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Alphacoronavirus Transmissible Gastroenteritis Virus nsp1 Protein Suppresses Protein Translation in Mammalian Cells and in Cell-Free HeLa Cell Extracts but Not in Rabbit Reticulocyte Lysate

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The nsp1 protein of transmissible gastroenteritis virus (TGEV), an alphacoronavirus, efficiently suppresses protein synthesis in mammalian cells. Unlike the nsp1 protein of severe acute respiratory syndrome coronavirus, a betacoronavirus, the TGEV nsp1 protein was unable to bind 40S ribosomal subunits or promote host mRNA degradation. TGEV nsp1 also suppressed protein translation in cell-free HeLa cell extract; however, it did not affect translation in rabbit reticulocyte lysate (RRL). Our data suggested that HeLa cell extracts and cultured host cells, but not RRL, contain a host factor(s) that is essential for TGEV nsp1-induced translational suppression.

Coronaviruses (CoVs) primarily cause respiratory and enteric diseases in vertebrates (24). Although most human CoVs cause mild respiratory tract diseases, severe acute respiratory syndrome (SARS) coronavirus (SCoV) is the etiologic agent of SARS. CoVs, which carry a large single-stranded positive-sense RNA genome of ~30 kb, are classified into the groups alpha, beta, and gamma. In infected cells, CoV gene expression is initiated by the translation of two large polyproteins encoded by gene 1 from the incoming viral genomic RNA. These polyproteins are processed into 16 mature proteins (nsp1 to nsp16) by two or three viral proteinases, in the case of alphacoronavirus and betacoronavirus (16, 18). Most of the gene 1 proteins play critical roles in viral RNA synthesis (3–5, 7–9, 14, 17, 18, 20), while some have other biological functions (1, 6, 11, 12, 19).

The nsp1 protein of betacoronaviruses inhibits host gene expression. SCoV nsp1 uses a two-pronged strategy to inhibit host translation/gene expression by first binding to the 40S ribosomal subunit and then inactivating the translation activity of these 40S subunits (10). The nsp1-40S ribosome complex then induces the modification of the 5′ regions of capped mRNA templates and renders these template RNAs translationally incompetent. Importantly, SCoV nsp1 suppresses host innate immune functions by inhibiting type I interferon (IFN) expression in infected cells (15) and host antiviral signaling pathways (23), suggesting its important role in SCoV virulence. Nsp1 proteins of bat CoVs also suppress host translation, while some exert host translational suppression without degrading host mRNAs (22). Mouse hepatitis virus (MHV) nsp1 protein also suppresses host gene expression. A recombinant MHV lacking the nsp1 gene is severely attenuated in infected mice, yet mutant virus replication and spread are restored to wild-type (wt) virus levels in type I IFN receptor-deficient mice (26), indicating that MHV nsp1 interferes efficiently with the type I IFN system.

Alphacoronaviruses encode nsp1 proteins of ~9 kDa, which are substantially smaller than the ~20-kDa nsp1 proteins of betacoronaviruses. The nsp1 proteins of alphacoronaviruses and betacoronaviruses have no amino acid sequence similarities to each other or with known host proteins. Nonetheless, the expression of nsp1 of human CoV 229E, an alphacoronavirus, suppresses the expression of reporter genes under the control of beta IFN (IFN-β), IFN-stimulated response element, and simian virus 40 (SV40) promoters (26). Within the alphacoronaviruses, the amino acid sequence of the human CoV 229E nsp1 protein is moderately similar to those of nsp1 proteins of human CoV NL63 (60%) and porcine epidemic diarrhea virus (52%), but it is different from that of transmissible gastroenteritis virus (TGEV) (32%). In contrast, the TGEV nsp1 protein has 97% and 93% amino acid sequence identities with the nsp1 proteins of porcine respiratory coronavirus and feline infectious peritonitis virus, respectively. The present study used TGEV nsp1 as a model system to explore how the nsp1 protein of alphacoronavirus suppresses host gene expression.

To determine if TGEV nsp1 suppresses host gene expression, we cotransfected human embryonic kidney 293 cells or swine testis (ST) cells, the latter of which supporting TGEV replication, with the plasmid pRL-SV40 expressing the SV40 promoter-driven Renilla luciferase (rLuc) gene, together with one of the following plasmids: pCAGGS carrying the gene for chloramphenicol acetyltransferase (CAT), the nsp1 protein of TGEV (Purdue strain), the SCoV nsp1 protein, or the SCoV nsp1 mutant (SCoV nsp1-mt) protein; the SCoV nsp1-mt does not suppress host gene expression (10, 15). All proteins, except CAT, contained a C-terminal myc epitope tag. Both TGEV nsp1 and SCoV nsp1 efficiently suppressed the reporter gene

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expression in both cell lines (Fig. 1A). The low-level accumulation of the nsp1 proteins of TGEV and SCoV suggested that these nsp1 proteins inhibited their own expression. Furthermore, transfection of RNA expressing the C-terminal myc epitope-tagged TGEV nsp1 or SCoV nsp1, but not CAT or SCoV nsp1-mt, in 293 cells resulted in potent suppression of host protein synthesis in the presence or absence of actinomycin D (Act D), an inhibitor of host transcription (Fig. 1B). Replication of TGEV in ST cells caused cytopathic effects in ~30% of cells and floating of ~10% cells at 14 h postinfection. Following metabolic radiolabeling of infected cells and mock-infected cells with [35S]methionine and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the same amounts of intracellular proteins, we

FIG. 1. Effect of TGEV nsp1 on host protein synthesis. (A) HEK293 cells (left) or ST cells (right) were cotransfected with plasmid pRL-SV40 encoding the rLuc reporter gene downstream of the SV40 promoter and one of the following plasmids: pCAGGS-CAT (CAT), pCAGGS-TGEV nsp1-myc (TGEV), pCAGGS-SCoV nsp1-myc (SCoV), and pCAGGS-SCoV nsp1-mt (SCoVmt), expressing the CAT, TGEV nsp1, SCoV nsp1, and SCoV nsp1-mt proteins, respectively. All the expressed proteins, except CAT, had a C-terminal myc epitope tag. At 24 h posttransfection, cell lysates were prepared and subjected to rLuc assay. Error bars show standard deviations (SD) of results from three independent experiments. Cell extracts were also subjected to Western blot analysis by using anti-myc antibody (top) or antiaxin antibody (bottom). (B) 293 cells were transfected with in vitro-synthesized capped and polyadenylated CAT-myc RNA (lane 1), TGEV nsp1-myc RNA (lane 2), SCoV nsp1-myc RNA (lane 3), and SCoV nsp1-mt–myc RNA (lane 4) using TransIT-mRNA (Mirus). At 1 h post-RNA transfection, cells were mock treated (Act D−) or treated with 4 μg/ml Act D (Act D+) for 7 h. Cells were metabolically labeled with 50 μCi/ml of [35S]methionine for 30 min, and cell lysates were subjected to SDS-PAGE (left). The accumulation of expressed myc-tagged proteins was examined by Western blot analysis using anti-myc antibody (right). (C) ST cells were mock infected (M) or infected (I) with the Purdue strain of TGEV at a multiplicity of infection of 5. Cells were metabolically labeled with 100 μCi/ml of [35S]methionine for 30 min at 4, 6, 8, 10, and 14 h postinfection (hpi). Cell extracts were resolved with 12% SDS-PAGE, and the gels were exposed to X-ray film (autoradiography) or stained with colloidal Coomassie blue (CCB staining). Densitometry analysis was performed to determine the levels of host protein synthesis. The boxes represent the regions of the gel used for densitometry analysis, and the numbers below the lanes of infected cells represent the relative radioactivity compared with that of mock-infected cells at the indicated time postinfection.
found host protein synthesis to be inhibited in the infected cells (Fig. 1C); we have performed two independent experiments and obtained similar results. These data are consistent with the possibility that TGEV nsp1 plays a role in suppressing host gene expression during a viral infection.

Next, we tested the effects of TGEV nsp1 expression on the stability of host mRNAs by transfecting 293 cells with RNA transcripts expressing TGEV nsp1 in the presence or absence of Act D. We used transcripts expressing CAT, SCoV nsp1, or the SCoV nsp1-mt as controls. As expected (11, 22), SCoV nsp1 induced extensive degradation of host mRNAs (Fig. 2A).

In contrast, TGEV nsp1 expression did not reduce the abundance of these mRNAs, in both the presence and absence of Act D, a finding indicating that TGEV nsp1 was unable to promote host mRNA degradation. Recombinant SCoV nsp1 induces RNA cleavage at or near the 3′ end of the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) in Ren-EMCV-FF RNA, a dicistronic RNA carrying the EMCV IRES between the upstream rLuc gene and the downstream firefly luciferase (fLuc) gene (10), in rabbit reticulocyte lysate (RRL). As shown in Fig. 2B, SCoV nsp1, but not TGEV nsp1, also induced cleavage of expressed Ren-EMCV-FF RNA (Fig. 2B) in 293 cells. These data suggested that TGEV nsp1 suppressed host gene expression without promoting extensive host mRNA degradation.

TGEV nsp1-mediated translational suppression was further examined using in vitro translation assays in RRL or HeLa S10 extract (2, 21). We expressed the TGEV nsp1 as a glutathione S-transferase (GST) fusion protein in Escherichia coli, followed by the removal of GST to generate recombinant TGEV nsp1. Recombinant SCoV nsp1 and SCoV nsp1-mt proteins were also generated (10). SCoV nsp1 efficiently suppressed the translation of capped and polyadenylated GLA mRNA, which has the 5′ noncoding region of β-globin mRNA upstream of the rLuc gene, in RRL (Fig. 3A) or in HeLa cell extracts (Fig. 3B). Surprisingly, TGEV nsp1 efficiently suppressed translation of GLA mRNA in HeLa cell extract but not in RRL (Fig. 3A and B). Longer incubation of the samples did not alter the outcome of the results in either of the extracts (data not shown). The recombinant TGEV nsp1 protein, generated in insect cells using baculovirus-based expression, also suppressed translation in HeLa cell extract but not in RRL (data not shown), suggesting that purified TGEV nsp1 proteins from both E. coli and insect cells had similar functional conformations. It was also possible that TGEV nsp1 might have induced phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) by activating the heme-regulated eIF2α kinase, leading to translational suppression in HeLa cell extract; in commercially available RRL, hemin is added to inhibit the activation of the heme-regulated eIF2α kinase. However, this possibility was unlikely because there was no increase in the level of phosphorylated eIF2α in HeLa cell extract that was incubated with TGEV nsp1 and GLA mRNA (Fig. 3C).

While translation initiation mediated by the cricket paralysis virus (CrPV) IRES requires only the 40S and 60S ribosomes (13, 25), both cap-dependent and CrPV IRES-mediated translation require the same factors for the subsequent steps after translation initiation. Incubation of Ren-CrPV-FF (10), a bicistronic RNA carrying the CrPV IRES between the upstream rLuc gene and the downstream fLuc gene, with TGEV nsp1 in HeLa cell extract resulted in the suppression of cap-dependent translation but not CrPV-mediated translation (Fig. 3D). These data suggest that TGEV nsp1 suppressed translation at the initiation step but did not affect the postinitiation steps in HeLa cell extract. Similar experiments using dicistronic RNA carrying the hepatitis C virus (HCV) IRES showed that TGEV nsp1 suppressed HCV IRES-mediated translation but less efficiently than cap-dependent translation (Fig. 3E).

Based on assigning the values obtained for HCV IRES-driven fLuc and cap-dependent rLuc activities in the presence of GST as 100%, SCoV nsp1 inhibited both fLuc and rLuc activities by ~99%. TGEV nsp1 inhibited rLuc activity by ~97% and fLuc activity by ~70%. In contrast, TGEV nsp1 suppressed EMCV IRES-mediated translation as efficiently as it suppressed cap-dependent translation (Fig. 3F).

We then performed a series of experiments to examine how TGEV nsp1 might induce translational suppression. In contrast to SCoV nsp1, which binds to 40S ribosomes to exert its
biological functions (10), TGEV nsp1 (expressed in 293 cells following transfection of mRNA transcripts) did not bind to 40S ribosomes (Fig. 4A). In HeLa cell extract, the translation levels of the uncapped GLA mRNA and the nonpolyadenylated GLA mRNA were about 1/4 (Fig. 4B) and 1/10 (Fig. 4C) of those of capped and polyadenylated GLA mRNA, respectively. TGEV nsp1 could suppress translation of both uncapped and nonpolyadenylated GLA mRNAs (Fig. 4B and C), demonstrating that the presence of the 5' cap and the 3' poly(A) tail in GLA mRNA were not required for the TGEV nsp1-mediated translational suppression. The data implied that the cap-binding protein eIF4E and the poly(A)-binding protein were not involved in TGEV nsp1-mediated translational suppression, which is consistent with the observation that TGEV nsp1 efficiently suppressed translation mediated by the EMCV IRES (Fig. 3F), which does not require eIF4E for translation initiation (13). TGEV nsp1 also suppressed translation in both a mixture of 80% RRL and 20% HeLa S10 extract and one of 80% RRL and 20% HeLa S100 postribosomal supernatant, the latter of which prepared by removing the ribosomes from the HeLa S10 extract by centrifugation at 100,000 × g for 3 h (Fig. 4D). These findings indicated that
In this communication, we showed that TGEV nsp1 suppressed the initiation step of translation without binding to 40S ribosomes. Unlike SCoV nsp1, TGEV nsp1 did not promote extensive host mRNA degradation. The TGEV nsp1-induced translational suppression did not require the presence of the 5' cap and 3' poly(A) tail in mRNAs. Remarkably, TGEV nsp1 suppressed translation in mammalian cells and in HeLa cell extract but not in RRL. The data showing that TGEV nsp1 failed to suppress translation in RRL strongly suggest that nsp1 neither directly inhibited the biological activities of the canonical translation initiation factor(s), 40S ribosomes, or 60S ribosomes nor bound to mRNA templates to make them translationally inactive. We hypothesize that RRL lacks a factor(s) that is needed for TGEV nsp1-mediated translational suppression, and this putative factor does not appear to be a canonical translation initiation factor and is present in 293 cells, HeLa S10 extract, and postribosomal HeLa S100 supernatant. Such a factor may bind to TGEV nsp1 and activate the translation suppression function of TGEV nsp1. Alternatively, this putative host factor may act like an inhibitor by interacting with a translation initiation factor. The binding of TGEV nsp1 to this putative host factor may result in the stabilization of the interaction between such a factor and the target translation initiation factor, leading to translational suppression.

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FIG. 4. Characterization of TGEV nsp1-induced translational suppression. (A) 293 cells were independently transfected with capped and polyadenylated GLA mRNA transcripts encoding CAT-myc (CAT), TGEV nsp1-myc (TGEV nsp1), and SCoV nsp1-myc (SCoV nsp1) proteins. At 7 h posttransfection, total cell lysates were prepared and immunoprecipitated with mouse anti-myc antibody (Millipore), followed by stringent washing with high-salt buffer (10 mM HEPES [pH 7.4], 500 mM KCl, 2.5 mM MgCl2 and 1 mM dithiothreitol [DTT]), as described previously (10). Samples were analyzed by Western blotting (WB), using an antibody against S6 protein (anti-S6), which is a component of the 40S ribosome (Cell Signaling). The myc-tagged proteins were detected by using rabbit anti-myc antibody (Cell Signaling) as the primary antibody and horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG light-chain-specific antibody as the secondary antibody (Jackson ImmunoResearch) (anti-myc). (B) Capped or uncapped GLA mRNA transcripts were incubated in HeLa S10 extracts for 30 min in the presence of 1 μg of purified GST, SCoV nsp1 (SCoV), or TGEV nsp1 (TGEV). The rLuc activities were measured for capped (gray bars) and uncapped (white bars) GLA mRNA. (C) Capped GLA mRNA transcripts with poly(A) tails (gray bars) or those lacking poly(A) tails (white bars) were incubated in HeLa S10 extracts for 30 min in the presence of purified GST, SCoV nsp1 (SCoV), or TGEV nsp1 (TGEV), and rLuc activities were measured. (D) Capped and polyadenylated GLA mRNA transcripts were incubated in RRL supplemented with 20% HeLa S10 extract (left) or with 20% HeLa S100 extract (right) for 10 min in the presence of 1 μg of purified GST, SCoV nsp1 (SCoV), or TGEV nsp1 (TGEV), and rLuc activities were measured. Error bars show SD of results from three independent experiments.