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Human cytomegalovirus TRS1 protein associates with the 7-methylguanosine mRNA cap and facilitates translation

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Abstract

Viruses rely on the host translation machinery for the synthesis of viral proteins. Human cells have evolved sensors that recognize viral RNAs and inhibit mRNA translation in order to limit virus replication. Understanding how viruses manipulate the host translation machinery to gain access to ribosomes and disable the antiviral response is therefore a critical aspect of the host:pathogen interface. In this study we used a proteomics approach to identify human cytomegalovirus (HCMV) proteins that might contribute to viral mRNA translation. The HCMV TRS1 protein (pTRS1) associated with the 7-methylguanosine (m^7G) mRNA cap, increased the total level of protein synthesis, and co-localized with mRNAs undergoing translation initiation during infection. pTRS1 stimulated translation of a non-viral reporter gene and increased the translation of a reporter containing an HCMV 5' untranslated region (5'UTR) to a greater extent. The preferential effect of pTRS1 on translation of an mRNA containing a viral 5'UTR required the pTRS1 RNA and PKR binding domains, and was likely the result of PKR inhibition. However pTRS1 also stimulated the total level of protein synthesis and translation directed by an HCMV 5'UTR in cells lacking PKR. Thus our results demonstrate that pTRS1 stimulates translation through both PKR-dependent and PKR-independent mechanisms.

Keywords

virology; translational control; PKR

1 Introduction

The regulation of mRNA translation is a critical interface between viruses and the infected host cell. Viral mRNAs are completely reliant on host machinery for the synthesis of viral

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

Most eukaryotic mRNAs initiate translation through the ordered assembly of translation initiation factors on their 5' terminus [1, 2]. The eIF4F complex binds to the 7-methylguanosine (m^7G) mRNA cap and recruits 40S ribosomal subunits to the 5' end of the transcript [3]. Bound 40S subunits then scan the 5' untranslated region (5'UTR) of the mRNA until reaching the translation initiation codon, where the eIF2 complex pairs a charged methionine tRNA to the AUG initiation codon triggering ribosome assembly and translation elongation. Multiple regulatory cues govern the initiation phase, which is the rate-limiting step in protein synthesis. The mTOR kinase promotes translation initiation by antagonizing the translation repressor 4EBP1, which blocks assembly of the host eIF4F translation initiation complex [4]. The phosphorylation of the eIF2α subunit of the eIF2 complex is an additional regulatory step in translation initiation. Cell stressors including ER stress, nutrient deprivation or viral infection activate kinases that phosphorylate and inactivate eIF2α, which inhibits its ability to recycle GDP to GTP and thus blocks its ability to participate in further rounds of translation initiation [5]. Together these regulatory steps ensure tight control of mRNA translation that is matched to the physiological state of the cell.

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

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

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

2 Materials and Methods

2.1 Cells, Viruses and Plasmids

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

pTRS1 expression plasmids were generously provided by A. Geballe (Univ. of Washington), and have been previously described [8, 9]. The full length or truncated UL99 5’UTR (nucleotides 144,872 to 145,348 or 145,115 to 145,348, respectively) was amplified by PCR from the HCMV AD169 genome (GenBank FJ527563.1) using primers containing HindIII or NotI sites flanking the viral sequence (UL99FL-F 5’ GATCATCAAGCTTGACGCCGCTGGCGGCGGCGCTGATC 3’. UL99trunc-F 5’ GATCATCAAGCTTATTTCCGCGACCTGCCTACCGTC 3’, UL99FL-R 5’ GATGATCCCATGGATCGGTAGGTTCGTCTTGCG 3’). The resulting PCR products were cloned into the HindIII and NotI sites of the pGL3 Control vector (Promega), and sequence verified. The UL44 5’UTR was PCR amplified and cloned using the following primers: UL44F 5’ GATCATCAAGCTTGGCTCGGCGCGGCTGTATTATTAG 3’, UL44R 5’ GATGATCCCATGGCCCGGACAGCGTGCAAGTCTC 3’. The IE1 5’UTR was amplified from infected cell cDNA using the following primers: IE1F 5’ GGAGGCCTAGGCTTTTGCAAAACAGATCGCCTGGAGACGCCATC 3’, IE1R 5’ TTATGTTTTTGGCGTCTTCCATCGTGTCAAGGACGGTGAGTCAC 3’.The PCR products was cloned by Gibson assembly into the Hind III and NotI sites of the pGL3-

Control vector. The GAPDH 5' UTR (accession# NM_002046.4) was synthesized (IDT) and cloned into the HindIII and NcoI sites of the pGL3-Control vector using Gibson assembly. Two oligonucleotides each containing half of the actin UTR (accession # NM_001101.3) were annealed and extended with Klenow to generate double stranded DNA, and then cloned into the HindIII and NcoI sites of pGL3-Control by Gibson cloning. The tubulin 5'UTR (accession # NM_178014.2) construct was generated in the same manner. All constructs were sequenced verified.

2.2 Analysis of m^7G-Sepharose-Bound Proteins

HFFs were infected with BAD*in*GFP virus at a multiplicity of 3 IU/cell. The affinity purification of proteins associated with the m^7G sepharose was performed similarly to that described in ref [18] with the following modifications. Cell lysates were prepared at 72 hours after infection and incubated with m^7G-sepharose for 1 hour in cap binding buffer (40mM HEPES, pH 7.6; 120mM NaCl; 1mM EDTA; 0.3% CHAPS). The beads were washed at room temperature three times in cap binding buffer, once in cap binding buffer containing 500 mM KCl and then incubated in elution buffer (100 mM m^7GTP, 100 mM KCl, 1 mM DTT, 50 mM HEPES, pH 7.6) for 1 hour to release proteins from the beads. Eluted proteins were reduced with DTT (10mM) and alkylated with iodoacetamide. The samples were then digested with trypsin (Promega), and desalted and purified on Zip-Tip C18 columns (Millipore). The peptides were then separated by reverse phase nanospray liquid chromatography on C18 resin with an Agilent 1200 series HPLC. Mass spectrometry was performed on a LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific). Protein identification was performed using Protein Discoverer software version 1.4 with the MASCOT or SEQUEST search algorithms against the most current version of the Uniprot Human database. A custom HCMV reference proteome was derived from the HCMV AD169 genome (Genbank # FJ527563). We allowed for 2 missed cleavages. Precursor mass tolerance was set to 10 ppm, and fragment mass tolerance to 0.6 Da. Dynamic modifications included oxidized methionine and carbamidomethylated cysteine. Target FDR was set to 0.05. To confirm the specificity we performed our capture with sepharose only (no m^7GTP) beads and identified co-purifying proteins by mass spectrometry as above. Proteins recovered from sepharose only beads that were also purified with m^7G-sepharose (Fig. S5) were considered likely contaminants, and are denoted as such in Table S1.

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

2.3 Partial Purification of pTRS1

293T cells were transfected with his epitope-tagged pTRS1 expression vector and harvested 48 hours after transfection. Cells were lysed in cap binding buffer (above) containing 5 mM imidazole and treated with micrococcal nuclease. pTRS1 was captured from the lysates using His-Select Affinity Gel (Sigma #114K70151) and eluted with 250mM imidazole in cap binding buffer. Protein concentration was determined using a BCA assay (Pierce). pTRS1 (5 pmol) in cap binding buffer was used in the m^7GTP agarose-binding assay.

2.4 Luciferase Assays

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

2.5 RNA Quantification

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

2.6 Protein Analyses

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

For immunofluorescence analysis, HFFs were infected with the indicated virus at a multiplicity of 0.5 IU/cell, and 72 hours later cells were fixed and stained as described previously [6]. In some cases cells were treated with thapsigargin (1 μM) or sodium arsenite (0.5 mM) for 1 hour prior to fixation to induce the formation of stress granules [21]. Images were captured on a Zeiss confocal microscope. Antibodies specific for G3BP1 (BD Biosciences), eIF4E, eF4G, or eIF4A (Cell Signaling; 1:100 dilution) and DAPI were used to visualize stress granules, translation initiation factors and nuclei, respectively.

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

2.7 Velocity Sedimentation Analysis of Ribosomal Subunits

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

2.8 Depletion or Disruption of PKR

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

pLX-sgRNA and pCW-Cas9 [24] vectors were obtained from Addgene. HeLa cells were transduced with pCW-Cas9 lentivirus and then clonally selected in the presence of puromycin (1μg/mL) to create a stable HeLa line expressing Cas9 under the inducible control of the Tet ON promoter (HeLa-Cas9). Oligonucleotides containing PKR-specific guide RNAs were (ref[24], 5' TTCAGCAGGTTTCTTCATGGAGG 3'; PAM motif underlined) cloned into pLX-sgRNA in the place of the AAVS1 target sequence to create pLX-sgPKR. pLX-sgPKR was transfected into HeLa-Cas9 and the cells were grown and passaged in the presence of doxycycline (1μg/ml) and blasticidin (1μg/ml). The Surveyor Assay (Transgenomic #706025) was used to confirm the introduction of mutations into the PKR gene. The cells were clonally selected by limiting dilution, and loss of PKR expression in individual clones was determined by Western blot.

3 Results

3.1 HCMV pTRS1 binds to the 5' mRNA cap

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

We confirmed the capture of both pTRS1 and pIRS1 on m^7G sepharose from infected cell lysates by Western blot assay. pTRS1 and pIRS1 bound to the m^7G resin throughout a time course of infection (Fig. 1*A*). pTRS1 and pIRS1 are identical in their first two thirds and appear to be functionally redundant [7, 9, 10], therefore we chose to focus our work on pTRS1. Free m^7GTP inhibited the retention of pTRS1 on the m^7G resin (Fig. 1*B*), demonstrating that pTRS1 recognized the m^7G moiety rather than non-specifically binding to the resin. pTRS1 expressed outside the context of infection in HeLa cells also bound the m^7G resin (Fig. 1*C*), demonstrating that additional viral proteins were not required for the

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

3.2 pTRS1 associates with mRNAs undergoing translation initiation

The association of pTRS1 with the mRNA cap suggested that pTRS1 might interact with the translation machinery during infection. We first determined if pTRS1 co-sedimented with ribosomal subunits in HCMV infected cells. Infected cells were treated with puromycin to dissociate ribosomes into 40 and 60S subunits, and cytoplasmic lysates were then subjected to centrifugation in linear sucrose gradients to separate the ribosomal subunits. pTRS1 co-sedimented with the 40S ribosomal protein rpS6 and the 18S rRNA in a 10 to 20% sucrose gradient (Fig. S1*B*), arguing that pTRS1 associates with a very large structure, presumably the 40S ribosomal subunit, during infection.

To determine if pTRS1 associates with active initiation complexes during infection, we asked if pTRS1 co-localized with stress granules in HCMV infected cells. Stress granules contain stalled translation initiation complexes bound to polyadenylated mRNA [21]. Translation initiation factors, the 40S ribosomal subunit, and mRNA all localize to stress granules upon exposure to stress-inducing agents that limit mRNA translation. However, 60S ribosomal subunits and active 80S ribosomes are excluded [29]. Stress granules were absent from cells infected with HCMV variants expressing a pTRS1-GFP or pIRS-GFP fusion protein as determined by the diffuse localization of the stress granule marker G3BP1 (Fig. 2*A*,*B*) [30]. However, stress granules could be induced to form in infected cells by the addition of the stress-inducing agents, sodium arsenite and thapsigargin, as judged by the re-localization of G3BP1 into discrete cytoplasmic puncta (Fig. 2 *A*,*B*). While pTRS1 displays a diffuse cytoplasmic localization in untreated cells, treatment with arsenite (Fig. 2*A*) or thapsigargin (Fig. 2*C*-*E*) induced pTRS1 re-localization into discrete puncta that co-stained for G3BP1. pIRS1 behaved similarly to pTRS1, displaying diffuse cytoplasmic localization in untreated cells, and co-localization with G3BP1 in discrete puncta in the presence of arsenite (Fig. 2*B*). The relocalization of GFP to stress granules was dependent on the fusion to pTRS1, as GFP did not co-localize with G3BP1 in cells infected with an HCMV strain expressing GFP that was not fused to a viral protein (Fig. 2*C*-*E*). We also found that host initiation factors including eIF4G, eIF4A, eIF4E, co-localized with pTRS1 in stress granules (Fig. 2*C*-*E*). Together these data suggest that pTRS1 associates with active translation initiation complexes in HCMV-infected cells.

3.3 pTRS1 stimulates translation

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

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

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

Previous work has shown that pTRS1 inhibits the double-stranded RNA-dependent protein kinase R (PKR) [7, 9]. Therefore an explanation for the preferential effect of pTRS1 on the

translation of the viral 5'UTR reporter could be that the full-length UL99 5'UTR activated PKR, while mRNAs transcribed from the pGL3-Control vector and the truncated viral 5'UTR vector did not. We therefore measured the effect of each reporter on PKR T446 auto-phosphorylation, a measure of its activation [31] in the presence or absence of pTRS1. Similar levels of phosphorylated PKR were observed following co-transfection of each reporter with a control plasmid expressing GFP, and in each case pTRS1 efficiently blocked PKR auto-phosphorylation (Fig. 3*F*).

We further explored this result by determining if the previously identified pTRS1 PKR binding domain was necessary for pTRS1 to stimulate translation and inhibit PKR activation. A pTRS1 mutant lacking its PKR binding domain (amino acids 679 to 795; TRS1-dPBD), [9] was less effective than wild-type pTRS1 at stimulating translation of the reporter mRNAs (Fig. 4*A*). A control experiment confirmed that RNA levels were not differentially affected (Fig. 4*A*). The pTRS1-dPBD mutant increased the overall rate of cellular protein synthesis similarly to wild-type pTRS1 (Fig. 4*B*), and bound the m^7G mRNA cap as well as wild type pTRS1, if not better (Fig. S3). In addition PKR auto-phosphorylation was inhibited equally as well by wild type pTRS1 or the pTR1-dPBD mutant (Fig. 4*C*). We conclude that in this setting the PKR binding domain is dispensable for inhibition of PKR auto-phosphorylation and increased levels of protein synthesis. However, the carboxyl-terminus of pTRS1 is necessary for the preferential stimulation of a reporter containing an HCMV 5'UTR.

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

We next determined if pTRS1 could stimulate translation in the absence of PKR. We first determined the effect of shRNA-mediated PKR depletion on pTRS1-stimulated reporter gene expression. In these experiments we used the UL99 5'UTR reporter, as the greatest of effect of pTRS1 was observed with this construct. pTRS1 expression resulted in a statistically significant increase in luciferase activity in PKR depleted cells (Fig. 5*A*, *C*). Interestingly wild type pTRS1 increased luciferase activity of all reporters tested to a similar extent in the absence of PKR (Fig. 5*B*), suggesting that a PKR-independent activity of pTRS1 results in a general increase in protein synthesis. In a second approach we measured the effect of pTRS1 on reporter gene expression and nascent protein synthesis in cells where

CRISPR/Cas9-mediated genome editing was used mutate the PKR gene, and thus abrogate its expression (Fig. 5*F*). pTRS1 increased luciferase activity and the overall level of protein synthesis in PKR null cells (Fig. 5*D*,*E*). We also compared the ability of pTRS1-dPBD and pTRS1-dRBD to stimulate translation in PKR null cells. Neither mutant stimulated translation to a significant extent (Fig. 5*D*). This data demonstrates that pTRS1 is capable of stimulating translation independent of its ability to inhibit PKR.

4 Discussion

In this study we found that HCMV pTRS1 stimulates mRNA translation through both PKR-dependent and PKR-independent mechanisms. pTRS1 associated with the mRNA m^7G cap (Fig. 1*A*-*E* and Table S1), co-sedimented with 40S ribosomal subunits (Fig. S1*B*) and co-localized with stress granules in HCMV infected cells (Fig. 2*A*-*E*). Expression of pTRS1 alone increased the overall level of protein synthesis (Fig. 3*B*, 4B, *4E and* 5*D*) and stimulated the translation of a reporter gene expressing an mRNA with a short, non-structured 5'UTR (Fig. 3*A*). pTRS1 stimulated the translation of reporters expressing mRNAs with different 5'UTRs to varying extents. In the comparison we studied, it increased expression from a reporter expressing an mRNA containing an HCMV 5'UTR to a two-fold (Fig. 3*C*, 3*E*, 4A and 4*D*) to 3-fold greater extent than the relatively short 5'UTR in a control reporter. Apparently, specific mRNA sequences or structures in viral transcripts influence responsiveness to pTRS1 expression. This preferential stimulatory effect requires the carboxyl terminal region of pTRS1 that contains its PKR binding domain (Fig. 4*A*) and a segment including the pTRS1 RNA binding domain (Fig. 4*D*). Inhibition of PKR activation contributed to the enhancement of translation by pTRS1, however pTRS1 also stimulated reporter gene expression and protein synthesis in cells lacking PKR (Fig. 5*A*, 5*C*, 5*D*). Thus pTRS1 enhances translation through both PKR-dependent and independent mechanisms.

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

Our finding that pTRS1 prevented PKR auto-phosphorylation (Fig. 4*B*) clearly supports previous studies demonstrating that pTRS1 is a potent PKR inhibitor [7, 9]. Binding to double-stranded RNA induces PKR homodimer formation, subsequent auto-phosphorylation and activation [32, 33]. The ability of pTRS1 to inhibit PKR auto-phosphorylation therefore

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

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

In either case, the fact that pTRS1 stimulates some reporters more than others in a PKR-dependent manner suggest that PKR preferentially inhibits the translation of specific mRNAs. Binding to double stranded RNA structures, rather than specific mRNA sequences, activates PKR [32]. Once activated PKR globally suppresses mRNA translation by inactivating the eIF2α initiation factor. Perhaps recruitment of PKR to structures in mRNA 5'UTRs results in the local activation of PKR, resulting in the preferential inhibition of PKR-bound RNAs. Such a scenario has previously been suggested for the preferential inhibition of specific cellular mRNAs by PKR [35, 36]. This possibility could explain how an undetectable level of PKR auto-phosphorylation might have a biologically significant effect. pTRS1 may bind similar RNA structures and prevent PKR recruitment, thus preventing local PKR activation. Additional studies to define the complement of RNAs bound by PKR and pTRS1 will be needed to clarify the mechanism of preferential effects of pTRS1 and PKR on mRNA translation.

We also found that pTRS1 stimulated mRNA translation in a PKR-independent manner. pTRS1 increased the expression of each of the tested reporters in PKR depleted or PKR deficient cells (Fig. 5*A*, *B*), and increased the overall level of protein synthesis in the absence of PKR (Fig. 5*C*). Thus pTRS1 stimulates translation in both a PKR-dependent and PKR-independent manner. How might pTRS1 stimulate translation independent of its antagonism of PKR? We propose that pTRS1 preferentially associates with viral mRNAs in a sequence-dependent manner and bridges their association with the translation machinery. Our data suggests that pTRS1 acts at the initiation step of mRNA translation, as pTRS1

associates with the m^7G mRNA cap (Fig. 1*A-E*), co-sediments with 40S ribosomal subunits (Fig. S1*B*) and co-localizes with stress granules containing stalled translation initiation complexes in infected cells (Fig. 2*A-E*). Perhaps pTRS1 acts in concert with host translation initiation complexes to stimulate translation. Alternatively, pTRS1 could be part of an uncharacterized initiation complex. Based on the known role of pTRS1 in limiting PKR activity this may also allow for the continued translation of viral mRNAs in the presence of the host antiviral response. However, our data do not exclude the possibility that pTRS1 may also regulate additional aspects of the antiviral response that might act to limit protein synthesis. For example pTRS1 could bind to dsRNA and limit OAS activation, and thus block RNaseL-dependent mRNA decay. Additional biochemical studies will be needed to define the molecular interactions underlying PKR-independent pTRS1 activity.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

HCMV	human cytomegalovirus
m^7G	7-methylguanosine
5'UTR	5' untranslated region
PKR	double stranded RNA-dependent protein kinase
eIF4F	eukaryotic translation initiation factor 4F
S	Svedberg units
eIF2	eukaryotic translation initiation factor 2
mTOR	mechanistic target of rapamycin
4EBP1	eukaryotic translation initiation factor 4E binding protein 1

eIF2α	eukaryotic translation initiation factor 2 subunit α
GMP	guanosine monophosphate
GTP	guanosine triphosphate
PERK	RNA-dependent protein kinase (PKR)-like ER kinase
HFFs	human foreskin fibroblasts
BAC	bacterial artificial chromosome
GFP	green fluorescence protein
TCID50	tissue culture infectious dose 50%
IU	infectious units
BCA	bicinchonic acid
TCA	trichloroacetic acid
G3BP1	GTPase activating protein (SH3 domain) binding protein 1
eIF4E	eukaryotic translation initiation factor 4E
eIF4G	eukaryotic translation initiation factor 4G
eIF4A	eukaryotic translation initiation factor 4A
PABP1	poly(A) binding protein, cytoplasmic 1
TRCN	The RNAi Consortium
CRISPR	clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein Cas9
Tet	tetracycline
PAM	protospacer adjacent motif
AAVS1	adeno-associated virus integration site 1
CBC	cap binding complex
RBD	RNA binding domain
PBD	PKR binding domain
MN	Micrococcal Nuclease
WCL	whole cell lysate

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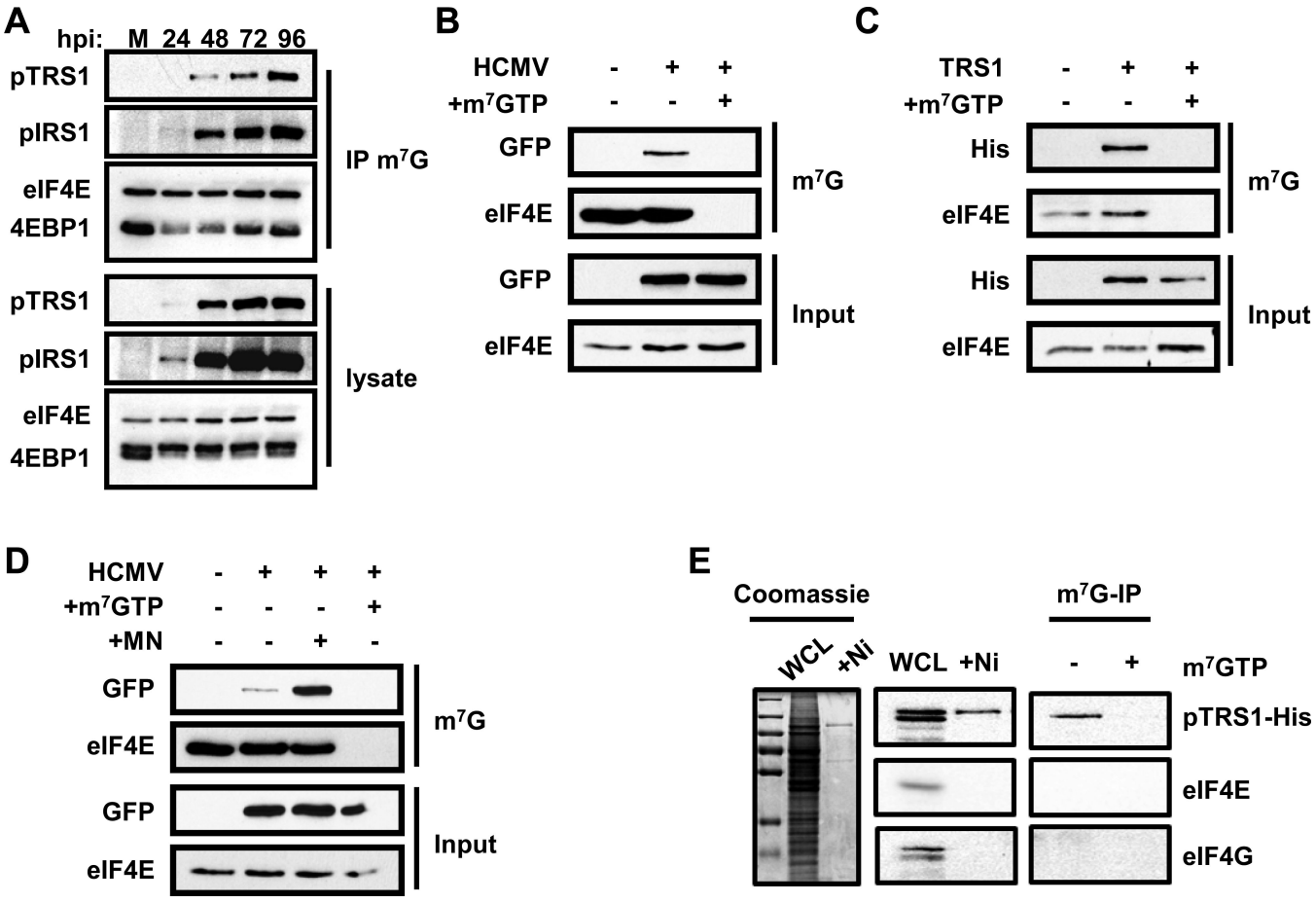

Figure 1.

HCMV pTRS1 associates with the m^{7}G mRNA cap. (*A*) Cells were infected with BAD*in*GFP (3 IU/cell) and proteins bound to m^{7}G sepharose were analyzed by Western blot with the indicated antibodies. Samples were harvested at the indicated time after infection. (*B*) Lysates from cells infected with BAD*in*TRS1GFP (72 hpi) were incubated with m^{7}G sepharose in the presence of free m^{7}GTP (+m^{7}GTP). (*C*) HeLa cells were transfected with a expression vector encoding pTRS1 fused to the his epitope and analyzed as in B. (*D*) Same as in B, except that one sample was treated with micrococcal nuclease (+MN) prior to incubation with m^{7}G sepharose. (*E*) Partially purified pTRS1 incubated with m^{7}G sepharose and analyzed as in B. (Left) Coomassie stained acrylamide gel of Whole Cell Lysate (WCL) and partially purified pTRS1 (+Ni) (Right).(Middle) Western blot of WCL and partially purified pTRS1 (Right) Western blot of m^{7}G-associated proteins following incubation with partially purified pTRS1.

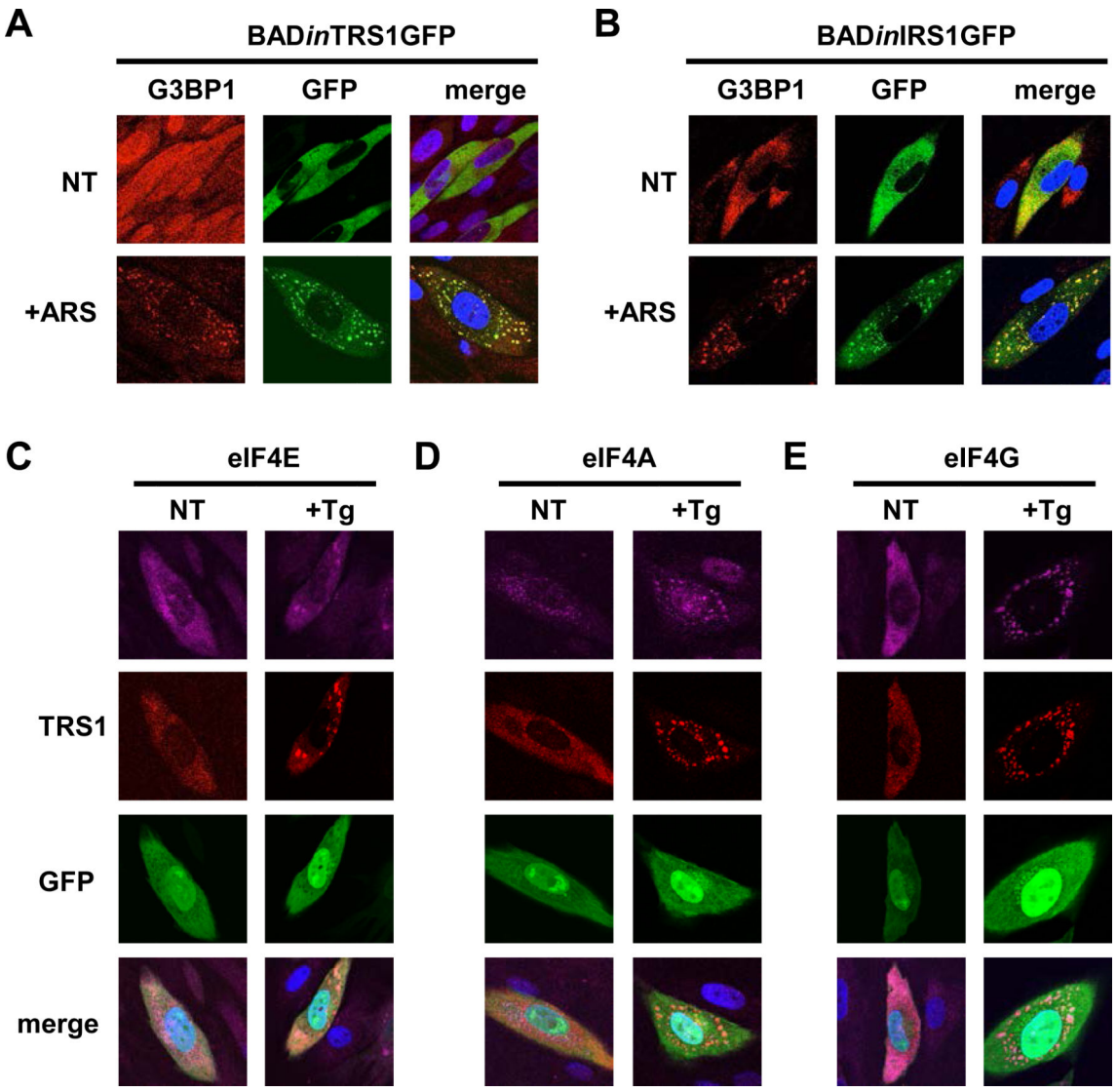

Figure 2.

pTRS1 associates with active translation initiation complexes during HCMV infection. HFFs were infected with BAD*in*TRS1GFP (*A*), BAD*in*IRS1GFP (*B*) or BAD*in*GFP (C-E) at a multiplicity of 0.5. (*A & B*) Cells were treated with sodium arsenite (+ARS, 0.5 mM) or vehicle for two hours at 72 hpi. Immunofluorescence confocal microscopy was used to visualize GFP (green), the stress granule marker protein G3BP1 (red) or nuclei (DAPI, blue). (*C-E*) Cells were infected with BAD*in*GFP and treated with thapsigargin (+Tg, 2 μg/ml) for one hour before fixation at 72 hpi. Confocal microscopy was used as above to visualize pTRS1 (red) together with eIF4E, eIF4G or eIF4A (purple). DAPI (blue) was used to visualize nuclei, and GFP (green) was used to identify infected cells.

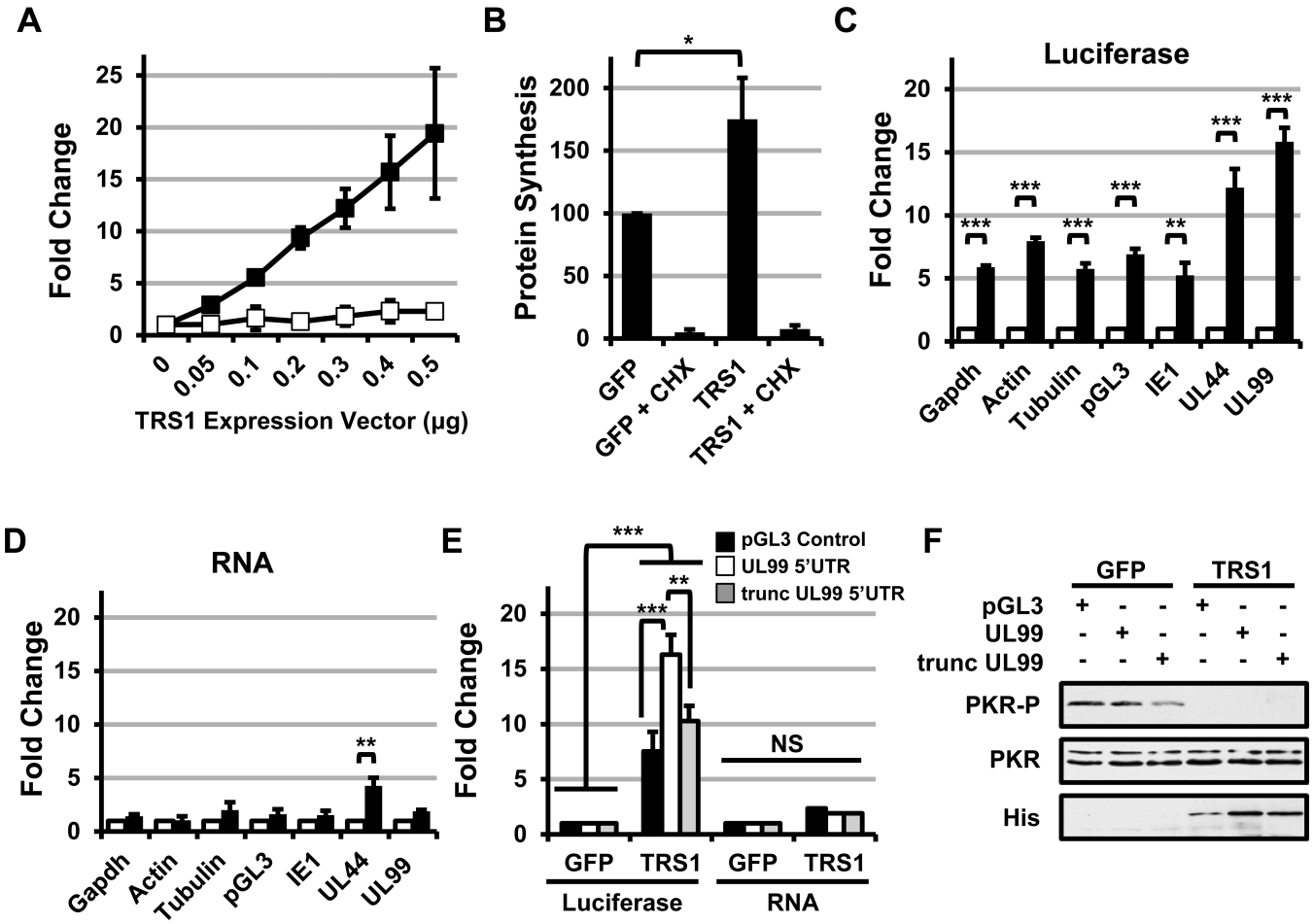

Figure 3.

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

blot using antibodies to PKR phosphorylated on T446 (PKR-P), total PKR, or the his epitope (TRS1). For all panels * = p-value < 0.05; ** = p value < 0.01; *** = p value < 0.001.

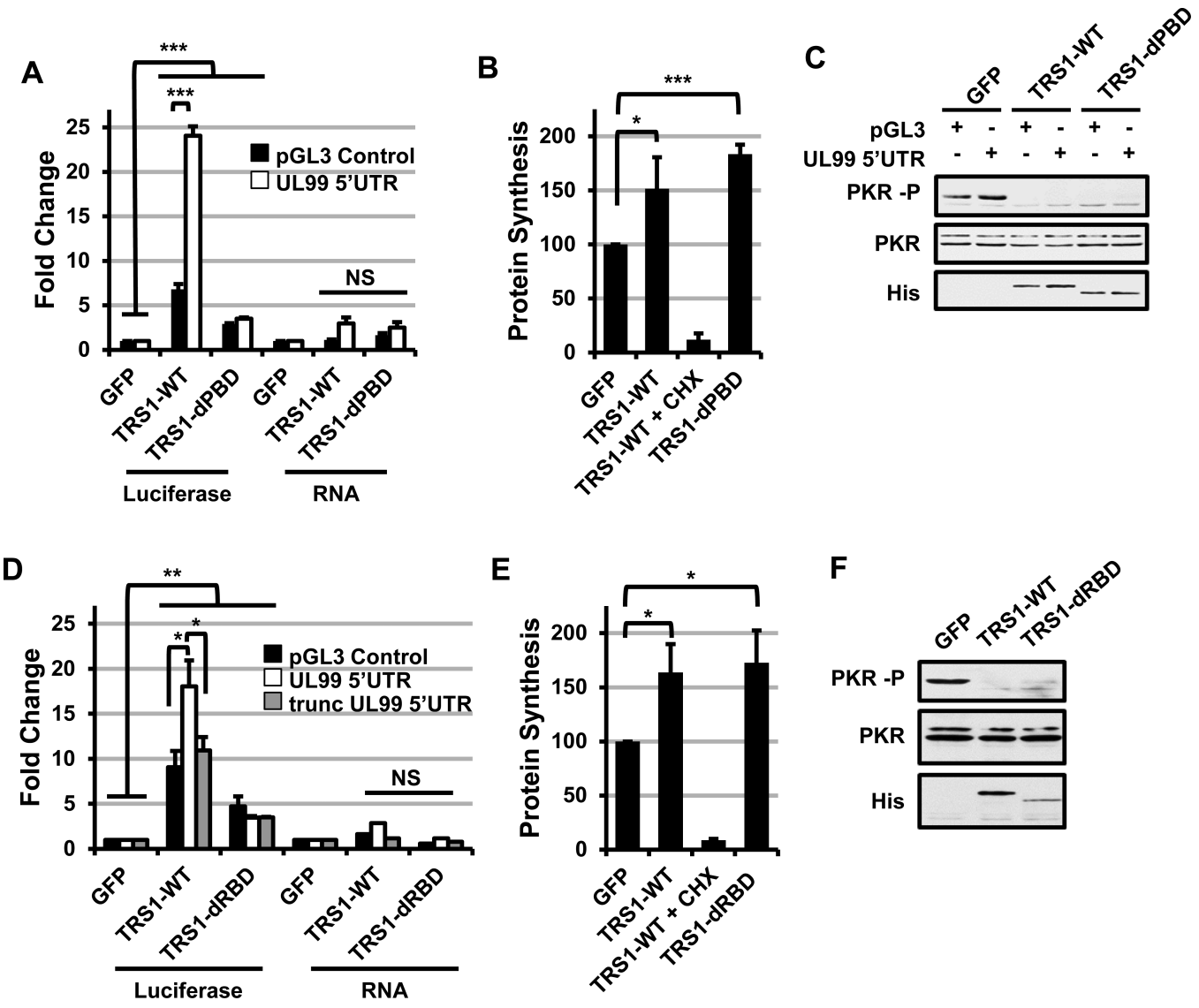

Figure 4.

The pTRS1 PKR binding and RNA binding domains are dispensable to inhibit PKR auto-phosphorylation, but necessary to preferentially stimulate translation. (*A*) Cells were transfected as in figure 3 with either pGL3-Control (black bars) or the UL99 5'UTR luciferase construct (open bars) together with either a control vector (GFP) or the indicated pTRS1 expression constructs. The graph shows the fold change in luciferase activity (left side) and RNA abundance (right side) in pTRS1 expressing cells relative to control cells expressing GFP (n=3). (*B*) The rate of nascent protein synthesis in cells expressing wild type pTRS1 or pTRS1 lacking the PKR binding domain was measured as in figure 3B (n=3). (*C*) Cells were transfected as in figure 3 with pGL3-Control vector or the UL99 5'UTR reporter together with a control vector (GFP) or the indicated pTRS1 constructs. PKR phosphorylation (PKR-P) was measured by Western blot. (*D*) Same as in *A*, except a pTRS1 mutant lacking the RNA binding domain (333ng) and the truncated UL99 5'UTR pGL3-Control construct was included (n=3). (*E*) The rate of nascent protein synthesis in cells

expressing wild type pTRS1 or pTRS1 lacking the RNA binding domain was measured as in figure 3B (n=3). (*F*) Cells were transfected with the UL99 5'UTR reporter together with a control vector (GFP) or the indicated pTRS1 construct. PKR auto-phosphorylation was measured by Western blot. For all panels * = p-value < 0.05; ** = p value < 0.01; *** = p value < 0.001.

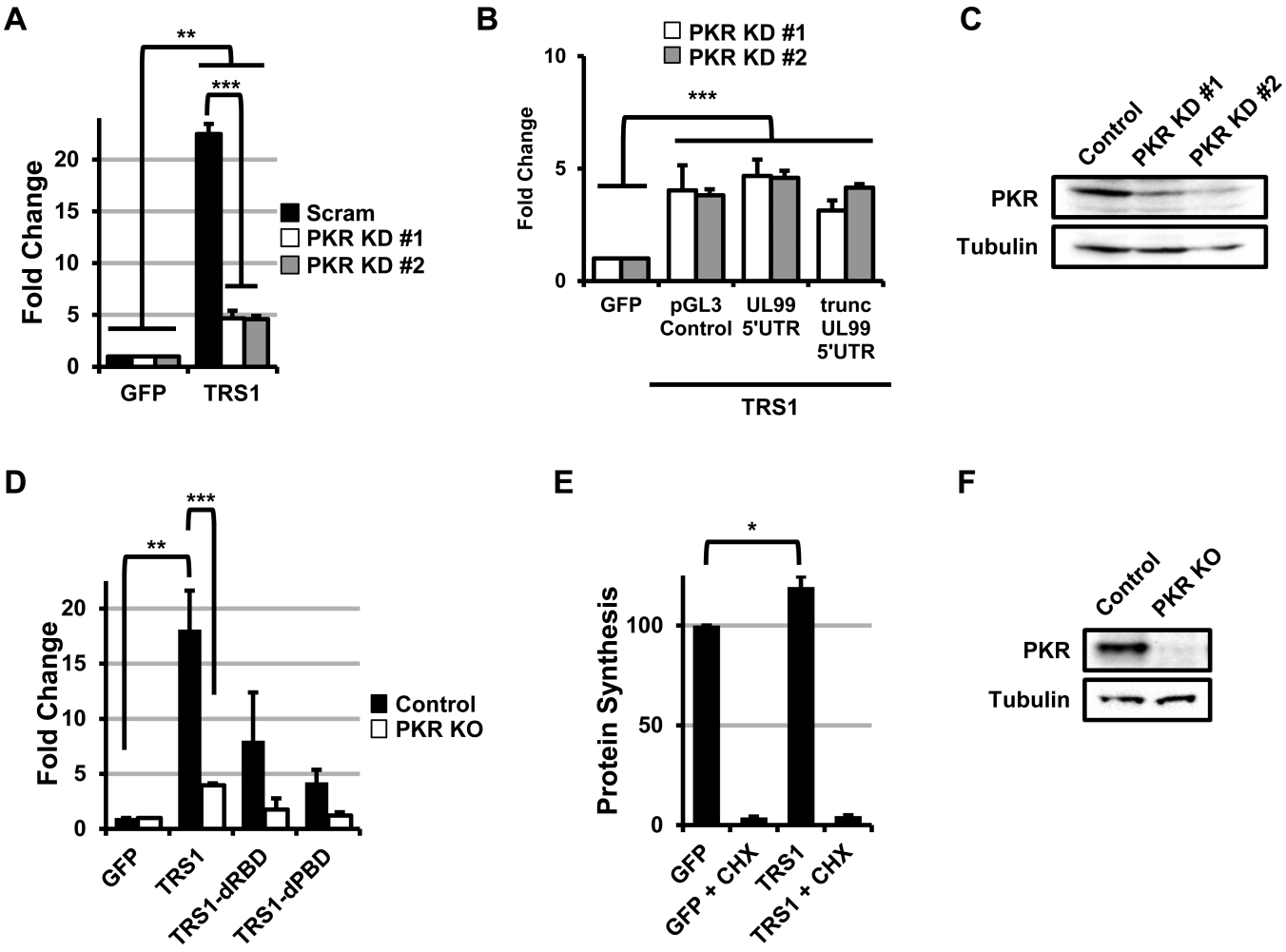

Figure 5.

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