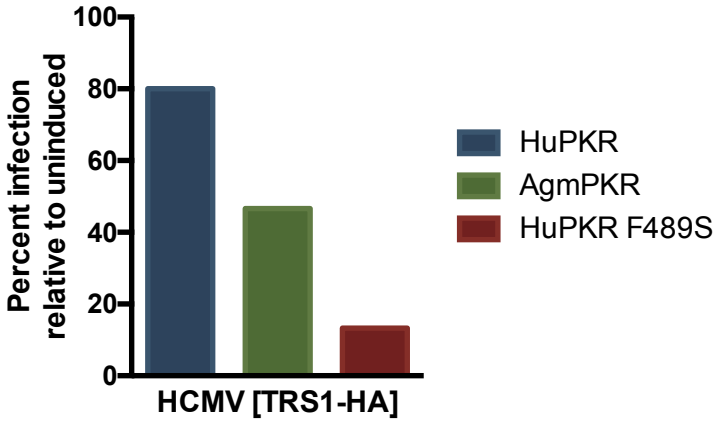

Figure 6.2 Replication of HCMV [TRS1-HA] in HF-PKR KO cells stably expressing PKR variants.
HF PKR-KO Cells stably expressing an HuPKR, AgmPKR, or HuPKR F489S were treated +/- doxycycline (1ug/ml) for 72 hours and then infected with HCMV [TRS1-HA] at an MOI of 0.1for 1 hour, after which the wells were refed +/- doxycycline. Media was replaced daily, and the supernatant of day 6 was harvested and tittered on HF PKR KO cells.

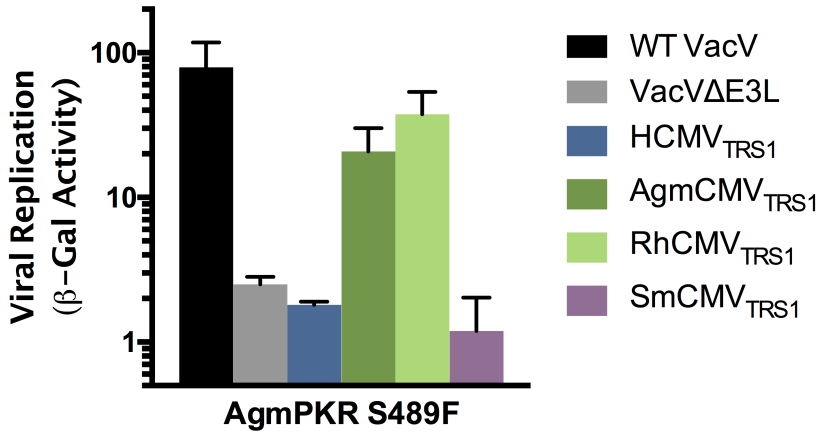

Figure 6.3 Replication of VacV recombinants in AgmPKR S489F cells
Duplicate wells of HeLa PKR KO cells inducibly expressing AgmPKR F489S were treated +/- doxycycline and infected (MOI 0.1) with a panel of VacVs. At 48hpi, viral replication was quantified by measuring β-gal activity and is reported as percent replication in doxycycline treated cells relative to replication in the same cells without induction of PKR expression (mean ± s.d.).

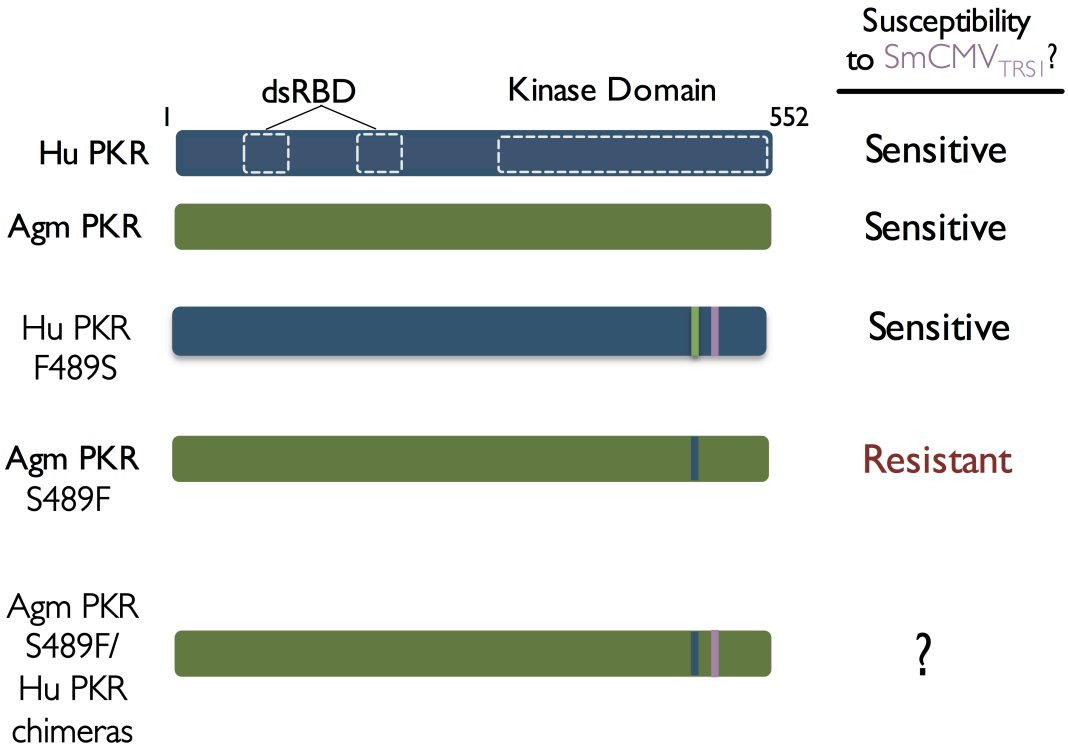

Figure 6.4 Schematic representation of the differential sensitivity of PKR variants to SmCMV$_{TRS1}$

HuPKR and AgmPKR are both sensitive to SmCMV$_{TRS1}$. While altering position 489 of HuPKR has no impact on SmCMV$_{TRS1}$ activity, mutating position 489 of AgmPKR (S489F) confers resistance to SmCMV$_{TRS1}$. This suggests there is an additional point of contact present in HuPKR (shown here as a purple line) that is missing from Agm PKR. Additional chimeras between HuPKR and AgmPKR S489F could facilitate mapping of the determinant of HuPKR's sensitivity to SmCMV$_{TRS1}$.

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between PKR Dimerization, Autophosphorylation, and eIF2α Substrate Recognition. Cell. 2005;122: 901–913. doi:10.1016/j.cell.2005.06.041

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Curriculum Vitae

Kathryn S. Carpentier

EDUCATION

PhD Candidate in Microbiology, (GPA: 3.87), University of Washington, Seattle, WA
October 2016
Advisor: Dr. Adam Geballe, FHCRC

B.A. in Biology, mathematics minor, summa cum laude (GPA: 4.0), Carroll College-Helena, MT
May 2010

RESEARCH EXPERIENCE

Graduate Student, University of Washington, Seattle, WA
September 2011- November 2016
Geballe Lab, Fred Hutchinson Cancer Research Center
Dissertation: "Evolution of Protein Kinase R Antagonism in Primate Cytomegaloviruses"

NIH Post-baccalaureate Fellow, Rocky Mountain Laboratories, Hamilton, MT
August 2010-July 2011
Bloom Lab, NIH, NIAID, RML

- Developed and optimized an efficient protocol to transfect ISE6 tick cells
- Produced polyclonal antibodies of Salp25D and Salp25C to be used in understanding the role of *Ixodes scapularis* salivary proteins in the transmission and acquisition of Tick-borne flaviviruses.

Undergraduate Researcher, Weigand Undergraduate Research Lab, Carroll College, Helena, MT
March 2009-May2010
Alvey Lab, Carroll College

- Participated in a research project focused on using spatial epidemiology to generate a risk map of West Nile Virus for Montana.
- Completed an Honors Thesis entitled, "Detection of West Nile Virus in Mosquitoes of Montana through RT-PCR and TaqMan Assays and Correlation to Reservoir Competent Avian Populations"

PUBLICATIONS

Carpentier KS, Esparo NM, Child SJ, Geballe AP. 2016. A single amino acid dictates Protein Kinase R susceptibility to unrelated viral antagonists. *PLoS Pathogens* 12:e1005966.

Carpentier KS, Geballe AP. 2016. An evolutionary view of the arms race between Protein Kinase R and large DNA viruses. *Journal of Virology* 90:3280–3283.

Bierle CJ, **Semmens KM**, Geballe AP. 2013. Double-stranded RNA binding by the human cytomegalovirus PKR antagonist TRS1. *Virology* 442: 28-37.

CONFERENCE PRESENTATIONS

2016 International Herpesvirus Workshop, Madison, WI
Oral and Poster Presentation: "A single amino acid dictates PKR susceptibility to unrelated viral antagonists"

2015 International Herpesvirus Workshop, Boise, ID
Oral and Poster Presentation: "Evolution of PKR antagonism in primate cytomegaloviruses"

2010 NIH, NCRR Third Biennial National IDeA Symposium of Biomedical Research Excellence, Bethesda, MD
Poster: "Detection of West Nile Virus in Mosquitoes of Montana through RT-PCR and TaqMan Assays and Correlation to Reservoir Competent Avian Populations"

2009 M. J. Murdock Undergraduate Research Conference; Gonzaga University, Spokane, WA
Poster: "Detection of West Nile Virus in Mosquitoes of Montana through RT-PCR and TaqMan Assays and Correlation to Reservoir Competent Avian Populations"

2009 Western Region COBRE-INBRE Scientific Conference, Big Sky, MT
Poster: "Detection of West Nile Virus in Mosquitoes of Montana through RT-PCR and TaqMan Assays and Correlation to Reservoir Competent Avian Populations"

TEACHING EXPERIENCE

Guest Lecturer, University of Washington, Seattle, WA
- Micro 431: Prokaryotic Recombinant DNA Techniques, January 2015
- Micro 442: Medical Bacteriology, two guest lectures, January 2016

Teaching Assistant, University of Washington, Seattle WA
September 2012 – March 2013
- Managed the following lab courses. Responsibilities included giving a brief introduction at the beginning of each session, supervising students as they completed each lab, and writing and grading exams.
  - Micro 402: Fundamentals of General Microbiology Laboratory
  - Micro 431: Prokaryotic Recombinant DNA Techniques

Biomedical Research After School Scholars Program, Hamilton Middle School, Hamilton, MT
December 2010
- Worked in a group of four to develop a lesson plan for middle school students and taught a lesson to three classes

Student Lab Teaching Assistant, Carroll College, Helena MT
August 2007 – May 2010
- Assisted professors in managing the following labs: Biological Principles I and II, Cell Biology, Genetics, Biochemistry, Animal Physiology, General Microbiology

GRANTS / SCHOLARSHIPS

- Cell & Molecular Biology Training Grant Awardee: 2014-2015
- Interdisciplinary Training Grant Awardee: 2012-2014
- Thomas Paul Research Scholarship: 2009

AWARDS

- Helen Riaboff Whiteley Fellowship Awardee: 2016
- International Herpesvirus Workshop Travel Awardee: 2016
- The Galloway Cup Human Biology Poster Award: 2015
- Neal B. Groman Award for Excellence in Teaching: 2013
- NSF GRFP Honorable Mention: 2012
- NSF GRFP Honorable Mention: 2011
- University of Washington Top Scholar Award: 2011
- Carroll College Manion Scholar: 2009, 2010

COMMUNITY WORK

- Hutch High, Activity Co-organizer, FHCRC, Seattle WA
- Biomedical Research After School Scholars Program Volunteer, Hamilton MT
- Exploration Works Science Museum Floor Staff, Helena MT