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MERS coronavirus nsp1 participates in an efficient propagation through a specific interaction with viral RNA

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ABSTRACT

MERS-CoV is the only lethal human CoV still endemic in the Arabian Peninsula and neither vaccine nor therapeutics against MERS-CoV infection is available. The nsp1 of CoV is thought to be a major virulence factor because it suppresses protein synthesis through the degradation of host mRNA. In contrast, viral RNA circumvents the nsp1-mediated translational shutoff for an efficient propagation. In this study, we identified amino acid residue in MERS-CoV nsp1 that differ from those of SARS-CoV nsp1, and that appear to be crucial for circumventing the translational shutoff. In addition, reverse genetics analysis suggested the presence of a *cis*-acting element at the 5′-terminus of the nsp1-coding region, which contributes to the specific recognition of viral RNA that is required for an efficient viral replication. Our results suggest the CoVs share a common mechanism for circumventing the nsp1-mediated translational shutoff.

1. Introduction

Coronaviruses (CoVs) are pathogens that infect a large variety of vertebrate animals, resulting in mainly respiratory and enteric diseases (Weiss and Navas-Martin, 2005). Most human CoVs are causative agents of mild illness and common cold (Bradburne et al., 1967; van der Hoek et al., 2006, 2004; Woo et al., 2005). However, an epidemic of severe acute respiratory syndrome (SARS) occurred in China in 2002, and the causative agent was designated as SARS-CoV (Drosten et al., 2003; Ksiazek et al., 2003). Ten years after the SARS outbreak, another highly pathogenic human CoV, designated as Middle East respiratory syndrome (MERS)-CoV, emerged in Saudi Arabia (Zaki et al., 2012). Many patients infected with MERS-CoV have been identified, most of them in the Arabian Peninsula, although MERS-CoV has also spread to several other countries in North Africa, Europe, and Asia (Bermingham et al., 2012; Buchholz et al., 2013; Cowling et al., 2015; Müller et al., 2014; Mailles et al., 2013; Perera et al., 2013; Reusken et al., 2014). In humans, MERS-CoV infection causes high fever, cough, and pneumonia, and is transmitted through close contact with infected dromedary camels (Memish et al., 2014; Reusken et al., 2013).

CoVs are enveloped viruses possessing a large single-stranded and positive-sense RNA genome (~ 32 kb). The 5′ two-thirds of the CoV genome consists of two overlapping open reading frames (ORFs 1a and 1b) that encode non-structural proteins (nsps). The other one-third of the genome consists of ORFs encoding structural proteins, including spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins, and accessory proteins (Woo et al., 2010). Upon infection of CoV into host cells, the translation of two precursor polyproteins, pp1a and pp1ab, occurs and these polyproteins are cleaved into 15 or 16 nsps by viral proteases, papain-like protease (PL^{pro}: nsp3) and 3C-like protease ($3CL^{pro}$: nsp5) (Prentice et al., 2004; Thiel et al., 2003). Alpha and beta CoVs possess 16 nsps, while gamma and delta CoVs, lacking nsp1, possess 15 nsps (nsp2 to nsp16) (Neuman et al., 2014). Although the amino acid sequences of nsp3 to nsp16 are predicted to be conserved among CoVs, those of nsp1 are highly divergent (Connor and Roper, 2007). However, the nsp1s of several CoVs, such as porcine transmissible gastroenteritis virus (TGEV), human CoV (HCoV)-229E, mouse hepatitis virus (MHV), and SARS-CoV, exhibit a similar function to induce translational suppression (Huang et al., 2011a; Kamitani et al., 2006; Wang et al., 2010; Züst et al., 2007). The nsp1 of SARS-CoV is the most studied among CoVs and is known to inhibit host gene expression via a two-pronged strategy (Kamitani et al., 2009)—i.e., translational shutoff through interaction with the 40 S ribosomal subunit, and host mRNA degradation through the recruitment of unidentified host nuclease(s) (Huang et al., 2011a; Kamitani et al., 2009; Lokugamage et al., 2012). This two-pronged strategy of nsp1 inhibits expression of the IFN gene (Kamitani et al., 2006; Narayanan et al., 2008; Wathelet et al., 2007). Murine models of SARS-CoV have

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revealed that the dysregulated type I IFN response is a key factor for inducing lethal pneumonia (Channappanavar et al., 2016; Kindler and Thiel, 2016). In addition, Züst and colleagues showed that a mutant MHV with partial deletion in nsp1 is highly attenuated (Züst et al., 2007). These accumulated data indicate that the nsp1 of CoV is a major virulence factor.

Although viral mRNAs of CoVs are capped and polyadenylated (Cencic et al., 2011; Chen et al., 2011; Decroly et al., 2011; Yount et al., 2000) like host mRNA, the viral mRNAs are resistant to the translational suppression induced by the expression of nsp1. Like many CoV nsp1s, expression of MERS-CoV nsp1 suppresses host protein synthesis (Lokugamage et al., 2015). The nsp1 of MERS-CoV targets nuclear transcribed host mRNAs for suppression, but mRNAs of cytoplasmic origin are resistant to the nsp1-meditated gene suppression (Lokugamage et al., 2015). However, the mechanisms of the interaction between nsp1 and viral RNA of MERS-CoV and the roles of this interaction on the viral replication are largely unknown.

In this study, we examined the biological significance of the interaction of viral RNA with the nsp1 of MERS-CoV. As we expected, MERS-CoV nsp1 also induced translational suppression and RNA degradation. Like SARS-CoV, MERS-CoV circumvents the nsp1-mediated translational suppression by inducing a specific interaction between SL1 in the 5′ UTR of viral RNA and nsp1. Interestingly, the amino acid residue of MERS-CoV nsp1 required for viral RNA recognition appeared to differ from those of SARS-CoV nsp1. In addition, reverse genetics analysis of MERS-CoV revealed that the specific interaction of nsp1 with SL1 is crucial for the efficient replication of MERS-CoV. These results indicate CoVs share a common mechanism of viral gene expression that is regulated by a specific interaction between SL1 in the 5′ UTR of viral RNA and nsp1.

2. Materials and methods

2.1. Cells

293 T cells (human embryonic kidney) and Huh7 cells (human hepatocellular carcinoma) were maintained in Dulbecco's modified minimum essential medium (DMEM) (Nacalai Tesque, Kyoto, Japan) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Nacalai Tesque). Vero cells (African green monkey kidney) were maintained in DMEM containing 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Nacalai Tesque). All cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C.

2.2. Plasmid constructions

The construction of a firefly luciferase-expressing plasmid, pcD-fLuc, has been described elsewhere (Tanaka et al., 2012). The PCR products of the CAT gene were cloned into pCAGGS-MCS-FLAG, pCAG-CAT. Using the supernatants of Vero cells infected with the MERS-CoV (EMC2012 Strain), first-strand cDNA was prepared by using a SuperScript II First-Strand Synthesis Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. The cDNA was used for construction of the expression plasmids. The PCR products of the MERS-CoV nsp1 sequence were cloned into pCAGGS-MCS-FLAG, yielding pCAG-nsp1-wt. An inverse PCR procedure using pCAG-nsp1 as the template was employed to generate pCAG-nsp1-Δ1, pCAG-nsp1-Δ2, pCAG-nsp1-Δ3, pCAG-nsp1-G11A, pCAG-nsp1-R13A, pCAG-nsp1-G14A, and pCAG-nsp1-T15A by using a KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan). The In-fusion HD cloning procedure (Clontech, Mountain View, CA) was employed to generate pCAG-nsp1-Δ4 according to the manufacturer's instructions. The 5′ UTR sequence of MERS-CoV was connected downstream of the cytomegalovirus (CMV) promoter by overlapping PCR using the same method as described previously (Tanaka et al., 2012; Yamshchikov et al., 2001). The fragment was cloned between the CMV promoter and firefly luciferase gene into pcD-fluc, yielding pcD-5′-fluc. For mutational analysis of the nucleotide sequence from position 1–190 in the MERS-CoV 5′ UTR, the inverse PCR procedure using pcD-5′-fluc was employed to generate pcD-ΔSL1-fluc, pcD-ΔSL2-fluc, pcD-ΔSL4-1-fluc, pcD-ΔSL4-2-fluc, and pcD-SL1-fluc by using a KOD Mutagenesis Kit (TOYOBO). All plasmid constructs were confirmed by sequence analysis using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

2.3. Luciferase assay

293T cells were transfected with reporter and expression plasmids by using TransIT LT1 (Mirus, Madison, WI) according to the manufacturer's instructions. At 24 h posttransfection, transfected cells were collected and luciferase activities were determined by using a luciferase assay system (Promega, Madison, WI) and AB-2200 luminometer (Atto, Tokyo, Japan). Luciferase activities were shown after standardization with those in cells expressing CAT.

2.4. Northern blot analysis

Intracellular RNA of transfected 293T cells was extracted using a PureLink RNA Mini Kit (ThermoFisher Scientific) according to the manufacturer's instructions and stored at −80 °C until use. RNA samples were diluted to 1 μg in 5 μl by UltraPure DW (Invitrogen, Waltham, MA) and then mixed with 5 μl of 2× Loading Dye (New England Biolabs, Ipswich, MA). After heating at 65 °C for 5 min, 10 μl RNA samples were electrophoresed through 1.2% denaturing agarose gel and then transferred onto a positively charged nylon membrane (Roche, Basel, Switzerland). Northern blot analysis was performed using a digoxigenin (DIG) Wash and Block Buffer Set and a DIG Luminescence Detection Kit (Roche). The DIG-labeled riboprobe to detect the firefly luciferase gene was generated by using a DIG RNA Labeling Kit (SP6/T7) (Roche) as described previously (Kamitani et al., 2006; Tanaka et al., 2012).

2.5. Western blot analysis

Transfected 293T cells were lysed using RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP40, 1% sodium deoxicholate, 0.1% sodium dodecyl sulfate (SDS) and 2.5U benzonase) and then centrifuged at 16,000×*g* for 10 min at 4 °C. Supernatants were collected and mixed with 2× sample buffer (0.1 M Tris-HCl (pH6.8), 4% SDS, 20% glycerol, 0.004% bromophenol blue and 10% 2-mercaptoethanol). Ten microliters of boiled samples was electrophoresed by SDS-poly acrylamide gel electrophoresis (PAGE). Electrophoresed gels were transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Billerica, MA). The transferred membranes were blocked by 3% skim milk in phosphate-buffered saline (PBS) containing 0.05% tween 20 (Nacalai Tesque) (PBS-T). Anti-DYKDDDDK (FLAG) mouse antibody (Wako, Osaka, Japan) or Anti-β-Actin mouse antibody (Sigma, St. Louis, MO) was used as a primary antibody, and goat anti-mouse IgG-horseradish peroxidase (HRP) (Sigma) was used as a secondary antibody. ChemiLumi One Ultra (Nacalai Tesque) was used for visualization.

2.6. Immunoprecipitation of reporter RNA

At 24 h posttransfection, the 293T cells were lysed with lysis buffer (1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing 40U RNase inhibitor (Takara, Shiga, Japan) and protease inhibitor (cOmplete, EDTA-free; Roche). After centrifugation, the supernatants of the cell lysates were collected and mixed with protein A/G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA), then incubated for 30 min at 4 °C. After centrifugation, the supernatants