Mutagenesis of Coronavirus nsp14 Reveals Its Potential Role in Modulation of the Innate Immune Response

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ABSTRACT

Coronavirus (CoV) nonstructural protein 14 (nsp14) is a 60-kDa protein encoded by the replicase gene that is part of the replication-transcription complex. It is a bifunctional enzyme bearing 3'-to-5' exoribonuclease (ExoN) and guanine-N7-methyltransferase (N7-MTase) activities. ExoN hydrolyzes single-stranded RNAs and double-stranded RNAs (dsRNAs) and is part of a proofreading system responsible for the high fidelity of CoV replication. nsp14 N7-MTase activity is required for viral mRNA cap synthesis and prevents the recognition of viral mRNAs as "non-self" by the host cell. In this work, a set of point mutants affecting different motifs within the ExoN domain of nsp14 was generated, using transmissible gastroenteritis virus as a model of *Alphacoronavirus*. Mutants lacking ExoN activity were nonviable despite being competent in both viral RNA and protein synthesis. A specific mutation within zinc finger 1 (ZF-C) led to production of a viable virus with growth and viral RNA synthesis kinetics similar to that of the parental virus. Mutant recombinant transmissible gastroenteritis virus (TGEV) ZF-C (rTGEV-ZF-C) caused decreased cytopathic effect and apoptosis compared with the wild-type virus and reduced levels of dsRNA accumulation at late times postinfection. Consequently, the mutant triggered a reduced antiviral response, which was confirmed by evaluating different stages of the dsRNA-induced antiviral pathway. The expression of beta interferon (IFN- β), tumor necrosis factor (TNF), and interferon-stimulated genes in cells infected with mutant rTGEV-ZF-C was reduced compared to the levels seen with the parental virus. Overall, our data revealed a potential role for CoV nsp14 in modulation of the innate immune response.

IMPORTANCE

The innate immune response is the first line of antiviral defense that culminates in the synthesis of interferon and proinflammatory cytokines to control viral replication. CoVs have evolved several mechanisms to counteract the innate immune response at different levels, but the role of CoV-encoded ribonucleases in preventing activation of the dsRNA-induced antiviral response has not been described to date. The introduction of a mutation in zinc finger 1 of the ExoN domain of nsp14 led to production of a virus that induced a weak antiviral response, most likely due to the accumulation of lower levels of dsRNA in the late phases of infection. These observations allowed us to propose a novel role for CoV nsp14 ExoN activity in counteracting the antiviral response, which could serve as a novel target for the design of antiviral strategies.

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses belonging to the *Coronaviridae* family within the *Nidovirales* order (1). CoVs are frequently associated with respiratory and enteric diseases in humans, livestock, and companion animals (2, 3). CoVs have been divided into four genera: *Alphacoronavirus*, such as *Human coronavirus 229E* (HCoV-229E) or the *Porcine transmissible gastroenteritis virus* (TGEV); *Betacoronavirus*, including the *Severe acute respiratory syndrome coronavirus* (SARS-CoV) and the recently emerged *Middle East respiratory syndrome coronavirus* (MERS-CoV), causing both pneumonia and a high mortality rate; *Gammacoronavirus*, including *Infectious bronchitis virus* (IBV); and *Deltacoronavirus* (4). CoVs contain the largest known genome among RNA viruses, consisting of a single-stranded, positive-sense, 5'-capped, and polyadenylated RNA molecule that is 27 to 31 kb in length (5). The replicase gene, which is comprised of two overlapping open reading frames (ORFs), ORF1a and ORF1b, is contained in the first two-thirds of the genome. After infection, viral genomic RNA (gRNA) is directly translated to yield two polyprotein precursors, pp1a and pp1ab, the latter requiring ribosomal frameshifting near the 3' end of ORF1a for its translation (6). Subsequently, the two polyproteins are cleaved by ORF1a-encoded proteases to release a total of 16 nonstructural proteins (nsp1 to nsp16) (7, 8). These nsps assemble together with cellular factors to form a large repli-

cation-transcription complex (RTC) associated with membrane structures derived from the endoplasmic reticulum (9–11). The RTC is required for CoV RNA synthesis, which is comprised of genome replication and transcription. CoV transcription is a discontinuous process that yields a nested set of subgenomic mRNAs (sg mRNAs) that serve as the templates for translation of the viral structural and genus-specific proteins (8, 12, 13). The CoV RTC is extremely complex, and, apart from the RNA-dependent RNA polymerase (RdRp) and helicase activities common to many RNA viruses, CoVs encode a unique set of RNA-modifying activities such as the 3'-to-5' exoribonuclease (ExoN) and the uridylylate-specific endoribonuclease (NendoU), a recently discovered nucle-

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otidyltransferase (14), or enzymatic activities related to the synthesis of the cap structure, such as RNA 5'-triphosphatase (RTPase), N7-methyltransferase (N7-MTase), and 2'-O methyltransferase (2O-MTase) (7, 15, 16).

CoV nsp14 is a bifunctional enzyme that harbors both ExoN and N7-MTase activities (17–19). The amino-terminal part of nsp14 includes the ExoN active core, which is divided into three motifs: I (DE), II (E), and III (D). Due to this characteristic, CoV nsp14 is included in the DEDD exonuclease superfamily, which comprises cellular enzymes that catalyze DNA proofreading (20). nsp14 ExoN has been proposed to have a critical role in CoV replication and transcription, as mutants lacking this activity showed an important reduction in viral RNA synthesis or accumulation (17, 21). nsp14 hydrolyzes single- and double-stranded RNA (ssRNA and dsRNA) to final products of 8 to 12 nucleotides (nt) and 5 to 7 nt, respectively, and its nucleolytic activity is enhanced up to 35-fold by interaction with nsp10 (17, 22). In addition, nsp14 ExoN activity was proposed to be part of the RNA proofreading machinery during CoV replication (20, 23), which would be required for the replication and maintenance of the large CoV genome. In fact, only larger-sized *Nidovirus* genomes encode ExoN activity (24). Mutations in the ExoN active core abolishing the exonuclease activity led to CoVs that exhibited a 15- to 20-fold increase in replication errors in *Betacoronavirus* such as mouse hepatitis virus (MHV) and SARS-CoV (21, 23, 25). As a proofreading component, ExoN should be involved in the removal of misincorporated nucleotides. Indeed, nsp14 activity efficiently removed mismatched 3'-end nucleotides mimicking RdRp misincorporation products (22). Moreover, mutants lacking ExoN activity showed greater sensitivity to the mutagen 5-fluorouracil, in contrast to CoVs with ExoN activity, which are considered resistant to lethal mutagenesis (26).

The carboxy-terminal part of nsp14 contains N7-MTase activity, involved in the addition of a methyl group to the cap guanosine at the N7 position, leading to formation of the cap-0 structure (18, 27). In general, this cap-0 is critical for efficient export, translation, and stability of mRNAs. In addition, the methylation of N7 would be required for the subsequent methylation at the O-2' position, which is essential for prevention of viral RNA recognition by the host immune system (28–30).

The bifunctional nsp14 is part of the large multisubunit polymerase complex described as the core of the CoV RTC, which integrates RNA polymerization, proofreading, and cap-modifying activities into a multifunctional protein assembly (16, 31).

The recent crystallization of the SARS-CoV nsp14 identified the presence of three zinc fingers (ZFs) within the nsp14 structure (32). A ZF is a small, independently folded domain that is structured around a zinc ion, which is coordinated through cysteine and histidine residues. ZFs are structurally diverse, with more than 40 types of annotated ZFs in UniProtKB, and are found in proteins that perform a broad range of functions, including essential cellular processes such as replication, signaling, cell proliferation, and apoptosis (33). ZFs usually function as interaction modules and bind to a wide variety of compounds, such as nucleic acids, proteins, and small molecules (34). While interaction of different ZF types with DNA has been well characterized in terms of affinity and specificity, ZF interactions with RNA remain poorly characterized (35). Nevertheless, it has been shown that modification of a ZF sequence may alter its binding to an RNA substrate (36).

Viral dsRNA production as a replication intermediate is a pathogen-associated molecular pattern (PAMP), which mediates the activation of well-characterized antiviral mechanisms leading to shutdown of protein synthesis, stimulation of host innate immunity for initial detection of pathogens, and subsequent activation of adaptive immunity (37, 38). CoVs have evolved mechanisms impairing the activation of the innate immune response at different stages. These mechanisms include the production of several viral proteins that interfere with type I interferon (IFN) and proinflammatory cytokine production (39) and the induction of double-membrane vesicles, where dsRNA is shielded from innate immune sensing by cytoplasmic receptors (10).

As a key enzyme in cap formation, CoV nsp14 was proposed as an IFN antagonist (39). In fact, overexpression studies have revealed that nsp14 acts as an IFN antagonist (40), although it has not been determined whether this activity is linked to its ExoN or N7-MTase domains. The second CoV-encoded RNase, NendoU, is also a robust IFN antagonist when overexpressed (41). The possibility that CoV ribonucleases could degrade dsRNA PAMPs has been suggested as an additional mechanism preventing IFN induction during CoV infection (42). Nevertheless, the role of these proteins in preventing the innate immune response has not been studied so far.

Current knowledge about CoV nsp14 is mainly derived from study of the betacoronaviruses. In contrast, there is limited information on the role of *Alphacoronavirus* nsp14 during infection, as HCoV-229E mutations which abrogated ExoN activity were lethal (17). In this work, we used another *Alphacoronavirus*, TGEV, to analyze the role of the nsp14 ExoN domain in terms of virus viability and induction of antiviral response. Our results show that mutations abolishing ExoN activity resulted in only a modest decrease in viral RNA synthesis but that recovery of infectious progeny failed, something that may be a general characteristic for alphacoronaviruses. A specific mutation in the ZF1 produced a viable virus causing a decrease in accumulation of dsRNA intermediates at late times postinfection (pi). Consequently, this mutant virus triggered a reduced antiviral response and apoptosis in comparison to the parental virus, indicating that CoV nsp14 plays a role in modulation of the innate immune response.

MATERIALS AND METHODS

Cells. Baby hamster kidney cells (BHK-21) (ATCC CCL-10) or BHK-21 cells stably transformed with the gene coding for porcine aminopeptidase N (BHK-pAPN) (43) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). Both cell lines were transfected with the Sindbis virus replicon pSINrep1 (44) expressing TGEV nucleocapsid (N) protein, obtaining BHK-N or BHK-pAPN-N cells, respectively. G418 (1.5 mg/ml) and puromycin (5 µg/ml) were used as selection agents for pAPN and pSINrep1, respectively. Recombinant TGEV viruses obtained in this work were grown in swine testis (ST) cells (45) using DMEM supplemented with 10% FCS.

Plasmid constructs. cDNAs of TGEV-derived replicons and infectious viruses (46, 47) were generated by PCR-directed mutagenesis. To generate nsp14-ExoI, nsp14-ExoIII, nsp14-ZF-H, nsp14-ZF-C, and nsp14-N7MTase mutant sequences, two overlapping PCR fragments were obtained by using as a template the plasmid pBAC-TGEV (47), containing the full-length TGEV genome (GenBank accession number AJ271965). The 5' fragment was obtained by using the forward primer nsp13-finVS (5'-CATGTGTGATAGAAGTATGTATGAGAATCTTG-3') and the specific reverse primer shown in Table 1. The 3' fragment was obtained by using the specific forward primer shown in Table 1 and, in all cases, the reverse primer nsp15prin-RS (5'-CCATTATTTGTGTCAGCAATAACAG

TABLE 1 Primers used for nsp14 mutagenesis

Mutant	Primer ^a	Sequence (5'–3') ^b
nsp14-ExoI	ExoI_VS ExoI_RS	CTTGGGTTTGCACTGCTGGTGCACATG CATGTGCACCAGCACTGCAACCCCAAG
nsp14-ExoIII	ExoIII_VS ExoIII_RS	GCTAGTGGTGCGGCTATCATGACTAG CTAGTCATGATAGCCGCCACCACTAGC
nsp14-ZF-H	CHCH_VS CHCH_RS	CAAAATGTGAACACGGCAAAAG CTTTTGCCGTGTTTACATTTTGTG
nsp14-ZF-C	CCCC_VS CCCC_RS	GCTTGCTTCAAGTGTGCATTAGGATG CATCCTAATGCACACTTGAAGCAAGC
nsp14-N7MTase	N7MTase_VS N7MTase_RS	GCTTGCTTCAAGTGTGCATTAGGATG GATTACCCACCGCTGAATTGCAGC

^a VS, forward primer; RS, reverse primer.^b Mutant nucleotides are in bold.

CAG-3'). Full-length amplicons were generated by overlap extension of 3' and 5' PCR products using primers nsp13-finVS and nsp15prin-RS. In all cases, full-length amplicons were digested with BstBI and XbaI restriction enzymes and cloned into the same sites of plasmid pSL-nsp14, which includes the TGEV genomic sequence from nt 15063 to 21504, leading to intermediate plasmids pSL-nsp14-ExoI, pSL-nsp14-ExoIII, pSL-nsp14-ZF-H, pSL-nsp14-ZF-C, and pSL-nsp14-N7MTase. All intermediate plasmids were digested with NheI and PacI restriction enzymes, and insertions were cloned into the same sites of pBAC-REP2 (46), generating the mutant replicons pBAC-REP2-nsp14-ExoI, pBAC-REP2-nsp14-ExoIII, pBAC-REP2-nsp14-ZF-H, pBAC-REP2-nsp14-ZF-C, and pBAC-REP2-nsp14-N7MTase. Alternatively, insertions digested with NheI and PacI restriction enzymes were cloned into the same sites of plasmid pBAC-TGEV-S_{7.1} (C. M. Sanchez, M. Becares, S. Zuñiga, and L. Enjuanes, unpublished results), leading to pBAC-TGEV-S_{7.1}-nsp14-ExoI, pBAC-TGEV-S_{7.1}-nsp14-ExoIII, pBAC-TGEV-S_{7.1}-nsp14-ZF-H, pBAC-TGEV-S_{7.1}-nsp14-ZF-C, and pBAC-TGEV-S_{7.1}-nsp14-N7MTase. All cloning steps were checked by sequencing of the PCR fragments and cloning junctions. For each mutant sequence, two independent cDNAs were constructed.

Production of a polyclonal antibody (Ab) specific for TGEV nsp14.

An nsp14 protein with a 6-His tag fused at its N terminus was expressed in the baculovirus-insect cell system, using a baculovirus obtained from the Fei Deng laboratory (Wuhan Institute of Virology, Wuhan, China). Recombinant nsp14 was purified to near-homogeneity by metal chelate affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Sigma-Aldrich, Madrid, Spain) following standard procedures set up in our laboratory (48). Specific polyclonal antisera were generated by Biogenes GmbH (Germany) after immunization of rabbits with purified recombinant nsp14.

Transfection and recovery of infectious recombinant TGEVs (rTGEVs) from cDNA clones. BHK-pAPN-N or BHK-N cells grown to 90% confluence in 35-mm-diameter plates (or 12-well plates for RNA synthesis experiments) were transfected using 3.3 µg of the corresponding pBAC plasmid and 10 µl of Lipofectamine 2000 (Invitrogen) per million cells according to the manufacturer's specifications. For recovery of infectious recombinant TGEVs (rTGEVs) from cDNA infectious clones, BHK-pAPN-N-transfected cells were trypsinized at 6 h posttransfection (hpt) and plated over confluent ST monolayers grown in 35-mm-diameter plates. After a 2-day incubation period, the cell supernatants were harvested (passage 0) (49).

Viral RNA presence at passages 0 and 1 was analyzed by reverse transcription-PCR (RT-PCR). Total cellular RNA was extracted using an RNeasy minikit (Qiagen) following the manufacturer's instructions. DNA was removed by treatment of 7 µg of each purified RNA with 20 U of DNase I (Roche) for 30 min at 37°C, and DNA-free RNAs were repurified

using an RNeasy minikit (Qiagen). Reverse transcription was performed with a High Capacity RNA-to-cDNA kit (Life Technologies) according to the manufacturer's instructions. PCRs were performed to analyze genomic RNA (gRNA) by amplifying nsp14 sequences (using the forward primer nsp13fin_VS and the reverse primer nsp15prin_RS) and mRNA of the N protein using the forward primer SP (5'-GTGAGTGTAGCGTGGCTATATCTCTTC-3') and the reverse primer N-479RS (5'-TAGATTGA GAGCGTGACCTTG-3').

Each rTGEV was cloned by three plaque purification steps, and the resultant virus was grown and titrated as previously described (50). Lysis plaque diameter was determined by measuring the area of the lysis plaques using the software ImageJ (51).

Full-genome sequencing of each rTGEV clone was performed using overlapping PCRs covering the entire TGEV genome (primer sequences available on request). Both strands of each PCR product were sequenced, and contigs were assembled using SeqMan software from the DNASTAR Lasergene package (SeqMan, version 8.0.2; DNASTAR, Madison, WI).

Analysis of viral RNA synthesis and cellular gene expression by quantitative RT-PCR (RT-qPCR). Total intracellular RNA was extracted at 24 hpt from transfected BHK-N cells or at the indicated times postinfection from ST cells infected with rTGEVs. Total RNA was purified with an RNeasy minikit (Qiagen) according to the manufacturer's specifications. For the analysis of viral RNA synthesis in transfected cells, an additional step was used in order to remove DNA from samples. For this purpose, 7 µg of each RNA was treated with 20 U of DNase I (Roche) for 30 min at 37°C. DNA-free RNA was repurified using an RNeasy minikit (Qiagen). In all cases, 100 ng of total RNA was used as the template for synthesis of cDNA with random hexamers using a High Capacity cDNA transcription kit (Life Technologies) following the manufacturer's instructions.

Viral RNA levels were evaluated by RT-qPCR using custom TaqMan assays (Life Technologies) specific for the TGEV genomic RNA (gRNA) and subgenomic mRNA 7 (mRNA-7), in both positive (+) polarity and negative (–) polarity (Table 2), following standard procedures set up in our laboratory (52). Cellular gene expression was analyzed using TaqMan gene expression assays (Applied Biosystems) specific for porcine genes encoding tumor necrosis factor (TNF) (Ss03391318_g1), beta IFN (IFN-β) (Ss03378485_u1), IRF-1 (Ss03388785_m1), MDA5 (Ss03386373_u1), RIG-I (Ss03381552_u1), or TFG-β (Ss03382325_u1), and porcine 2',5' oligoadenylate synthetase 1 (OAS1) was analyzed by using a custom TaqMan gene expression assay (53). The β-glucuronidase (GUSB) gene (TaqMan code Ss03387751_u1) was used as a reference housekeeping gene, since its expression remains constant in both infected and noninfected cells (54). Data were acquired with a 7500 real-time PCR system (Applied Biosystems) and analyzed with 7500 software v2.0.6. The relative quantifications were performed using the threshold cycle (2^{–ΔΔCT}) method (55). All experiments and data analyses were Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) compliant (56).

Protein analysis by Western blotting. Transfected BHK-N cells and infected ST cells were collected at the indicated times, and cell lysates were obtained by solubilizing cells in 1× SDS gel-loading sample buffer (57). Cell lysates were resolved by denaturing electrophoresis in NuPAGE 4% to 12% Bis-Tris gels with 3-morpholinopropane-1-sulfonic acid (MOPS) SDS running buffer (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P PVDF; Merck-Millipore) employing a Trans-Blot Turbo transfer system (Bio-Rad), using the manufacturer's recommended conditions. Membranes were blocked for 1 h at room temperature with 5% skim milk–TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) and then incubated with monoclonal antibodies (MAbs) specific for TGEV N protein (MAB-3DC10) (58) or β-actin (Abcam) (1:10,000 dilution). Polyclonal antibodies (pAb) against active caspase 3 protein (Abcam) (1:1,000) or TGEV nsp3 (59) were also used. The blots were then incubated with a horseradish peroxidase-conjugated secondary antibody diluted in TBS supplemented with 0.1% Tween 20 (TTBS) and

TABLE 2 TaqMan assays used for RT-qPCR of viral gRNA and sg mRNA

Amplicon and primer or probe	TaqMan primer or probe ^a	Sequence (5'–3') ^b
gRNA(+)		
Forward primer	RepVS	TTCTTTTGACAAAACATACGGTGAA
Reverse primer	RepRS	CTAGGCAACTGGTTTGTAAACATCTTT
Probe	Rep-MGB	FAM-AGGGCACC GTTGTCA-MGB
gRNA(–)		
Forward primer	RepRS	CTAGGCAACTGGTTTGTAAACATCTTT
Reverse primer	RepVS	CTAGGCAACTGGTTTGTAAACATCTTT
Probe	cRep-MGB	FAM-CTGTTCCACCGTATGTTT-MGB
mRNA7(+)		
Forward primer	Ldrt-VS	CGTGGCTATATCTCTTCTTTTACTTTAACTAG
Reverse primer	7 (38)-RS	AAAACTGTAATAAATACAGCATGGAGGAA
Probe	mRNA7-MGB	FAM-CGAACTAAACGAGATGCT-MGB
mRNA(–)		
Forward primer	7 (38)-RS	AAAACTGTAATAAATACAGCATGGAGGAA
Reverse primer	Ldrt-VS	CGTGGCTATATCTCTTCTTTTACTTTAACTAG
Probe	cmRNA7-MGB	FAM-AGCATCTCGTTTAGTTTCGAGT-MGB

^a VS, forward primer; RS, reverse primer; MGB, minor groove binder group.^b FAM, 6-carboxyfluorescein.

3% skim milk for 1 h at room temperature. After extensive washing with TTBS, the immune complexes were detected using Clarity Western ECL blotting substrate (Bio-Rad) and a ChemiDoc XRS⁺ System (Bio-Rad), according to the manufacturer's instructions.

Protein amounts were estimated by densitometric analysis using ImageLab 4.1 software (Bio-Rad). At least three different experiments and appropriate gel exposures were used in all cases with similar results. In addition, different exposures of the same experiment were analyzed to ensure that data were obtained within the linear range.

Immunofluorescence analysis. ST cells were grown on 12-mm-diameter glass coverslips in DMEM–10% FCS to a confluence of 30% to 50%, and then cell cultures were synchronized by serum deprivation (60). Briefly, cells were rinsed with phosphate-buffered saline (PBS) and incubated with DMEM–0.1% FCS for 48 h, as it had been previously determined that this incubation time allows for 85% to 90% of ST cells in G₀ phase. After serum starvation, cells were released into the cell cycle by incubation in DMEM–10% FCS for 4 h and were subsequently mock infected or infected at a multiplicity of infection (MOI) of 2 with each rTGEV. At 8 or 16 h postinfection (hpi), cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with cold methanol for 10 min, and blocked in PBS with 10% FCS for 45 min at room temperature. MAb specific for dsRNA (SCICONS English and Scientific Consulting kit) (1:200 dilution) and pAb specific for TGEV nsp14 (1:1,000; see above) were used. Bound primary antibodies were detected with Alexa Fluor 488- or 594-conjugated antibodies specific for mouse or rabbit IgG, respectively (Invitrogen) (1:500). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) (1:200). Confocal microscopy was performed using a Leica SP5 laser scanning microscope, and images were collected and processed with LAS AF software (Leica, Wetzlar, Germany). Quantification of the intensity of the fluorescence was done by measuring the mean gray fluorescence of individual cells from gray-scale projections of microscopy images. The variance of the intensity was also determined, and the normalized optical density variance was calculated as an indicator of signal dispersion, as previously described (61). Data represent averages of 30 individual cell measurements.

Poly(I:C) treatment. ST cells were grown to confluence in 24-well plates and infected with rTGEVs at an MOI of 1. At 12 hpi, infected cells were transfected with 0.5 µg of polyinosinic poly(C) [poly(I:C)] (Sigma-Aldrich) per well using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. At 4 hpi, total intracellular RNA was ex-

tracted and analyzed by RT-qPCR, as described above. Total cell RNA integrity was evaluated with a Bioanalyzer 2100 (Agilent Technologies) following the manufacturer's recommendations and was analyzed with 2100 Expert software (Agilent Technologies).

Statistical analysis. Two-tailed, unpaired Student's *t* tests were used to analyze the differences in mean values between groups. All results were expressed as means ± standard deviations; *P* values of <0.05 were considered significant.

RESULTS

Analysis of RNA synthesis by nsp14-ExoN mutants. CoV nsp14 has been proposed to play a role in RNA synthesis, although the specific mechanism has not been determined (17, 20). In addition, there is limited information on the role of *Alphacoronavirus* ExoN in the infection context. Therefore, a set of four TGEV ExoN domain mutants was engineered by reverse genetics using a TGEV-derived replicon (46). nsp14 is highly conserved among different CoV genera, and all the previously described motifs were identified in TGEV nsp14 (Fig. 1A). Two of the mutants were designed to affect the catalytic residues DE and D within the conserved exonuclease motifs ExoI and ExoIII, respectively (Fig. 1A). These mutations are well characterized *in vitro* as abolishing ExoN activity in the case of HCoV-229E and SARS-CoV (17, 18). The recent publication of the SARS-CoV nsp14 protein crystal structure has revealed the presence of two ZFs in the ExoN domain of the protein which seem essential for the function of nsp14 (Fig. 1A) (32). Two mutants affecting ZF1, designed to alter the ZF type but theoretically still allowing zinc coordination, were engineered. The ZF2 motif was not modified as it overlaps the catalytic core, and its modification would most likely influence nsp14 catalytic activity. An additional TGEV mutant replicon modifying the N7-MTase domain was designed as a control, as it was previously shown that this mutation has no effect on ExoN activity (32). The mutant replicons were transfected into BHK-N cells (52), and replication and transcription levels were analyzed at 24 hpi by RT-qPCRs measuring the accumulation of gRNA and mRNA-7, respectively. RNA levels were compared in all cases with those obtained from a

FIG 1 RNA synthesis of TGEV nsp14 mutants. (A) Schematic representation of TGEV nsp14. Exonuclease (ExoN) and N7 methyltransferase (N7-MTase) domains are indicated. Motifs I, II, and III conferring the ExoN active site are shown (gray boxes). The three zinc fingers (ZF1, ZF2, and ZF3) are indicated (black boxes), as are the amino acids forming the S-adenosylmethionine (SAM) binding pocket in the N7-MTase domain (white box). Within the partial sequence alignments, positions of key amino acids in each motif (white letters in black boxes) are indicated. Those mutated in each rTGEV construct with respect to the wild-type (WT) sequence are shown as black letters in gray boxes. (B) Quantification of replication and transcription levels in each rTGEV mutant replicon. Nonreplicative (NR) and wild-type (WT) replicons were included as controls. Genomic RNA (gRNA) and subgenomic mRNA from gene 7 (mRNA-7) were analyzed by RT-qPCR using specific TaqMan assays after transfection of each replicon in BHK-N cells. Mean values from eight independent transfection experiments are plotted; error bars represent standard deviations. *, P value < 0.05 ; **, P value < 0.01 ; ***, P value < 0.001 .

nonreplicative replicon, which served to establish background reference levels of replication and transcription due to the cytomegalovirus promoter. As expected, the engineered mutation in the N7-MTase domain did not have any impact on RNA synthesis (Fig. 1B). The ExoI and ExoIII mutations led to a modest (approximately 3-fold) reduction that was barely statistically significant in both replication and transcription compared to the wild-type (WT) replicon, indicating that these mutants were competent in RNA synthesis (Fig. 1B). Interestingly, the engineered mutations

in ZF1 led to two different outcomes. Mutant ZF-H showed a modest reduction in replication in comparison to WT levels, while the level of mRNA-7 was comparable to that of the nonreplicative replicon, indicating that the ZF-H mutation completely abolished transcription (Fig. 1B). In contrast, the ZF-C mutant was competent in both replication and transcription (Fig. 1B).

Mutations abolishing ExoN activity resulted in a lethal phenotype. To study the role of TGEV ExoN activity during infection, the ExoI and ExoIII mutant infectious viral cDNAs were gener-

ated. The mutant cDNAs were transfected into BHK-pAPN cells expressing TGEV N protein that were plated over ST cells, susceptible to viral infection. At 48 hpt, cell supernatants were harvested (passage 0) and used to infect ST cells (passage 1), following the standard protocol set up in the laboratory (49). No cytopathic effect was observed after ExoI or ExoIII mutant cDNA transfection (data not shown). In order to analyze whether viral RNA synthesis occurred, total RNA was extracted from passage 0 and passage 1 and the presence of viral RNA was evaluated by RT-PCR. Viral genomic RNA (gRNA) corresponding to nsp14 sequences, as well as the mRNA of N protein (mRNA-N) produced by WT virus, was detected at both passage 0 and passage 1, indicating efficient virus recovery (Fig. 2A). In contrast, gRNA and mRNA-N synthesized from rTGEV ExoN mutants were amplified only from passage 0 samples and not detected at passage 1, indicating a failure in mutant virus recovery (Fig. 2A). Sequencing of the nsp14 gRNA fragment confirmed that each rTGEV carried the desired engineered mutations.

In order to determine whether a defect in RNA synthesis prevented recovery of mutant viruses, RNA accumulation was analyzed by RT-qPCR 24 h after transfection of the ExoN mutant cDNAs into BHK-N cells. Results indicated a decrease in replication (15-fold reduction) and transcription (2-fold reduction, not statistically significant), compared to WT cDNAs (Fig. 2B). A similar reduction in the accumulation of viral RNA was observed for both the positive and negative strands (Fig. 2B). These data are in agreement with the decrease in viral RNA synthesis observed for HCoV-229E, MHV, and SARS-CoV (17, 21). Nevertheless, in the case of MHV and SARS-CoV, this reduction in viral RNA synthesis did not prevent virus recovery. Moreover, experiments performed using previous rTGEV mutants obtained in our laboratory that had a higher reduction in RNA synthesis led to the recovery of viable viruses (62), suggesting that there is another cause for the lethal phenotype of TGEV ExoI and ExoIII mutants.

As nsp14 also has a role in cap synthesis, mutations could have an effect on viral protein accumulation, although it has been demonstrated that SARS-CoV ExoI and ExoIII mutants were competent in N7-MTase activity (19). Variation in protein synthesis of the rTGEV mutants was evaluated by Western blotting. Accumulation of nsp3, which is directly translated from gRNA, did not vary significantly with respect to WT virus despite the reduction of gRNA levels (Fig. 2C), suggesting that viral protein synthesis was not affected in the ExoI and ExoIII mutants. Unfortunately, detection of other viral structural proteins after cDNA transfection failed even for WT virus, due to low bacterial artificial chromosome (BAC) transfection efficiency.

Taken together, the results indicate that ExoN activity is required for TGEV viability. Similar results were obtained with HCoV229E (17); therefore, the requirement of ExoN activity may be common to all members of the *Alphacoronavirus* genus.

Effect of mutations in nsp14 zinc finger 1 on viral phenotype.

In order to study the functional role of nsp14 ZF1 in the context of CoV infection, rTGEV infectious clones harboring the ZF-H and ZF-C mutations were engineered. As expected, the ZF-H virus was not recovered (data not shown), probably due to viral RNA transcription defects similar to those observed with the replicon system (Fig. 1B). Interestingly, MHV substitution of two Zn-coordinating residues by alanine also led to a lethal phenotype (63), suggesting an important role for ZF1 in nsp14 function.

The rTGEV-ZF-C virus was successfully recovered with a titer

of 4.3×10^7 PFU/ml, similarly to the WT virus (8.7×10^7 PFU/ml). The introduced mutations were present in the recovered mutant virus, even after 10 passages in cell cultures of plaque-purified viruses, indicating that they were stably maintained in the rTGEV genome. Moreover, the full-length genomes of two independent clones of rTGEV-WT and rTGEV-ZF-C were sequenced, and the only differences found between the rTGEV-WT and the rTGEV-ZF-C were the desired engineered mutations.

Infection with rTGEV-ZF-C induced a delayed cytopathic effect (CPE) in comparison to rTGEV-WT, and lysis plaques formed by rTGEV-ZF-C were significantly smaller than (i.e., were half the diameter of) those from rTGEV-WT (Fig. 3A). This plaque size reduction was observed in analyzing both uncloned viruses obtained from transfection and plaques formed by six individual clones (data not shown). Viral growth was analyzed at different times postinfection, at both a low (0.05) and a high (5) MOI. The infectious titer of rTGEV-ZF-C was up to 10-fold higher than that of rTGEV-WT at early times postinfection, although the maximum titer observed from 24 hpi was identical to that of the rTGEV-WT (Fig. 3B).

rTGEV-ZF-C and rTGEV-WT showed the same gRNA accumulation kinetics at early times postinfection, corresponding to the active RNA synthesis stage of the TGEV infectious cycle (Fig. 3C), in agreement with our observations using TGEV replicons (Fig. 1B). Nevertheless, at late times postinfection, rTGEV-ZF-C accumulated lower levels of gRNA than rTGEV-WT (Fig. 3C). These data, together with the reduced CPE and a smaller-plaque phenotype, could be attributed to the dissemination of rTGEV-ZF-C being less efficient than that of rTGEV-WT.

Antiviral response elicited by rTGEV-ZF-C. Previous results from our laboratory indicated that CPE produced by TGEV and lysis plaque size could correlate with apoptosis induction as a consequence of the host antiviral response (53). TGEV induces apoptosis following a caspase-dependent pathway that involves the processing of two initiator proteases (caspase 8 and caspase 9), as well as three downstream effector caspases (caspases 3, 6, and 7) (64, 65). To evaluate whether rTGEV-ZF-C CPE and lysis plaque size reduction were due to reduced apoptosis, caspase 3 activation was evaluated by Western blotting using specific antibodies. At 24 hpi, when rTGEV-ZF-C and rTGEV-WT infectious titers were similar (Fig. 3B), rTGEV-ZF-C was found to induce lower levels of active caspase 3 than rTGEV-WT (Fig. 4, left panels). It has been found that TGEV N protein is cleaved by caspases within the host cell (66). In agreement with the reduced level of caspase 3 activation, decreased cleavage of N protein by caspases was observed during rTGEV-ZF-C infection in comparison to rTGEV-WT results (Fig. 4, right panels). Taken together, these results indicated that the rTGEV-ZF-C virus triggered reduced apoptosis.

TGEV-induced apoptosis is linked to the antiviral response produced during infection (53, 54). Therefore, accumulation of IFN- β and TNF mRNAs was measured as a marker of the activation of the IRF-3 and NF- κ B pathways, respectively, during the dsRNA-triggered antiviral response. rTGEV-ZF-C infection led to significantly reduced levels of IFN- β (up to 25-fold) and TNF (up to 17-fold) in comparison to rTGEV-WT infection (Fig. 5A). The induction of interferon-stimulated genes (ISGs) was also evaluated, by measuring the accumulation of IRF-1, OAS, RIG-I, and MDA-5 mRNAs. In agreement with the reduced IFN- β production, a significant decrease in expression of these genes was ob-

FIG 2 Analysis of rTGEV ExoN mutants. (A) RT-PCR analysis of viral RNA from passage 0 and passage 1 of the rTGEV-ExoI and rTGEV-ExoIII mutants, compared with the rTGEV-WT. Genomic RNA (gRNA) and subgenomic mRNA of gene N (mRNA-N) were analyzed. Lanes A and B indicate duplicate infectious cDNAs, tested individually for each mutant. Weight-average molecular weight markers (M_w) are shown on the right. (B) Quantification of genomic RNA (gRNA) and mRNA of gene 7 (mRNA-7). Both negative and positive strands were measured by RT-qPCR using specific TaqMan assays 24 h posttransfection of infectious cDNAs into BHK-N cells. Means of results from four independent transfection experiments are plotted; error bars represent standard deviations. (C) Detection of viral proteins by Western blotting. Total protein was extracted 24 h after transfection of BHK-N cells with infectious cDNAs from ExoI, ExoIII, WT, nonreplicative rTGEV (NR), or mock-transfected (M) cells. Viral protein nsp3 and β -actin (as a loading control) were detected using specific antibodies. The right panel represents the quantification of the bands by densitometry, corrected by the amount of β -actin. Means of results from two independent transfection experiments are plotted; error bars represent standard deviations. *, P value < 0.05; ***, P value < 0.001.

FIG 3 Growth of nsp14 mutant rTGEV-ZF-C in tissue cultures. (A) Lysis plaques produced by mutant rTGEV-ZF-C and rTGEV-WT at 48 h postinfection in ST cells. The right panel represents the mean diameter of 10 individual lysis plaques; error bars indicate standard deviations. (B) Growth kinetics of rTGEV-WT and rTGEV-ZF-C viruses. ST cells were infected at low (0.05; left) and high (5; right) MOIs with mutant rTGEV-ZF-C (ZF-C) or rTGEV-WT (WT); supernatants were collected at different times postinfection, and infectious titers were determined by plaque titration on ST cells. Means of results from three independent experiments are plotted; error bars represent standard deviations. (C) RNA synthesis of rTGEV-WT and rTGEV-ZF-C viruses. ST cells were infected at an MOI of 1 with mutant rTGEV-ZF-C or rTGEV-WT; intracellular RNA was collected at different times postinfection, and genomic RNA (gRNA) was quantified by RT-qPCR using specific TaqMan assays. Means of results from three independent experiments are plotted; error bars represent standard deviations. *, P value < 0.05; **, P value < 0.01; ***, P value < 0.001.

served in rTGEV-ZF-C-infected cells compared to rTGEV-WT-infected cells (Fig. 5B). In contrast, the levels of accumulation of TGF β mRNA, whose expression did not change with TGEV infection (54), were similar in the two cases (Fig. 5B), discarding a general decrease of host cell transcription after rTGEV-ZF-C infection.

Overall, these results indicated that rTGEV-ZF-C triggered an antiviral response that was reduced in comparison to that induced by rTGEV-WT.

dsRNA accumulation in rTGEV-ZF-C-infected cells. In non-immune cells, RIG-I and MDA-5 cytoplasmic sensors mainly mediate the production of cytokines and IFN. These proteins recog-

FIG 4 Induction of apoptosis by mutant rTGEV-ZF-C. (A) Detection of active caspase 3 (casp3) and TGEV N protein (N) by Western blotting. Total protein was extracted from ST cells 24 h after infection with mutant rTGEV-ZF-C, infection with rTGEV-WT, or mock infection (M). Lanes A and B indicate duplicate viral clones that were tested individually. Casp3, N, and β -actin (loading control) were detected using specific antibodies; procaspase 3 (procasp3) and the form of the TGEV N protein cleaved by caspases (N-cl) are also indicated. (B) Quantification of the bands by densitometry, corrected by amount of the β -actin. Relative levels of protein (r.u.) were based on comparison with the WT virus protein level, which was considered to be 100%. Means of results from three independent experiments are plotted; error bars represent standard deviations. *, P value < 0.05; **, P value < 0.01.

nize the presence of dsRNAs in the cytoplasm, activating transcription factors IRF-3 and NF- κ B, which finally leads to the expression of type I IFN and proinflammatory cytokines, respectively (67). Because rTGEV-ZF-C infection led to reduced IFN- β and TNF accumulation, dsRNA levels were analyzed by immunofluorescence in synchronized and infected ST cells. nsp14 was also detected by immunofluorescence to analyze the effect of ZF1 mutation on nsp14 protein subcellular location. The nsp14-ZF-C mutant protein localized similarly to the WT protein (Fig. 6A), with a pattern similar to that of other viral proteins present in the CoV RTC such as nsp3 (data not shown) (59).

At early times postinfection, no differences in dsRNA distribution patterns were observed in rTGEV-ZF-C-infected cells and rTGEV-WT-infected cells (Fig. 6A; 8-hpi panels). Colocalization between nsp14 and dsRNA was observed, probably corresponding to active sites of viral RNA synthesis (Fig. 6A; lower 8-hpi panels). At late times postinfection, dsRNA labeling produced a scattered pattern in rTGEV-WT-infected cells, uniformly distributed throughout the cytoplasm (Fig. 6A; middle 16-hpi panels). This dsRNA pattern was previously observed in CoV infection at late times postinfection (10, 68). In contrast, dsRNA was still concentrated in well-defined perinuclear granules in rTGEV-ZF-C-infected cells (Fig. 6A; 16-hpi panels). In order to quantify the cytoplasmic dsRNA accumulation and distribution, the mean intensity of fluorescence and the normalized variance of the intensity of fluorescence were measured in 30 individual cells. In the case of a uniform distribution of the signals, the variance is minimal, while its value increases when the signals are irregularly distributed (61). An unbiased analysis showed that, at early times postinfection, no significant differences were observed in either mean intensity or variance of intensity between rTGEV-ZF-C-

and rTGEV-WT-infected cells (Fig. 6B). In contrast, a significant reduction in the intensity of fluorescence was observed in rTGEV-ZF-C-infected cells compared to the rTGEV-WT-infected cells (Fig. 6B). Moreover, the quantification of the signal distribution revealed a significant increase in the normalized variance in the case of rTGEV-ZF-C-infected cells, which meant a reduction in the spreading of the signal in comparison to that seen with the rTGEV-WT-infected cells (Fig. 6B). This result suggested that the nsp14-ZF-C mutation interfered with dsRNA accumulation at late times postinfection, which may have been the cause of the reduced induction of subsequent antiviral responses.

Modulation of innate immune response by nsp14-ZF-C. To further investigate whether rTGEV-ZF-C actively inhibits the cytoplasmic accumulation of dsRNA and, as a consequence, the activation of the subsequent antiviral response, the dsRNA-sensing pathway was activated by the addition of exogenous dsRNA in the context of viral infection.

In order to evaluate the potential of rTGEV-WT or rTGEV-ZF-C in the inhibition of dsRNA-triggered antiviral responses, ST cells were infected with rTGEV-ZF-C or rTGEV-WT at an MOI of 1 and were transfected at 12 hpi with the dsRNA analog poly(I-C). At 4 hpt, viral RNA accumulation and the induction of the innate immune response were analyzed by measuring gRNA, IFN- β , and TNF mRNA accumulation by RT-qPCR. In the absence of poly(I-C), IFN- β and TNF mRNA production levels were significantly reduced in rTGEV-ZF-C-infected cells, as described above (Fig. 7A). Poly(I-C) treatment did not significantly affect virus production, as similar gRNA levels were detected in the absence and presence of poly(I-C) (Fig. 7A). rTGEV-WT virus, together with poly(I-C), triggered an exacerbated innate immune response, as expected from the combined action of rTGEV-WT infection

FIG 5 Innate immune response induced by mutant rTGEV-ZF-C. ST cells were infected with rTGEV-ZF-C or rTGEV-WT virus at an MOI of 1, and intracellular RNA was collected at different times postinfection. (A) Quantification of IFN- β and TNF mRNA was performed by RT-qPCR using specific TaqMan assays; relative mRNA levels were based on comparison with mock-infected cells. Means of results from three independent experiments are plotted; error bars represent standard deviations. (B) Induction of interferon-stimulated genes by rTGEV-ZF-C. Quantification of IRF-1, OAS, RIG-I, MDA5, and TGF- β mRNAs was performed by RT-qPCR using specific TaqMan assays; relative mRNA levels were based on comparison with mock-infected cells. Numbers below asterisks indicate the fold change in induction by rTGEV-WT relative to that of mutant rTGEV-ZF-C. Means of results from four independent experiments are plotted; error bars represent standard deviations. *, P value < 0.05; **, P value < 0.01; ***, P value < 0.001.

and the dsRNA analog poly(I-C) (Fig. 7A). In contrast, this response was significantly reduced in the case of rTGEV-ZF-C infection, despite the exogenous activation by poly(I-C) (Fig. 7A).

To test whether the decreased dsRNA levels led to reduced recognition and, subsequently, to a diminished innate immune response during rTGEV-ZF-C mutant virus infection, RNase L activation was analyzed. As previously described, rTGEV-WT infection produces minimal RNase L activation and RNA degradation (53). In agreement with these data, no significant differences in RNA degradation were observed for the rTGEV-ZF-C mutant-infected cells compared with those infected with the parental virus (Fig. 7B). In contrast, when RNase L activation was triggered by exogenous dsRNA [poly(I-C)] transfection together with infection, a significant decrease in RNA degradation during rTGEV-ZF-C infection compared with rTGEV-WT infection was observed (Fig. 7B). These results indicated that there was indeed decreased dsRNA recognition during rTGEV-ZF-C infection, as expected due to the lower dsRNA accumulation, which, in consequence, caused reduced IFN production.

In a complementary approach, coinfections with rTGEV-ZF-C and rTGEV-WT were performed in order to evaluate whether the

antiviral response elicited by rTGEV-WT infection was modulated by rTGEV-ZF-C. ST cells were infected at a different MOI of each virus, and IFN- β accumulation and TNF mRNA accumulation were evaluated by RT-qPCR at 16 hpi. A slight difference in gRNA levels between rTGEV-ZF-C and rTGEV-WT, which, as shown above, does not influence infectious titers, was also observed during independent infections (Fig. 8). No significant differences in gRNA accumulation were detected during coinfections (Fig. 8). Moreover, the nsp14 region was sequenced and the proportion of each rTGEV at the moment of the analysis was estimated to be similar to the one in the inoculum (data not shown). A decreased innate immune response was observed in cells coinfecting with rTGEV-ZF-C and rTGEV-WT at a 1:1 ratio, and, more significantly, in coinfections performed at a 5:1 ratio, compared to the same dose of rTGEV-WT alone (Fig. 8). This result suggests that the nsp14-ZF-C mutation could be acting in *trans*, reducing the level of dsRNA produced during rTGEV-WT infection and, as a consequence, decreasing the subsequent innate immune response. Furthermore, these data strongly suggest that the mutant virus actively reduced the level of the antiviral response triggered by the parental virus, as the

FIG 6 Accumulation of dsRNA in cells infected with mutant rTGEV-ZF-C. Confocal microscopy analysis was performed on synchronized ST cells infected with the rTGEV-ZF-C or rTGEV-WT virus at an MOI of 1. (A) Immunofluorescence images of ST cells subjected to mock infection (mock) or to infection with rTGEV-ZF-C (ZF-C) or rTGEV-WT at 8 and 16 h postinfection. nsp14 was detected using a specific polyclonal antisera and a secondary antibody staining green; dsRNA was detected using monoclonal antibody MAb-J2 and a secondary antibody staining red; DAPI (4',6-diamidino-2-phenylindole) (blue) was used to stain the nuclear DNA. Colocalization is indicated by yellow pixels in the merge panels. (B) Quantification of the mean intensity of fluorescence and normalized standard deviation of fluorescence intensity (intensity variance) of nsp14 and dsRNA. Means of 30 individual cells are plotted; error bars represent standard deviations. ***, P value < 0.001 .

levels of IFN- β and TNF accumulation were not the sum of those induced by each virus independently and, in fact, the reduction was dependent on the MOI used for the rTGEV-ZF-C virus.

Taken together, these data indicate that rTGEV-ZF-C infection leads to a reduction in the accumulation of exogenous or infection-produced dsRNA. Our results indicate that CoV nsp14 modulates the innate immune response, most likely by affecting dsRNA accumulation.

DISCUSSION

Previous studies have shown that CoV ExoN activity is required for the high-fidelity replication of the CoV genome (21, 25). Although there is extensive knowledge of *Betacoronavirus* ExoN functions during infection, the function of *Alphacoronavirus* ExoN during infection has not been analyzed apart from bio-

chemical characterization *in vitro*. In this study, we generated a set of mutants with mutations in different domains of TGEV ExoN, based on the published structure of nsp14 (32). Mutants with abrogated ExoN activity were lethal, despite being competent in viral RNA and protein synthesis. The reduction in viral RNA synthesis reported here for TGEV and previously for HCoV-229E (17) was similar to that observed for the analogous equivalent viable mutants in *Betacoronavirus*, such as SARS-CoV (21). Therefore, the reduction in RNA synthesis is most likely not itself the causative factor for the lethality of the ExoN mutations.

Mutagenesis of a residue in the ZF1 motif, included in the ExoN domain of nsp14, led to viable viruses reaching an infectious titer similar to that of the parental virus. The rTGEV-ZF-C mutant accumulated smaller amounts of dsRNA in the cytoplasm and subsequently triggered a reduced antiviral response, as evidenced

FIG 7 Modulation of dsRNA-induced antiviral response by mutant rTGEV-ZF-C. (A) ST cells were mock infected (white bars) or infected with mutant rTGEV-ZF-C (gray bars) or rTGEV-WT (black bars) at an MOI of 1 and were subsequently transfected at 12 h postinfection with poly(I-C). At 4 h posttransfection, total intracellular RNA was collected, and quantification of viral genomic RNA (gRNA), IFN- β , and TNF mRNAs was performed by RT-qPCR using specific TaqMan assays. Relative mRNA levels were based on comparison with mock-infected, nontransfected cells. Means of results from three independent experiments are plotted; error bars represent standard deviations. (B) Cellular RNA integrity. Total RNA was extracted from mock-infected (M) or rTGEV-WT (WT)- and rTGEV-ZF-C (ZF)-infected ST cells that were left untreated (-PolyIC) or treated (+PolyIC) with poly(I-C). The RNA was then analyzed using a Bioanalyzer. The positions of 28S and 18S rRNAs are indicated (left panel). The data in the graph represent 28S rRNA integrity, as measured by the Bioanalyzer. Error bars indicate standard deviations of results from four independent experiments. f.u., fluorescence units. *, P value < 0.05 ; **, P value < 0.01 ; ***, P value < 0.001 .

by decreased levels of IFN- β and TNF mRNAs. This work showed for the first time that the CoV ExoN domain, beyond its well-known role in proofreading, participates in innate immune response modulation.

The relevance of the nsp14 ZF1 motif was previously suggested by biochemical studies (32), as well as by reverse-genetics analyses, which led to nonrecovery of a virus carrying mutations of alanine of the ZF1 domain residues responsible for zinc coordination (63). Structural data, together with the fact that those mutations severely altering the ZF were lethal in MHV whereas MHV mutants lacking ExoN activity were viable (21, 63), strongly suggest that this domain is not directly involved in ExoN catalytic activity. Although the ZF1 motif may have a role in the maintenance of specific interactions of the nsp14 protein with its RNA substrate or other RTC proteins, our data support the idea of the relevance of this domain, as conservative mutations theoretically allowing zinc

coordination, such as the ZF-H mutant, completely abolished transcription in both replicon and virus systems. In addition, our data were also in agreement with the ZF1 motif not being directly involved in ExoN catalytic activity, as preliminary sequencing data have shown similar mutation rates for rTGEV-WT and rTGEV-ZF-C viruses (data not shown).

As with other ZF motifs, the ExoN ZF1 domain may have a role in RNA-nsp14 interaction, and therefore mutations in ZF1 may impact its properties in terms of affinity and/or specificity. *In vitro* studies have revealed that CoV nsp14 binds to both ssRNA and dsRNA with no sequence specificity, although data indicated that ExoN activity is much higher in substrates with dsRNA structure (22). ZFs are typically involved in protein binding to nucleic acids (35), though the recent nsp14 crystallographic structure unfortunately does not include an RNA substrate, so the specific RNA-protein interactions involving each ZF motif were not elucidated

FIG 8 Modulation of rTGEV-WT-induced antiviral response by rTGEV-ZF-C mutant. ST cells were mock infected or individually infected with mutant rTGEV-ZF-C or rTGEV-WT or coinfecting with each virus at two different ratios (1:1 or 5:1 ZF-C/WT ratio; the MOIs of each virus used are indicated by numbers on the x axis). At 16 h postinfection, total intracellular RNA was collected, and quantification of viral genomic RNA (gRNA), IFN- β , and TNF mRNAs was performed by RT-qPCR using specific TaqMan assays. Relative mRNA levels were based on comparison with mock-infected cells. Means of results from three independent experiments are plotted; error bars represent standard deviations. *, P value < 0.05; **, P value < 0.01.

(32). The molecular bases for the mechanism by which ZF proteins recognize dsRNA are complex, as they are highly dependent on contact with phosphates and hydrophobic interactions (69). This complexity makes it difficult to predict how mutations might impact the properties of binding of a ZF-containing protein to its substrate. Further analyses of the nsp14 ZF-C mutant protein exonuclease or N7-MTase activities, or RNA binding ability, are required to decipher the role of nsp14 in the dsRNA response modulation. This would require *in vitro* analysis with the purified WT and ZF-C nsp14 proteins (17, 28). Moreover, to determine its role in ExoN activity, purified nsp10 should also be included (22). This would represent an extensive biochemical characterization that would be the aim of future work.

In spite of reaching similar levels of infectious titer and gRNA accumulation, cells infected with mutant rTGEV-ZF-C accumulated lower levels of dsRNA than the rTGEV-WT-infected cells. Two alternative explanations are compatible with this fact: (i) the ZF-C mutant degrades dsRNA more efficiently than the WT virus, or (ii) the ZF-C mutant generates smaller amounts of dsRNA intermediates during infection. Nevertheless, the biochemical activity of nsp14 as an RNase, which has been clearly reported *in vitro* (17, 22), is hardly compatible with a role in generating dsRNA. Moreover, the fact that rTGEV-ZF-C reduced the antiviral response triggered both by exogenous dsRNA [poly(I-C)] and by infection-produced dsRNA (during coinfections of WT and mutant viruses) makes the first hypothesis the more feasible. Therefore, our data suggest that nsp14 ExoN plays a role in degrading dsRNA intermediates that could act as PAMPs, triggering the subsequent immune response. In this working model (Fig. 9), the engineered rTGEV-ZF-C mutant was more efficient at this task, leading to reduced amounts of dsRNA and, subsequently, inducing a very weak innate immune response.

Theoretically, it would be expected that a reduced antiviral response should lead to an increase in viral replication, eventually leading to higher virus titers. On the other hand, as viruses evolve, leading to optimum replication in a cell host, it is quite frequently

the case that disturbances in the host antiviral response do not cause a significant effect on virus titers in cell cultures. This seems to be the case for TGEV virus infecting porcine ST cells, as mutant viruses causing an increased antiviral response replicate to titers similar to those seen with the wild-type virus in tissue cultures (53). In line with that observation, the decreased IFN production caused by the rTGEV-ZF-C mutant virus did not lead to an increased viral titer. It is worth noting that rTGEV-WT infection of ST cells produces around 35 pg/ml of IFN- β (54). This concentration is much lower than that required to affect TGEV replication. In fact, we have found that a concentration of 9,000 pg/ml of IFN- β is required to cause a 20-fold decrease in viral titer (M. Becares, S. Zúñiga, and L. Enjuanes, unpublished data). Therefore, it was not surprising that it was not possible to detect an effect on virus titers of the ZF-C mutation under our experimental conditions.

To our knowledge, this work represents the first linking of CoV ExoN activity and degradation of dsRNA leading to innate immune suppression, although this function of CoV nsp14 ExoN was recently hypothesized (42). Beyond CoVs, only arenaviruses encode a protein with exonuclease activity within their genome.

FIG 9 Working model for the role of nsp14 in the counteracting of antiviral responses during CoV infection. A schematic overview of the host cell dsRNA-induced antiviral pathway is shown. The proposed mechanism of action for CoV nsp14 is indicated.

Similarly to CoV nsp14, the arenavirus nucleoprotein (NP) has cap binding function and carries 3'-5' exonuclease activity, degrading short RNA molecules and inhibiting IFN production in arenavirus-infected cells. Studies revealed that mutants lacking the 3'-5' exonuclease activity do not inhibit IFN induction, strongly implicating it in modulation of cellular antiviral responses (70–72). Additionally, it has been shown that the arenavirus NP directly interacts with the cytosolic RNA sensors RIG-I and MDA-5 (73), giving support to the hypothesis that degradation of dsRNA PAMPs by the arenavirus exonuclease avoids further recognition by RIG-I or MDA5. In the case of CoVs, which efficiently inhibit IFN induction during infection, nsp14 could also be acting by degrading dsRNA PAMPs. Interestingly, CoV nsp14 is known to interact with the cellular helicase DDX1, which has recently been described as a cytosolic dsRNA sensor that activates type I IFN responses (74).

In agreement with our working model hypothesis (Fig. 9), CoV mutants lacking ExoN activity would trigger an exacerbated innate immune response. An enhanced antiviral response, together with the reported defects in viral RNA synthesis and the increased mutation rate, could contribute to the lethal phenotype observed for alphacoronaviruses lacking ExoN activity. In fact, SARS-CoV ExoN mutants were rescued in Vero cells, defective in IFN synthesis, and MHV ExoN mutant rescue was initially impaired when IFN-competent cells were used (63) (M. Denison, personal communication). SARS-CoV lacking ExoN activity is attenuated in young and aged diseased mice, showing faster clearance than the WT virus (75). This phenotype could be compatible with the triggering of a higher innate immune response by these mutants, although this aspect was not analyzed.

Further studies are needed to understand the role of ribonucleases, both ExoN and NendoU, in antagonizing the activation of the dsRNA-induced antiviral response in the different CoV genera. These proteins may counteract the innate immune response by degrading dsRNA and limiting activation of antiviral pathways by cytosolic sensors, and therefore they may serve as targets for the design of novel antiviral strategies.

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