

Human Cytomegalovirus pTRS1 and pIRS1 Antagonize Protein Kinase R To Facilitate Virus Replication

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ABSTRACT

Human cytomegalovirus (HCMV) counteracts host defenses that otherwise act to limit viral protein synthesis. One such defense is the antiviral kinase protein kinase R (PKR), which inactivates the eukaryotic initiation factor 2 (eIF2) translation initiation factor upon binding to viral double-stranded RNAs. Previously, the viral TRS1 and IRS1 proteins were found to antagonize the antiviral kinase PKR outside the context of HCMV infection, and the expression of either pTRS1 or pIRS1 was shown to be necessary for HCMV replication. In this study, we found that expression of either pTRS1 or pIRS1 is necessary to prevent PKR activation during HCMV infection and that antagonism of PKR is critical for efficient viral replication. Consistent with a previous study, we observed decreased overall levels of protein synthesis, reduced viral protein expression, and diminished virus replication in the absence of both pTRS1 and pIRS1. In addition, both PKR and eIF2 α were phosphorylated during infection when pTRS1 and pIRS1 were absent. We also found that expression of pTRS1 was both necessary and sufficient to prevent stress granule formation in response to eIF2 α phosphorylation. Depletion of PKR prevented eIF2 α phosphorylation, rescued HCMV replication and protein synthesis, and reversed the accumulation of stress granules in infected cells. Infection with an HCMV mutant lacking the pTRS1 PKR binding domain resulted in PKR activation, suggesting that pTRS1 inhibits PKR through a direct interaction. Together our results show that antagonism of PKR by HCMV pTRS1 and pIRS1 is critical for viral protein expression and efficient HCMV replication.

IMPORTANCE

To successfully replicate, viruses must counteract host defenses that limit viral protein synthesis. We have identified inhibition of the antiviral kinase PKR by the viral proteins TRS1 and IRS1 and shown that this is a critical step in HCMV replication. Our results suggest that inhibiting pTRS1 and pIRS1 function or restoring PKR activity during infection may be a successful strategy to limit HCMV disease.

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

Perhaps the best-studied antiviral defense targeting viral mRNA translation is the RNA-dependent protein kinase R (PKR). PKR binds to double-stranded RNAs (dsRNAs) produced during viral infections, resulting in PKR dimerization and activating autophosphorylation (1–4). Activated PKR in turn inhibits mRNA translation by phosphorylating its substrate the eukaryotic initiation factor 2 alpha (eIF2 α) (5–8). eIF2 α plays a critical role in translation initiation as a regulatory subunit of the trimeric eIF2 complex, which mediates binding of the ternary complex, consisting of eIF2, GTP, and tRNA^{Met}, to the ribosome (9). eIF2 α phosphorylation by PKR prevents recycling of the ternary complex after initiation, resulting in an overall decrease in translation initiation and diminished viral protein synthesis and replication (10).

Phosphorylation of eIF2 α further limits protein synthesis by sequestering actively translating mRNAs into cytoplasmic ribonucleoprotein complexes called stress granules (11). During viral infection, stress granules are most often induced by activated PKR;

however, additional virus-induced stressors such as the accumulation of unfolded proteins and/or nutrient depletion are also involved (12). Prolonged periods of stress lead to the degradation of stress granule-associated mRNAs, which further inhibits viral protein expression (13). Despite the induction of stress response pathways known to trigger stress granule formation, stress granules do not form in HCMV-infected cells (14, 15). This suggests that HCMV encodes viral proteins that inhibit stress granule formation. However, a role for HCMV proteins in the inhibition of stress granule formation has not been described.

Many viruses generate dsRNA ligands recognized by PKR during infection, and thus viruses commonly encode PKR antagonists. Human cytomegalovirus encodes two PKR antagonists, the TRS1 and IRS1 proteins (pTRS1 and pIRS1, respectively). The amino-terminal 550 amino acids of pTRS1 and pIRS1 are encoded by the short-repeat regions of the viral genome and are therefore identical, while the remainder of pTRS1 and pIRS1 are encoded by

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the unique short segment of the genome and thus diverge. However, the unique regions of pTRS1 and pIRS1 are highly similar, sharing approximately 50% amino acid conservation. Both proteins limit PKR activation outside the context of HCMV infection (16), and the expression of either pTRS1 or pIRS1 is necessary for HCMV replication (17). Several functional domains have been identified in pTRS1 and pIRS1, including an RNA binding domain between amino acids 86 and 246 (18) and a PKR binding domain in the unique carboxyl terminus (19). While both domains are necessary for PKR antagonism in heterologous systems and *in vitro*, the contribution of each domain to PKR inhibition during HCMV infection is currently unknown.

Efforts to study pTRS1 functions in the context of HCMV infection have been complicated by the functional redundancy of pIRS1. Expression of either protein is sufficient to support HCMV replication at high multiplicities of infection (MOI) (17, 20), although a TRS1 deletion virus is attenuated after infection at low MOI (20). However, deletion of both TRS1 and IRS1 results in a replication-deficient virus. As a result, the majority of studies defining functional roles for pTRS1 and pIRS1 have been performed *in vitro* or in heterologous systems outside the context of HCMV infection. Both pTRS1 and pIRS1 complement the growth defect of vaccinia virus mutants lacking the E3L protein (16, 18, 21–24), a known PKR inhibitor, and pTRS1 complements the growth of herpes simplex virus (HSV) mutants lacking the ICP34.5 protein (25), which stimulates eIF2 α dephosphorylation through the recruitment of a cellular phosphatase (26, 27). Similarly, pTRS1 complements the replication of murine cytomegalovirus (MCMV) strains lacking the PKR antagonists m142 or m143 (28, 29). While these studies show that pTRS1 and pIRS1 act as PKR antagonists, the role of PKR in suppressing HCMV replication has not been defined. In addition, the requirement for pTRS1 and pIRS1 to prevent PKR activation during HCMV infection has not been described.

A previous study by Marshall et al. (17) generated an HCMV mutant with TRS1 and IRS1 deleted and found that in the absence of both pTRS1 and pIRS1, eIF2 α is phosphorylated and HCMV protein synthesis and replication are dramatically inhibited. However, this study did not measure PKR activation in the absence of both pTRS1 and pIRS1, nor was it determined if depleting PKR could prevent eIF2 α phosphorylation or restore viral protein synthesis and replication. Fibroblasts express three additional eIF2 α kinases, GCN2, HRI, and PERK, which phosphorylate eIF2 α in response to amino acid stress, reactive oxygen species (ROS), and activation of the unfolded protein response (UPR), respectively (30, 31). Several studies show that HCMV induces ROS (32–34) and activates PERK as part of the virus-induced UPR (35, 36). In addition, HCMV potentially induces amino acid stress due to high levels of ongoing host and viral protein synthesis. Consistent with this idea, mice lacking GCN2 are more susceptible to infection with the related murine cytomegalovirus (MCMV) (37). The role of these additional eIF2 α kinases in HCMV replication is unknown, as is the ability of pTRS1 to regulate their activity. In addition, pTRS1 and pIRS1 have other functions potentially unrelated to PKR antagonism, including suppression of autophagy (38, 39), transactivation of gene expression (40), and packaging of viral DNA into capsids (41). pTRS1 also interacts with the HCMV DNA polymerase accessory subunit and promotes ori-Lyt-dependent DNA replication through an unknown mechanism (42–46).

How these functions contribute to the replication defect observed in the absence of pTRS1 and pIRS1 has not been tested.

In this study, we used a combination of short hairpin RNAs (shRNAs) and viral mutants to evaluate the role of antagonism of PKR by pTRS1 and pIRS1 during HCMV infection. Our results confirm previous studies showing that either IRS1 or TRS1 is sufficient for HCMV replication (17). We found that expression of either pTRS1 or pIRS1 is both necessary and sufficient to suppress PKR activation and eIF2 α phosphorylation and maintain overall levels of protein synthesis in HCMV-infected cells. In addition, we found that expression of either pTRS1 or pIRS1 is necessary to prevent stress granule formation during HCMV infection and that pTRS1 alone is sufficient to prevent stress granule formation in transfected cells. Using a pTRS1 mutant lacking a portion of the unique carboxyl terminus, we found that the pTRS1 PKR binding domain is necessary to prevent PKR activation during HCMV infection. Depletion of PKR restored virus replication and viral protein synthesis in the absence of pTRS1 and pIRS1 and prevented stress granule formation. Thus, our findings demonstrate that antagonism of PKR by pTRS1 and pIRS1 is critical for efficient HCMV protein expression and replication.

MATERIALS AND METHODS

Viruses, cells, and reagents. MRC-5 fibroblasts, human foreskin fibroblasts (HFFs), and HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. MRC-5 fibroblasts and HFFs were used between passages 8 and 20. HCMV strain AD169 lacking the TRS1 open reading frame (ORF) was a generous gift of Adam Geballe (University of Washington) and has been described previously (17). All mutations in the HCMV genome were generated in the AD169BADinGFP bacterial artificial chromosome (BAC) using recombinering in *Escherichia coli* strain DY380 as previously described (47, 48). To generate the PKR binding domain deletion mutant (HCMV Δ PBD), a FLAG-FRT-KAN-FRT cassette was PCR amplified using primers FRT FWD (5' TTAGATTATA AAGATGATGATGATAAA 3') and PBD FRT REV (5' TCCCGGAGAA GATTCCTGGTACGACTTGGACGAGACTTCTGGGTTCTTTAGA TTATAAAGATGATGATGATAAA 3') containing 50 nucleotides of homology flanking the insertion site at nucleotide 228885 (corresponding to amino acid 679; GenBank accession number FJ527563.1). Following recombination, expression of the Flp recombinase was induced with arabinose to excise the kanamycin cassette. The resulting recombinant contains a FLAG epitope tag followed by a translation stop codon in frame with the 3' end of the TRS1 ORF. The gross integrity of the recombinant viral genomes was confirmed by restriction digest, and the sequences of the entire TRS1 open reading frame and the flanking 500 nucleotides were confirmed by Sanger sequencing. For each mutant, two independent recombinants were isolated, sequenced, and characterized to control for potential spurious mutations introduced during recombination. BAC DNA was purified using the Nucleobond Midiprep kit (Macherey-Nagel) according to the manufacturer's directions. One microgram of BAC DNA together with one microgram of pCGN pp71 (49) was electroporated into MRC-5 fibroblasts to reconstitute infectious virus. Viral stocks were propagated on MRC-5 fibroblasts, and their titers were determined using the tissue culture infective dose (TCID₅₀) method. Unless otherwise noted, all infections were performed at a multiplicity of infection of 1 in 300 μ l of medium. The amount of cell-free virus in the supernatant was quantified by the TCID₅₀ method on MRC-5 fibroblasts as previously described (50).

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

siRNA- and shRNA-mediated depletion. Small interfering RNA (siRNA) targeting the PKR transcript (Dharmacon SMARTpool ON-TARGETplus EIF2AK2 siRNA) was transfected at a final concentration of 20 nM in 100 μ l Opti-MEM using 6 μ l Mission siRNA transfection reagent (Sigma). Complexes were incubated at room temperature for 15 min prior to dropwise addition to 30 to 50% confluent MRC-5 cells. Cells were used in subsequent experiments at 72 h after transfection. Efficient knockdown was routinely monitored by Western blotting. MRC-5 cells stably expressing scrambled or IRS1-specific shRNAs were generated using the pSUPERretro system (Oligoengine). An shRNA hairpin (5' GGAGTTCATGTTTCGCGAACATTCAAGAGATGTTTCGCGAAA CATGAATCC 3'), which generates the mature shRNA (5' GGAGTTC ATGTTTCGCGAACA 3') targeting the unique 3' end of the IRS1 ORF was cloned into the pSUPERretro vector. Retrovirus stocks were generated by transfecting the Phoenix packaging cell line (51) with the pSUPERretro plasmids. Supernatants were collected at 48 h posttransfection, filtered through a 0.45- μ m filter, and used to transduce cells in the presence of Polybrene (4 μ g/ml). Stable cell lines were obtained by selection in puromycin (1 μ g/ml) for at least 1 week prior to use.

Western blot analysis. Cells were collected by scraping at the indicated times and frozen as dry pellets at -80°C until use. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 1 mM EDTA) containing protease and phosphatase inhibitors (Roche), and the protein concentration was determined by the Bradford assay (Amresco). Equivalent amounts of protein were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham). For monoclonal antibodies, membranes were blocked for 1 h at room temperature in 1% bovine serum albumin (BSA) in TBS-T (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) before incubation with primary antibody in TBS-T with 1% BSA for 1 h at room temperature or overnight at 4°C . For polyclonal antibodies, membranes were blocked in 1% BSA followed by incubation with primary antibody overnight at 4°C in 5% BSA in TBS-T. For Western blots using the phospho-eIF2 α (Ser51) antibody, membranes were blocked in 5% BSA in TBS-T overnight at 4°C , followed by overnight incubation at 4°C with primary antibody diluted in TBS-T containing 5% BSA. Antibodies to the following proteins were used in this study: IE1 (52) (1:10,000), pUL44 (1:1,000; Virusys), pp28 (53) (1:5,000), pTRS1 (40) (1:100), pIRS1 (40) (1:100), P99 antibody against pTRS1 and pIRS1 (17) (1:10,000), tubulin (1:50,000; Sigma T6199), total PKR (1:1,000; Santa Cruz sc-707), phospho-PKR (Thr446) (1:1,000; Abcam ab32036), total eIF2 α (1:1,000; catalog no. 3072; Cell Signaling), and phospho-eIF2 α (Ser51) (1:1,000; catalog no. 3398; Cell Signaling).

Indirect immunofluorescence. Cells were seeded into wells containing glass coverslips. Where noted, cells were infected at a multiplicity of infection of 0.5. At the time of harvest, coverslips were washed three times for 5 min with 37°C phosphate-buffered saline (PBS) and then fixed in 2% paraformaldehyde (PFA) for 15 min at 37°C . The cells were washed three times in room temperature PBS and subsequently permeabilized with 0.1% Triton X-100. The cells were again washed three times with PBS containing 0.2% Tween 20 (PBS-T) at room temperature and then incubated overnight in blocking buffer (2% BSA in TBS-T). The cells were stained for 1 h at room temperature with the following primary antibodies diluted in blocking buffer: IE1 (1:10); pTRS1 (1:10); G3BP1 (Santa Cruz sc-98561; 1:50). The coverslips were washed three times with PBS-T and then incubated with fluorescent secondary antibody (Invitrogen goat anti-mouse or rabbit; 1:500). The coverslips were again washed three times, mounted on glass slides in VectaShield containing 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei (Vector Laboratories), and sealed with nail polish. Images were captured using a Zeiss 710 confocal microscope with the help of the UNC Microscopy Core.

Quantification of stress granule formation. Control or PKR-deficient HeLa cells were transfected with either a green fluorescent protein (GFP) or pTRS1 expression vector. Twenty-four hours after transfection, cells were treated with arsenite for 1 h and then processed for immuno-

fluorescence microscopy as above. Transfected cells (i.e., GFP or pTRS1 positive) were identified, and the relative fluorescence intensity of the GFP or pTRS1 signal in each cell was quantified using ImageJ software. The relative pTRS1 or GFP expression level in each cell was scored on a scale of 1 to 100, with 100 representing the highest observed expression. The presence of stress granules in each transfected cell was then determined by measuring the formation of G3BP1 puncta. Transfected cells containing two or more G3BP1 puncta were considered positive for stress granules, while those with fewer than two puncta were considered negative. The range of fluorescence intensity for GFP and pTRS1 was divided into quartiles (1 to 25% of maximum, 26 to 50% of maximum, etc.), and the percentage of cells containing stress granules within each expression quartile was calculated. A minimum of 100 GFP- or pTRS1-positive cells were analyzed. The data are displayed as the percent transfected cells containing stress granules within each quartile of GFP or pTRS1 expression.

Metabolic labeling of nascent proteins. Nascent proteins were metabolically labeled and quantified as described previously (54). Briefly, cells were incubated in methionine- and cysteine-free medium (Sigma) for 15 min. ^{35}S -labeled methionine and cysteine (125 μCi ; PerkinElmer EasyTag Express Labeling Mix) were added and allowed to incorporate for 30 min. Cells were then washed twice in ice-cold PBS, scraped, and collected by centrifugation. Cell pellets were lysed in RIPA medium containing protease inhibitors (Roche), and protein concentrations were determined by the Bradford assay (Amresco). Trichloroacetic acid (TCA) was added to a final concentration of 20%, and precipitated proteins were captured on glass microfiber filters by filtration under vacuum. The filters were washed twice with 20% TCA and once with 100% ethanol and allowed to air dry. The filters were then transferred to vials containing scintillation fluid (EcoScint), and radioactivity was quantified using a scintillation counter. The amount of radioactivity was normalized to the protein concentration for each sample.

RESULTS

HCMV mutant strains lacking either pTRS1 or pIRS1 replicate to levels similar to those of wild-type virus (20, 55), while a mutant lacking both pTRS1 and pIRS1 replicates poorly (17). Previous studies of a pTRS1/pIRS1 double knockout virus were complicated by the inability to efficiently complement virus replication by expression of pTRS1 *in trans* (17). This likely reflected insufficient expression of the pTRS1 transgene or improper timing of expression. We reasoned that the failure of the pTRS1 transgene to fully complement the replication of a pTRS1/pIRS1 double deletion virus would limit our ability to generate and study recombinant viruses lacking domains of pTRS1 required for virus replication. Thus, we sought to develop a system that allowed us to control pIRS1 expression and express pTRS1 mutants to wild-type levels with wild-type kinetics from the context of the viral genome. We therefore used a combination of viral genetics and shRNA-mediated gene silencing to manipulate pTRS1 and pIRS1 expression during HCMV infection. We first generated primary human fibroblasts expressing either a scrambled shRNA (control cells) or an shRNA specific for the pIRS1 transcript (shIRS1-HFs). Infecting the cells with wild-type virus allowed us to study the contribution of pTRS1 to HCMV infection in the absence of pIRS1. Conversely, infection of control cells with a pTRS1 deletion virus (HCMV Δ TRS1, described in reference 17; kindly provided by Adam Geballe) provided a system to study specific roles for pIRS1, while infection of shIRS1-HFs with HCMV Δ TRS1 allowed us to determine how the absence of both proteins affected HCMV replication. Importantly, in this system both pTRS1 and pIRS1 were expressed from their native location in the viral genome under the control of their endogenous promoters, allowing wild-type kinetics and expression levels of the pTRS1 and pIRS1 proteins.

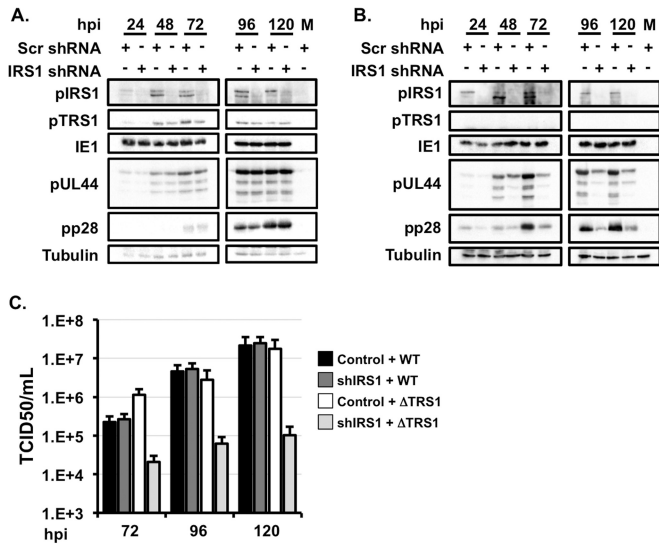


FIG 1 Expression of either pTRS1 or pIRS1 is necessary for efficient HCMV replication. (A) Control cells (Scr) or shIRS1-HFs were infected with wild-type HCMV at an MOI of 3. Cells were harvested at the indicated times and analyzed by Western blotting ($n = 3$). (B) Control cells and shIRS1-HFs were infected with HCMV lacking TRS1 (HCMV Δ TRS1) at an MOI of 3. Cells were harvested at the indicated times and analyzed by Western blotting ($n = 3$). (C) Cells were infected as described for panel A, and the amount of virus in cell free supernatants was measured by the TCID₅₀ method ($n = 3$).

Infection of shIRS1-HFs with wild-type HCMV resulted in a significant reduction in pIRS1 expression at all times after infection (Fig. 1A). Importantly, pTRS1 expression was not affected by the IRS1-specific shRNA. Consistent with previous reports showing that pIRS1 is dispensable for HCMV replication (20), wild-type virus replicated to equivalent titers in the two cell types despite efficient depletion of pIRS1 in shIRS1-HFs (Fig. 1C). These data also demonstrate that the IRS1-specific shRNA did not target additional host or viral proteins necessary for HCMV replication. Both wild-type and HCMV Δ TRS1 replicated to similar levels fol-

lowing infection of control cells. However, HCMV Δ TRS1 infection on shIRS1-HFs reduced viral replication by >2 orders of magnitude (Fig. 1C). These data demonstrate that our system confirms previous results showing that expression of either pTRS1 or pIRS1 is necessary for efficient HCMV replication.

We next examined viral protein expression after infection of control HF cells or shIRS1-HFs with wild-type or HCMV Δ TRS1 virus. Similar levels of representative immediate early (IE), early, and late proteins were expressed after infection of control cells or shIRS1-HFs with wild-type virus. Viral proteins of each kinetic class were also efficiently expressed after infection of control cells with HCMV Δ TRS1 virus (Fig. 1A and B); however, HCMV early and late protein expression was reduced after infection of shIRS1-HFs with the HCMV Δ TRS1 virus (Fig. 1B). While the immediate early protein IE1 was expressed equivalently in shIRS1-HFs and control cells, we observed decreased expression of the early protein pUL44 and the late protein pp28 after infection of shIRS1-HFs with HCMV Δ TRS1 (Fig. 1B). Consistent with previous results (17), we conclude that the expression of either pTRS1 or pIRS1 is necessary for the efficient expression of HCMV early and late proteins.

pTRS1 or pIRS1 prevents PKR activation during HCMV infection. pTRS1 and pIRS1 inhibit the antiviral kinase PKR in the context of vaccinia virus infection and in transfected cells (16, 24, 56). To determine if pTRS1 and pIRS1 similarly limit PKR activation during HCMV infection, we measured PKR autophosphorylation and phosphorylation of the PKR substrate eIF2 α after infection of control cells or shIRS1-HFs with wild-type or HCMV Δ TRS1 virus. Infection with wild-type virus did not induce PKR or eIF2 α phosphorylation in either cell type (Fig. 2A), demonstrating that the expression of pTRS1 is sufficient to limit PKR activation. Similarly, neither PKR nor eIF2 α was phosphorylated in control cells infected with HCMV Δ TRS1, showing that pIRS1 expression is sufficient to suppress PKR. However, infection of shIRS1-HFs with HCMV Δ TRS1 resulted in robust PKR and eIF2 α phosphorylation (Fig. 2A). Consistent with the inhibition of protein synthesis induced by eIF2 α phosphorylation, we

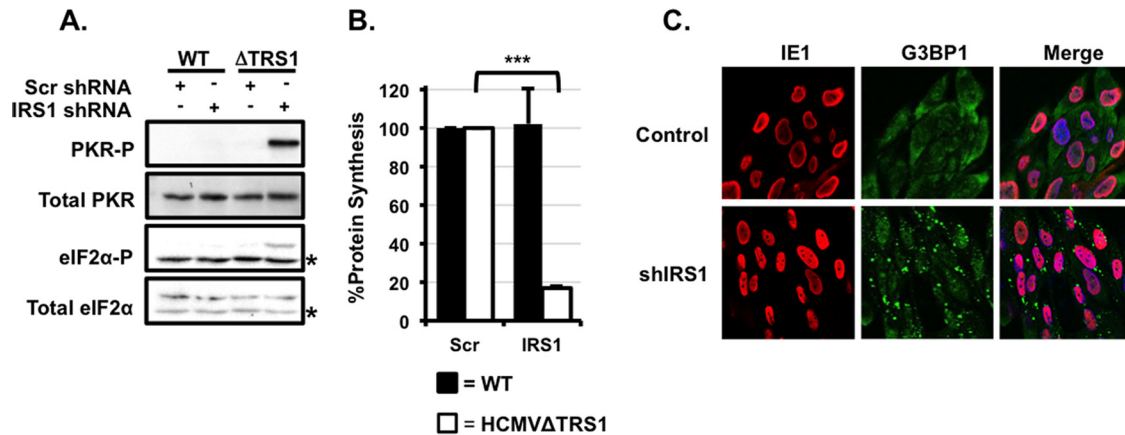


FIG 2 HCMV pTRS1 or pIRS1 is necessary to antagonize PKR, maintain infected-cell protein synthesis, and inhibit stress granule formation. (A) Control cells (Scr) or shIRS1-HFs (shIRS1) were infected with either wild-type virus or HCMV Δ TRS1 at an MOI of 3. Cells were harvested at 24 h after infection and analyzed by Western blotting ($n = 3$). Asterisks indicate nonspecific background bands. (B) Cells were infected as described for panel A, and the amount of radiolabeled amino acids incorporated into acid-insoluble protein in 30 min was quantified at 24 h after infection ($n = 3$; $P < 0.05$). Filled bars indicate wild-type infection, and open bars indicate HCMV Δ TRS1 infection. (C) Control cells or shIRS1-HFs were infected with HCMV Δ TRS1, and the formation of G3BP1 puncta was measured by indirect immunofluorescence at 24 h after infection. A representative image from one of three independent experiments is shown.

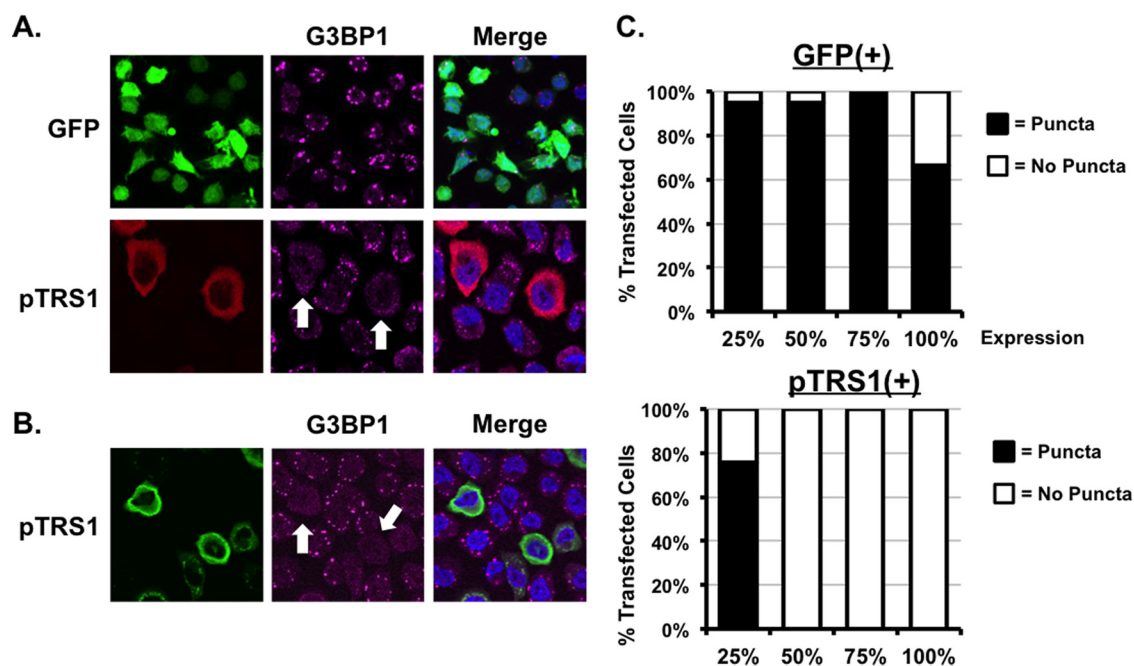


FIG 3 pTRS1 is sufficient to inhibit stress granule formation. (A) HeLa cells were transfected with a GFP or pTRS1 expression vector. Twenty-four hours after transfection, cells were treated with sodium arsenite (0.5 mM) for 1 h, and G3BP1 punctum formation was measured by indirect immunofluorescence. (B) PKR-deficient HeLa cells were transfected with a GFP or pTRS1 expression vector. Cells were treated with arsenite as described for panel A and analyzed by indirect immunofluorescence as above. (C) Western blotting was used to measure PKR expression in PKR-deficient HeLa cells. PKR-deficient cells were transfected and treated as described for panel A. The presence of G3BP1-positive puncta in transfected cells was determined by indirect immunofluorescence. The range of transgene expression was divided into quartiles (1 to 25% of maximum, 26 to 50% of maximum, etc.), and the results are shown as the percentage of GFP-expressing (top panel) or pTRS1-expressing (bottom panel) cells containing two or more G3BP1 puncta.

observed an 80% decrease in the overall levels of protein synthesis when shIRS1-HFs were infected with HCMV Δ TRS1 (Fig. 2B). These results demonstrate that the expression of either pTRS1 or pIRS1 is necessary to antagonize PKR activation, limit eIF2 α phosphorylation, and maintain protein synthesis in HCMV-infected cells.

pTRS1 inhibits stress granule formation. eIF2 α phosphorylation results in the redistribution of actively translating mRNAs into cytoplasmic puncta termed stress granules (11, 57). While stress granules do not form after infection with wild-type HCMV (14), the increase in eIF2 α phosphorylation after infection in the absence of both pTRS1 and pIRS1 suggested that pTRS1 and pIRS1 might limit stress granule formation during HCMV infection. In response to cellular stress, the G3BP1 protein localizes to cytoplasmic stress granules (58); therefore, we used the formation of G3BP1-positive cytoplasmic puncta as a measure of stress granule formation. We found that G3BP1 puncta do not accumulate in control cells or shIRS1-HFs infected with wild-type virus (data not shown). Similarly, few if any G3BP1 puncta formed after infection of control cells with the HCMV Δ TRS1 virus (Fig. 2C). In contrast, infection of shIRS1-HFs with HCMV Δ TRS1 resulted in the accumulation of G3BP1 puncta (Fig. 2C). Therefore, the expression of either pTRS1 or pIRS1 is necessary to prevent stress granule formation during HCMV infection.

To determine if pTRS1 was sufficient to prevent stress granule formation, we measured the accumulation of stress granules in cells transfected with a pTRS1 expression vector. Cells were transfected with either a pTRS1 expression vector or a control vector expressing GFP and then treated with arsenite to induce stress

granule accumulation. G3BP1 puncta readily accumulated in control cells expressing GFP. However, very few G3BP1-positive granules were found in cells expressing pTRS1 (Fig. 3A). Interestingly, arsenite induces stress granule formation through activation of the eIF2 α kinase HRI (59), suggesting that pTRS1 could inhibit stress granule formation induced by eIF2 α kinases other than PKR. To confirm that PKR was dispensable for arsenite-induced stress granule formation, we measured the formation of G3BP1 puncta after arsenite treatment in cells lacking PKR expression. The PKR gene in these cells was mutated using CRISPR/Cas9-mediated mutagenesis (56). Arsenite readily induced stress granule formation in PKR-deficient cells, and this induction was inhibited by pTRS1 expression (Fig. 3B). In order to measure the effect of pTRS1 expression on stress granule formation, we determined the number of cells containing stress granules among cells expressing pTRS1 or GFP. Stress granules formed in >90% of cells expressing GFP regardless of GFP expression levels (Fig. 3C, top panel). In contrast, a limited number of stress granule-positive cells (<45%) were found in cells expressing low levels of pTRS1. Higher levels of pTRS1 expression were sufficient to completely inhibit stress granule formation (Fig. 3C, bottom panel). Together our results demonstrate that pTRS1 is necessary and sufficient to prevent stress granule formation and suggest that pTRS1 also prevents stress granule formation induced by additional eIF2 α kinases.

PKR depletion prevents eIF2 α phosphorylation and restores HCMV protein expression and replication in the absence of pTRS1 and pIRS1. The increase in eIF2 α and PKR phosphorylation observed in the absence of pTRS1 and pIRS1 during infection

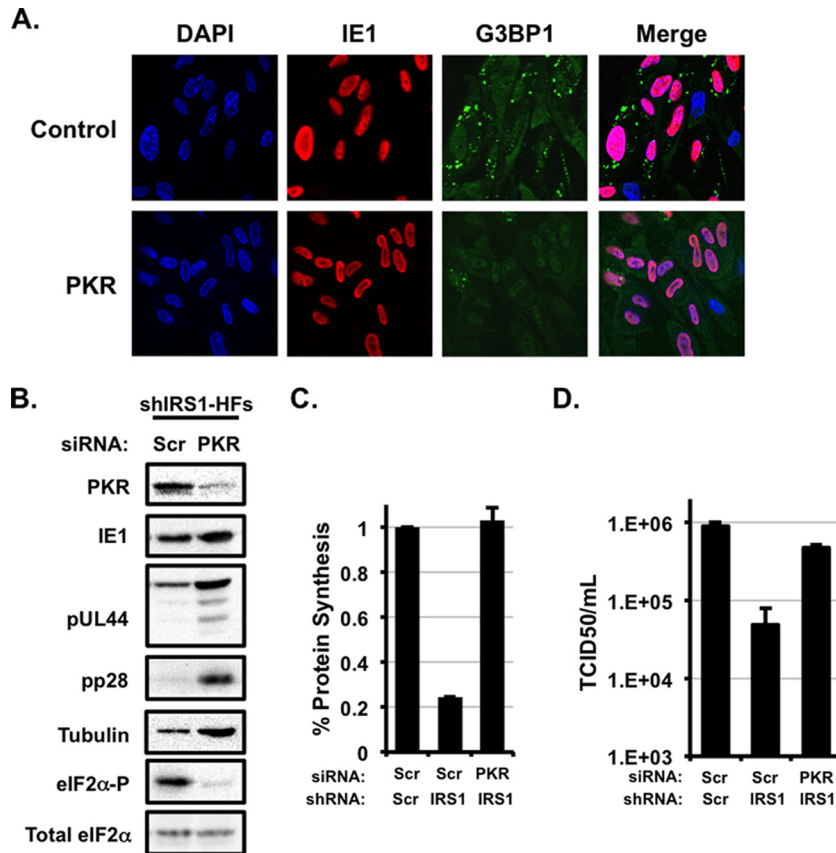


FIG 4 PKR inhibits viral protein synthesis and induces stress granule formation in the absence of pTRS1 and pIRS1. (A) shIRS1-HFs were transfected with scrambled or PKR-specific siRNAs prior to infection with HCMVΔTRS1. The formation of G3BP1 (+) puncta was monitored by indirect immunofluorescence at 24 h after infection. A representative image from one of three independent experiments is shown. (B) shIRS1-HFs were transfected with scrambled (scr) or PKR-specific (PKR) siRNAs prior to infection with HCMVΔTRS1. Expression of the indicated proteins was measured by Western blotting at 96 h after infection, except for eIF2α-P and total eIF2α, which were measured at 24 h after infection ($n = 3$). (C) Cells stably expressing scrambled (Scr) or pIRS1-specific (IRS1) shRNAs were transfected with scrambled (scr) or PKR-specific (PKR) siRNAs and then infected with HCMVΔTRS1. The amount of radiolabeled amino acids incorporated into acid-insoluble protein in 30 min was quantified at 24 h after infection ($n = 3$). (D) Cells were treated and infected as described for panel C. The amount of virus in the culture supernatants at 96 h after infection was determined by the TCID₅₀ method ($n = 3$).

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

The pTRS1 PKR binding domain is required during infection to antagonize PKR. As discussed above, several pTRS1 functional domains have been described outside the context of HCMV infection (18, 21) including a PKR binding domain in the carboxyl-terminal 116 amino acids of pTRS1 (19). As our system allows

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

We then measured the replication of the HCMVΔPDB virus over a single round of virus replication. HCMVΔPDB replicated to lower titers than HCMVΔTRS1 on control cells, and virus replication was further reduced 10-fold in the absence of pIRS1

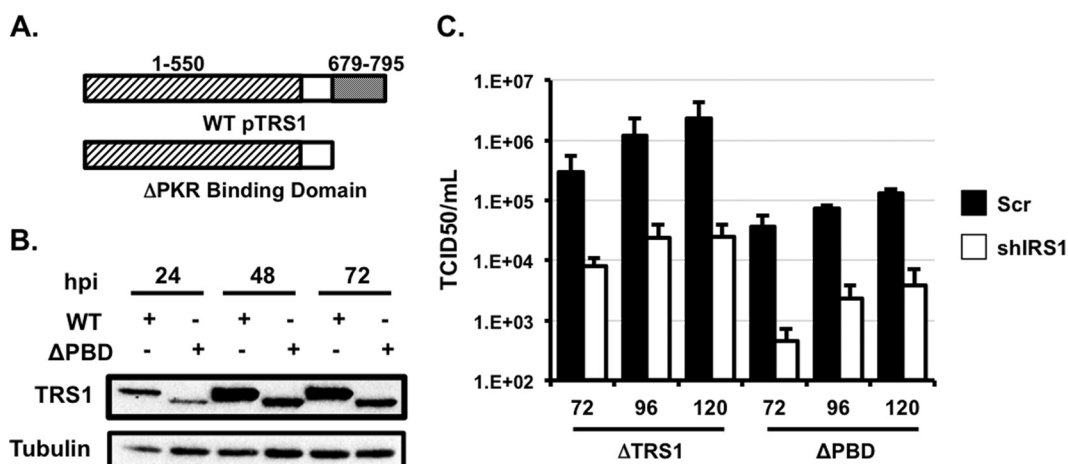


FIG 5 The pTRS1 PKR binding domain is required for efficient virus replication. (A) Cartoon showing the region of pTRS1 deleted in the HCMV Δ PBD virus. (B) Western blot showing expression of pTRS1 after infection with wild-type HCMV or HCMV Δ PBD virus at 72 h after infection. (C) Control HFs (scr; filled bars) or shIRS1-HFs (open bars) were infected with HCMV Δ TRS1 or HCMV Δ PBD virus at an MOI of 1, and the amount of cell-free virus in the culture supernatants was quantified by the TCID₅₀ method ($n = 3$).

(Fig. 5C). While the HCMV early protein pUL44 and late protein pp28 were expressed in control cells during HCMV Δ PBD infection, the expression of both viral proteins was significantly decreased in shIRS1-HFs (Fig. 6A). Interestingly, the expression of the mutant pTRS1 protein itself was also significantly reduced in pIRS1-depleted cells. To determine if PKR activation might account for diminished viral protein expression, we measured PKR and eIF2 α phosphorylation after infection of control or shIRS1-

HFs with the HCMV Δ PBD virus. We found no increase in PKR or eIF2 α phosphorylation after infection of control cells with HCMV Δ PBD (Fig. 6B), indicating that truncation of the pTRS1 carboxyl terminus did not result in PKR activation in the presence of pIRS1. However, the HCMV Δ PBD virus induced robust PKR and eIF2 α phosphorylation when pIRS1 was depleted (Fig. 6B), with a concomitant reduction in the overall levels of protein synthesis (Fig. 6C). In addition, HCMV Δ PBD infection induced

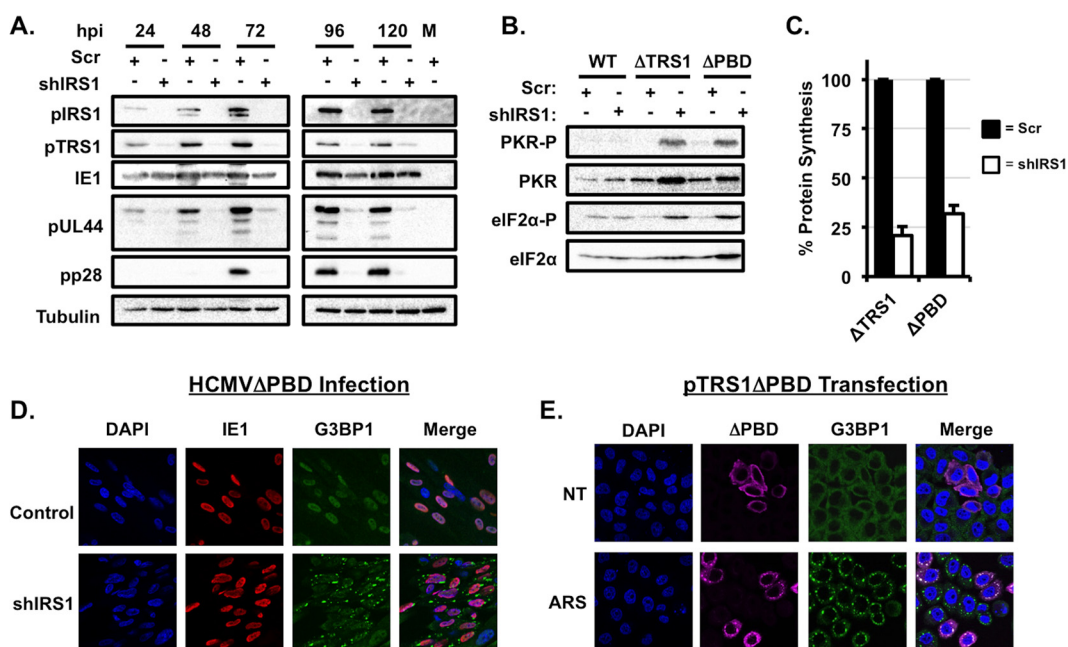


FIG 6 The pTRS1 PKR binding domain is necessary to antagonize PKR in the absence of pIRS1. (A) Control HFs (Scr) or shIRS1-HFs (shIRS1) were infected with the HCMV Δ PBD virus at an MOI of 1. Cells were harvested at the indicated times after infection, and the expression of the indicated proteins was measured by Western blotting ($n = 3$). (B) Control HFs or shIRS1-HFs were infected with the indicated virus as described for panel A. Cells were harvested at 24 h after infection and analyzed by Western blotting ($n = 3$). (C) Control HFs (filled bars) or shIRS1-HFs (open bars) were infected with HCMV Δ TRS1 or HCMV Δ PBD virus, and the amount of radiolabeled amino acids incorporated into acid-insoluble protein in 30 min was quantified 24 h after infection ($n = 3$). (D) Control HFs or shIRS1-HFs were infected with HCMV Δ PBD, and the presence of G3BP1 puncta was determined by indirect immunofluorescence 24 h after infection. Representative images from one of three independent experiments are shown. (E) Cells were transfected with a pTRS1 Δ PBD expression vector and treated and analyzed as described for Fig. 3. Representative results from one of three independent experiments are shown.

stress granule accumulation in the absence of pIRS1, but not in control cells (Fig. 6D), and expression of the pTRS1 Δ PBD protein was not sufficient to prevent arsenite-induced stress granule formation in PKR-deficient cells (Fig. 6E). We conclude that the carboxyl terminus of pTRS1 is required for efficient virus replication, even in the presence of pIRS1. Furthermore, our results suggest that the pTRS1 PKR binding domain is necessary to antagonize PKR during HCMV infection.

DISCUSSION

In this study, we developed a novel system for the study of pTRS1 and its functional domains in the context of viral infection. Using this system, we confirmed the results of a previous study (17) by showing that expression of either pTRS1 or pIRS1 is necessary for efficient HCMV replication and protein expression. We also found that in the absence of pTRS1 and pIRS1, HCMV infection activates the antiviral kinase PKR. We further show that antagonism of PKR by pTRS1 or pIRS1 is critical for ongoing protein synthesis during HCMV infection, as PKR depletion prevented eIF2 α phosphorylation and the shutoff of protein synthesis in the absence of pTRS1 and pIRS1. In addition, depletion of PKR restored the expression of HCMV proteins and rescued virus replication, in the absence of pTRS1 and pIRS1. We also identified a novel role for pTRS1 as an inhibitor of stress granule formation. Expression of either pTRS1 or pIRS1 was sufficient to suppress PKR-dependent stress granule accumulation during HCMV infection, and pTRS1 alone was sufficient to prevent PKR-independent stress granule formation in transfected cells. The ability of pTRS1 to antagonize PKR and prevent stress granule formation during infection required the previously identified PKR binding domain, suggesting that a direct interaction between pTRS1 and PKR is critical for efficient virus replication.

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

Our data also suggest pTRS1 may have additional roles in HCMV replication. The HCMV Δ PDB virus replicated less efficiently than wild-type virus even in the presence of pIRS1 (Fig. 5C). The HCMV Δ PDB mutant did not induce PKR or eIF2 α phosphorylation or stimulate stress granule formation when pIRS1 was present (Fig. 6B and D), and thus the truncated pTRS1 does not appear to act in a dominant negative manner toward pIRS1, at least in regard to PKR inhibition. These results suggest that pTRS1 may have additional roles in HCMV replication. Previous studies found that replication of an HCMV pTRS1/pIRS1 double mutant virus was rescued by the vaccinia virus E3L protein, a known PKR antagonist (17). However, E3L also suppresses additional aspects of the antiviral response (60–65). Perhaps pTRS1 shares these PKR-independent activities with E3L. The truncation could also impact the ability of pTRS1 to facilitate

packaging of viral DNA into nascent nucleocapsids (41) or perhaps limit the ability of pTRS1 to regulate autophagy (38, 39) or transactivate viral gene expression (40). Alternatively, the truncated pTRS1 isoform might partially inhibit pIRS1 function, resulting in low levels of PKR activation that are sufficient to limit viral mRNA translation. Additional studies in primary human cells completely devoid of PKR expression will be needed to determine what, if any, role pTRS1 plays in HCMV replication in addition to inhibiting PKR activation.

We also identified a novel role for pTRS1 in preventing stress granule formation. Previous studies found that stress granules do not form during HCMV infection (14, 56), despite the presence of dsRNAs (17). Our results provide a molecular mechanism for the lack of stress granules during HCMV infection, namely, the antagonism of PKR by pTRS1 and pIRS1. Stress granules did not form in infected cells expressing either pTRS1 or pIRS1; however, the absence of both proteins from infected cells triggered robust stress granule accumulation (Fig. 2C). Thus, expression of either pTRS1 or pIRS1 was necessary to prevent stress granule accumulation during HCMV infection. PKR depletion limited stress granule accumulation in infected cells in the absence of pTRS1 and pIRS1 (Fig. 4A), demonstrating that PKR activation triggers stress granule formation during HCMV infection. In addition, expression of pTRS1 outside the context of infection was sufficient to prevent stress granule accumulation in response to arsenite treatment (Fig. 3A), showing that additional viral proteins are not required for this activity. pTRS1 also blocked stress granule accumulation in response to arsenite treatment in PKR-deficient cells (Fig. 3B and C), suggesting that pTRS1 can prevent stress granule accumulation independent of its ability to antagonize PKR. Stress granules inhibit virus replication by sequestering viral RNAs and preventing their translation (12, 13, 57). While additional studies using depletion of specific stress granule components will be required to understand the impact of stress granule accumulation on HCMV infection, our results suggest that stress granules could similarly regulate HCMV protein synthesis.

Our data also provide new insight into the pTRS1 functional domains necessary for PKR inhibition during HCMV infection. The carboxyl-terminal 116 amino acids of pTRS1 contains a PKR binding domain that is necessary to complement growth in a vaccinia virus mutant lacking its major PKR antagonist, the E3L protein (18). Deletion of the pTRS1 PKR binding domain resulted in PKR activation, decreased viral protein expression, and limited virus replication in the absence of pIRS1 (Fig. 6). It was previously suggested that pTRS1 inhibits PKR in part by competing with PKR for binding to dsRNA (21). Our finding that PKR was autophosphorylated after infection with the HCMV Δ PDB virus does not contradict this conclusion but rather suggests that during HCMV infection the ability of pTRS1 to bind dsRNA is not sufficient to antagonize PKR. However, we cannot rule out that these mutations alter pTRS1 folding to abrogate its function. Structural analysis of pTRS1 and PKR will likely provide a better understanding of how these domains contribute to PKR inhibition. Nevertheless, in light of the failure of the HCMV Δ PDB pTRS1 mutant to antagonize PKR, it seems likely that pTRS1 binding to PKR is critical for PKR inhibition during HCMV infection. Confirmation of this hypothesis will require careful mapping of pTRS1 point mutants that specifically disrupt pTRS1 binding to PKR and the analysis of recombinant viruses carrying these mutations.

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