Chikungunya Virus nsP2 Impairs MDA5/RIG-I-Mediated Induction of NF-κB Promoter Activation: A Potential Target for Virus-Specific Therapeutics

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Introduction

Chikungunya virus (CHIKV) was first identified in 1952 as a causative agent of outbreaks. CHIKV is transmitted by two mosquito species, Aedes aegypti and A. albopictus. Symptoms after CHIKV infection in human are typically fever and joint pain, but can also include headache, muscle pain, joint swelling, polyarthralgia, and rash. CHIKV is an enveloped single-stranded, positive-sense RNA virus with a diameter of approximately 70 nm. The pathogenesis of CHIKV infection and the mechanism by which the virus evades the innate immune system remain poorly understood. Moreover, little is known about the roles of CHIKV-encoded genes in the viral evasion of host immune responses, especially type I interferon (IFN) responses. Therefore, in the present study, we screened CHIKV-encoded genes for their regulatory effect on the activation of nuclear factor kappa B (NF-κB), a critical transcription factor for the optimal activation of IFN-β. Among others, non-structural protein 2 (nsP2) strongly inhibited melanoma differentiation-associated protein 5 (MDA5)-mediated induction of the NF-κB pathway in a dose-dependent manner. Elucidation of the detailed mechanisms of nsP2-mediated inhibition of the MDA5/RIG-I signaling pathway is anticipated to contribute to the development of virus-specific therapeutics against CHIKV infection.

Keywords: Chikungunya virus, nsP2, type I interferon, evasion

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especially type I IFN responses. Therefore, in this study, we screened CHIKV-encoded genes for their regulatory effect on the activation of NF-κB, a critical transcription factor for the optimal activation of IFN-β. It is interesting to note that CHIKV-encoded nsP2, E1, and E2 proteins strongly downregulate almost all signaling molecules involved in the MDA5/RIG-I pathway, namely, MDA5, RIG-I, MAVS, IKKe, and TBK1. Subsequent studies show that nsP2 inhibits MDA5-induced activation of the NF-κB promoter activities in a dose-responsive manner while its inhibitory activity is retained regardless of the presence/absence of tag (3X FLAG) and its position (either N-term or C-term), suggesting that nsP2 is a strong antagonist of IFN-β induction upon viral infection in the cells. Further studies are warranted to fathom the underlying mechanisms of nsP2-mediated inhibition of IFN-β expression and will contribute to the development of vaccines and therapeutics against CHIKV.

Materials and Methods

Cells
HEK293T cells were acquired from the American Type Culture Collection (USA). Cells were maintained at 37°C in a humidifying 5% CO₂ incubator with Dulbecco’s modified Eagle’s medium (Welgene, Republic of Korea) supplemented with 10% fetal bovine serum (Welgene) and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA).

Reagents
Opti-MEM and the Pierce BCA Assay Kit were purchased from Thermo Fisher Scientific. Polyethylenimine (PEI) and complete Mini Protease Inhibitor Cocktail were purchased from Millipore Sigma (USA). The Luciferase Assay and Beta-Glo Assay systems were obtained from Promega (USA). EZ-Cytox was obtained from DoGenBio (Republic of Korea). The 4x Laemmli sample buffer and 2-mercaptoethanol were obtained from Bio-Rad (USA). Amersham ECL western blotting detection reagent, Amersham ECL Prime western blotting detection reagent, and Amersham Protran 0.45 NC (nitrocellulose) western blotting membranes were purchased from GE Healthcare Life Sciences (USA) [17, 18]. Mouse monoclonal anti-FLAG was purchased from Sigma-Aldrich (USA). Mouse monoclonal anti-hemagglutinin (HA), rabbit monoclonal anti-HA, and rabbit anti-GAPDH antibodies conjugated with horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (USA). Anti-mouse IgG conjugated with HRP and anti-rabbit IgG conjugated with HRP were obtained from Santa Cruz Biotechnology (USA). Monoclonal anti-CHIKV-nsP2 and anti-MAVS antibody were purchased from Abgenex (India) and Cell Signaling (USA), respectively. Pfu Plus DNA polymerase was obtained from Elpis Biotech (Republic of Korea). The restriction enzymes BglII and ApaI were purchased from Enzyme (Republic of Korea). T4 DNA ligase was obtained from New England Biolabs (USA).

Plasmid Construction
The multiple cloning site (MCS) of the pcDNA3.1-Hygro (+) vector was modified by introducing the linkers for 3X FLAG and 3X GGGGS using NheI and Pmel, respectively. The modified vector was named pcDNA3.1-Hygro-3X FLAG-GS3 and had the following sequence in the multi-cloning site: 5’-GCTAGCGCCACCATGGACTACAAGGACCACGACCATCGACTACAAGGACCACGACATCGACTACAAGGACGACGACGACAAGCTTTCTGTTGGCGTGCTCGGGCGGGAGGTGGGGTCGGGTGGCGGCGGATCCTGCAGGCGCGCCGAATTCGA (Dotted underline: restriction enzyme sequences, solid underline: CHIKV sequences. Note that when CHIKV gene sequences were codon-optimized for Homo sapiens, gene names are prefixed with “op-”).

Table 1. Primer sequences used in this study.

| Primer name          | Sequence (5'-3')                                      |
|----------------------|-------------------------------------------------------|
| ChiKV-nsp1-F         | GGCCTGCAAGGATGGAATCCGTGTACAGTGGGA                    |
| ChiKV-nsp1-R         | GCCGCCCTTATGCGGCCCTCTGTCC                             |
| ChiKV-op-nsp2-F      | GCCCTGCAAGGGAAATAATAGAAGACTCCAGAGAGG                   |
| ChiKV-op-nsp2-R      | CGGCCCTTACGGTACCTCAGGAAAAG                           |
| ChiKV-nsp3-F         | GCCCTGCAAGGGAGAGATCTGCAATCGAGAGAGG                    |
| ChiKV-nsp3-R         | CGCCGCCCTTACGTCTGAATCAGGAAAG                          |
| ChiKV-nsp4-F         | GCCCTGCAAGGGAGAGATCTGCAATCGAGAGAGG                    |
| ChiKV-nsp4-R         | CGCCGCCCTTATTTAGAAGCCCGCTCAAAAG                       |
| ChiKV-C-F            | GGCCTGCAAGGATGGAATCCGTGTACAGTGGGA                    |
| ChiKV-C-R            | CGGCCCTTACGCTTACCCACGTGGGCC                          |
| ChiKV-op-E3-F        | GGCCTGCAAGGCTTGCACCATGCTTAGATGTC                    |
| ChiKV-op-E3-R        | CGGCCCTTACCGGCCAGCGACGCAAGACG                         |
| ChiKV-op-E2-F        | GGCCTGCAAGGAAAGAACACTTCAATGATGTC                    |
| ChiKV-op-E2-R        | CGGCCCTTATTTAGCTGTCTGACGGACG                         |
| ChiKV-6K-F           | GGCCTGCAAGGGAGGGCCCATACACAGAAG                        |
| ChiKV-6K-R           | CGGCCCTTATTTAGCTGTCTGACGGACG                         |
| ChiKV-op-E1-F        | GGCCTGCAAGGAAACGTAACAGTACAGATGACG                    |
| ChiKV-op-E1-R        | CGGCCCTTACGCTTACCCACGTGGGCC                          |
AAGCGCTATCGATATCGATGGCGCCTGGCCAGACCATCAGTCGAGTGGCGCCACTGGACTAATGGTCCGTACGCTCGACTGTACAGGCCGGCCTCAGGTTAACACCGGTACCTCAGCCCGGGCGGCCGCATGGGGGGCCCCTCGAGTCTAGAGTTTAAAC-3’. Complementary DNA (cDNA) was prepared using M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions. CHIKV genes were amplified from the cDNA by polymerase chain reaction (PCR) using Pfu polymerase (Elpis Biotech, Republic of Korea). Each amplified gene was cloned into pcDNA3.1-Hygro-JY4-GS3, pcDNA3.1-Hygro-JY4-3X FLAG-GS3, and pcDNA3.1-Hygro-JY4-3X FLAG-GS3 vectors using the SbfI and ApaI restriction enzymes (Enzynomics). The sequences of the primers used in this study are provided in Table 1.

**Transfection and Luciferase Reporter Assays**

HEK293T cells were seeded into a 6-well plate and incubated at 37°C for 24 h. Next, transfection with complexes containing 1,000 ng CHIKV-encoded gene-expressing plasmids, 500 ng IFN-β-Luc vectors, 100 ng β-galactosidase (β-gal)-expressing vectors, and 500 ng each of signaling molecule-expressing plasmid was performed using PEI at a DNA:PEI ratio of 1:2 in a total of 200 μl of Opti-MEM (Thermo Fisher Scientific). At 24 h post-transfection, the transfected cells were lysed with 1× reporter assay lysis buffer (Promega) containing 1× protease inhibitor cocktail (Millipore Sigma). After incubation on ice for 5 min, lysates were harvested and centrifuged at 15,000 xg for 15 min. Then, 25 μl of lysate supernatant and firefly luciferase assay solution were mixed, and the luciferase activity was measured. Similarly, a 25-μl sample and Beta-Glo assay substrate were mixed and incubated at 37°C for 30 min, and then β-gal activity was estimated. Luciferase activity was normalized with the β-gal activity of each sample, which was used to determine the fold induction of the luciferase activity compared to the vector alone control.

**Western Blotting**

The lysates were centrifuged at 4°C and 15,000 xg for 15 min, and the supernatants were harvested for the subsequent assays. The amount of protein in a 10-μl sample was quantified using the Pierce BCA Protein Assay Kit.

Fig. 1. dsRNA-sensing molecules (MDA5 and RIG-I)-mediated induction of NF-κB promoter activities is regulated by Chikungunya virus (CHIKV)-encoded genes. Each individual gene of CHIKV was co-transfected into HEK293T cells with NF-κB-luc, β-gal, and either MDA5 (A), RIG-I-FL (B), or RIG-I-1-228 (C). At 24 h post-transfection, cells were lysed for luciferase activity and western blot, as described in Materials and Methods. Fold induction of the NF-κB promoter over empty vector control is plotted as mean ± standard deviation (upper panels) and its statistical significance was analyzed by two-tailed Student’s t-tests (*p < 0.05). Proteins were resolved by SDS-PAGE and immunoblotted using the indicated antibodies (lower panels). Viral proteins were detected using anti-HA antibodies. Asterisks indicate viral genes tagged with N-terminal 3X FLAG. HA-tagged MDA5, RIG-I, and RIG-I-1-228 were detected using anti-HA antibodies. Expression levels of MDA5, RIG-I, and RIG-I-1-228 were normalized to those of GAPDH. Representative data are shown from two independent experiments.
Thereafter, 4× Laemmli sample buffer and 2-mercaptoethanol were mixed at a 9:1 ratio, and 15 μl of each sample containing the same amount of protein was added. The mixtures were incubated at 100°C for 5 min. Each protein sample was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE Healthcare Life Science). The transferred membranes were blocked in 5% skim milk (BD, USA) for 1 h and washed three times with 1× TBS-T for 10 min. The membranes were blotted with primary antibodies (1:5,000 mouse monoclonal anti-FLAG, 1:1,000 mouse or rabbit anti-HA, and 1:2,000 anti-GAPDH) at 4°C overnight. Then, the membranes were washed three times with 1× TBS-T and incubated with anti-mouse IgG conjugated with HRP and anti-rabbit IgG conjugated with HRP for 1 h at room temperature. The membranes were treated with either Amersham ECL western blotting detection reagent or Amersham ECL Prime western blotting detection reagent (GE Healthcare Life Science) and exposed on X-ray film (Agfa–Gevaert, Belgium).

**Cell Viability Testing**

Transfected cells were treated with EZ-Cytox (DoGenBio, Republic of Korea) with 1/10th of the total culture volume at 24 h post-transfection and incubated at 37°C for 2 h. Then, the optical absorbance of each sample was measured at a wavelength of 450 nm using a SpectraMax iD3 (Molecular Device, USA).

**Statistical Analysis**

Data were expressed as the mean ± standard deviation. Representative data of two independent experiments are shown. Statistical significance was analyzed by two-tailed paired Student’s t-tests.

**Results**

**CHIKV-Encoded Genes Downregulated MDA5 and RIG-I-Mediated Induction of NF-κB Promoter Activities**

To investigate whether CHIKV-encoded genes regulate MDA5- or RIG-I-mediated induction of NF-κB, all CHIKV proteins were cloned into an expression vector fused with a tag (3X FLAG) and a spacer (3X GGGGS). As the protein expression levels of nsP2, E1, and E2 were initially low to undetectable, it was enhanced by codon...
Many CHIKV-encoded proteins seem to inhibit MDA5- and RIG-I-mediated activation of NF-κB promoter. Among others, both nsP2 and glycoproteins (E1 and E2) strongly downregulated MDA5-induced NF-κB promoter activities by over 80%, presumably by decreasing the protein levels of MDA5 by 40% to 80%, respectively (Fig. 1A). Interestingly, E1, but not nsP2, also decreased the protein levels of RIG-I-FL by 40% while both of them seemed to be able to inhibit RIG-I-mediated induction of NF-κB promoter activities to a similar extent (Figs. 1B and 1C). In addition, nsP1 and E2 glycoproteins also seem to be involved in the RIG-I-like receptors (RLR)-mediated induction of NF-κB promoter activation albeit in a differential manner; MDA5-mediated activation of the NF-κB promoter was decreased by 70% by nsP1 and 85% by E2 according to the luciferase assay whereas RIG-I-FL-induced activation of the NF-κB promoter was inhibited by neither nsP1 or E2.

As E1 and E2 form heterodimers and E3 functions to stabilize them in the host cells, dimeric (E1/E2 or E2/E3) or trimeric (E1/E2/E3) combinations were also screened for their effect(s) on the MDA5- or RIG-I-FL-mediated induction of NF-κB. The E1/E2 heterodimer and the trimer (E1/E2/E3) were able to inhibit the activation of NF-κB by MDA5 and RIG-I-FL over 90% (Fig. 1A). RIG-I-1-228 is a constitutively active form of RIG-I that only expresses the CARD domain of the full-length RIG-I (RIG-I-FL) [19]. RIG-I-1-228-mediated NF-κB activation was suppressed by nsP1 (50%), nsP2 (95%), E2 (80%), and E1 (95%). In addition, RIG-I-1-228 protein levels were greatly reduced by E1 (80%). Therefore, it is conceivable that nsP2 and E1/E2 target the CARD domains of RIG-I (Fig. 1C).

MAVS-Mediated Induction of the NF-κB Promoter Was Effectively Suppressed by nsP2, E2, and E1

To determine whether MAVS-induced NF-κB is suppressed by CHIKV proteins, we transfected each CHIKV-encoded gene, MAVS, NF-κB-luc, and β-gal into HEK293T cells. The results show that MAVS-mediated activation of NF-κB was strongly inhibited by nsP2 (95%), E2 (65%), and E1 (70%) (Fig. 2). In addition, combinations of E1/E2, E2/E3, and E1/E2/E3 significantly inhibited NF-κB promoter activation by MAVS. It is
interesting to note that MAVS protein levels were completely inhibited by nsP2 while E1 and E2 were able to significantly downregulate them as well (80% and 40%, respectively).

**TBK1/IKKe-Induced NF-κB Responses Were Inhibited by CHIKV-Encoded Genes**

Activation of TBK1 and IKKe has been shown to promote type I IFN responses. To determine if CHIKV-encoded proteins regulate TBK1/IKKe-mediated activation of IFN-β responses, HEK293T cells were transfected with TBK1- or IKKe-expressing construct, each of the viral genes, NF-κB promoter-expressing plasmid, and β-gal (control). Interestingly, many CHIKV-encoded proteins were able to inhibit TBK1-mediated (nsP2 (95%), E2 (80%), E1 (90%)) and IKKe-mediated activation of the NF-κB promoter (nsP2 (75%), E2 (85%), E1 (95%)) (Fig. 3). Levels of inhibition by CHIKV proteins were correlated with reductions of TBK1/IKKe protein levels, suggesting that CHIKV-encoded proteins seem to downregulate TBK1 and IKKe expression levels by as yet unknown mechanisms.

**MDA5-Induced Activation of the IFN-β and NF-κB Promoter Was Suppressed by nsP2 in a Dose-Dependent Manner**

To examine the kinetics of nsP2-mediated inhibition of the MDA5/RIG-I pathway, HEK293T cells were co-transfected with 0.1, 0.3, or 1.0 μg of nsP2 and MDA5 (Figs. 4A and 4B). Induction of IFN-β and NF-κB promoter activities was determined by luciferase assays. In the presence of an increasing amount of nsP2 proteins, IFN-β and NF-κB promoter activities were inhibited in a dose-responsive manner. More importantly, MDA5 protein levels were downregulated accordingly (Fig. 4), strongly suggesting that nsP2 may directly or indirectly suppress MDA5, leading to reduction of its protein levels, and thus, decrease in the downstream signaling. Ez-Cytox assays demonstrated that overexpression of nsP2 did not adversely affect cell viability (Figs. 4C-4E), suggesting that nsP2-mediated suppression of the signaling pathway may have specific underlying mechanisms that are now being investigated.
Inhibitory Activity of nsP2 Was Not Affected by the Presence/Absence and Position of a 3X FLAG Tag

We used three types of nsP2-expressing constructs: no tag, N-term (3FN), and C-term (3FC) tag (Figs. 5A and 5B). It is known that the presence/absence or position of a tag may have influence on the functions of protein to a varying degree. Our results clearly show that nsP2 was able to inhibit MDA5-mediated activation of NF-κB promoter activities regardless of the position of the 3X FLAG tag (Fig. 5), suggesting that nsP2 is a bona fide antagonist of the MDA5/RIG-I pathway.

Discussion

Pattern-recognition receptors (PRRs) play a leading role in the innate immune system. PRRs recognize conserved molecular motifs of pathogens such as bacteria and viruses through their distinctive molecular patterns. PRRs are divided into four families: toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), C-type lectin receptors (CLRs), and RLRs [20]. These receptors on the cell surface recognize pathogens, activating innate immune responses. PRR recognition of viral dsRNA by the RLRs (MDA5 and RIG-I) [21] induces the expression of IFNs and pro-inflammatory cytokines via a cascade of signaling events. MDA5 and RIG-I activation upon the recognition of viral dsRNA induces conformational changes of MAVS proteins through CARD–CARD interactions, leading to the interaction with IKKe and TBK1 [22] and then eventually phosphorylation of the transcription factors IRF3 and IRF7. Phosphorylated IRF3 and IRF7 form dimer complexes that are translocated into the nucleus to activate NF-κB, thereby inducing the production of IFNs. The IKK complex is a required component of the canonical NF-κB signaling pathway and consists of two catalytic subunits [23]. TBK1 acts as a downstream kinase mediating dsDNA-mediated IRF3 and NF-κB signaling [24].

The inhibition or downregulation of NF-κB activation through viral proteins has been reported: 1) hepatitis C virus protein is known to diminish NF-KB activation [25], 2) the classical swine fever virus non-structural 5A protein suppresses the poly(I:C)-induced NF-κB signaling pathway [26], 3) the Middle East respiratory syndrome coronavirus-encoded accessory proteins ORF4a and ORF4b or ORF8b have been reported to antagonize NF-κB activation [7], 4) the Myxoma virus M013 protein has been demonstrated to inhibit the NF-kB signaling pathway via direct binding to ASC1 and NF-κB1 [27].

Alphavirus nsP2 has been shown to antagonize antiviral responses including host transcriptional shut-off [28] and downstream type I/II IFN-induced JAK-STAT signaling, resulting in the inhibition of STAT1

Fig. 5. The presence/absence or the position of a tag (3X FLAG) on nsP2 did not affect its antagonism of NF-kB promoter activation. Native nsP2 was used or it was fused to a FLAG either at the N- or C-terminus. Fold induction of MDA5-induced IFN-β (A) or NF-κB (B) promoter activity in the presence/absence of an expression tag is plotted (upper panels). Protein expression levels were assessed by western blotting (bottom panels). Arrowheads indicate the nsP2 protein. Transfection of the native nsP2 without fusion with an expression tag displayed weak expression, and thus it was over-exposed and shown in the inlet (lower panels). One representative experiment is shown from two independent experiments. Statistical significance was determined by two-tailed Student's t-tests (*p < 0.05; **p < 0.01; ***p < 0.001).
phosphorylation and nuclear translocation [29, 30] and ultimately the blockage of type I IFN expression [1]. However, the effects of alphavirus interruption of the canonical NF-κB signaling pathway remain poorly understood. In this study, we screened CHIKV genes for antagonism of NF-κB signaling.

Previously, MDA5 and RIG-I have been shown to inhibit alphavirus replication [31]. Therefore, we hypothesized that CHIKV-nsP2 might be involved in downregulating the activities of those cytosolic receptors. We demonstrated that nsP2 and E1/E2 mediated strong inhibition of MDA5- and RIG-I-induced NF-κB (Figs. 1-3) without cell cytotoxic effects (Fig. 4). CHIKV nsP2 suppressed cytosolic immune receptor-induced NF-κB in a dose-dependent manner (Fig. 4) regardless of the presence/absence of an expression tag (3X FLAG), and its position (whether or not N-term or C-term) or C-term (Fig. 5).

Like nsP2, CHIKV-E1 and -E2 and their combination (E1/E2) were found to interfere with NF-κB induction as well. E1 and E2 are glycoproteins that form heterodimers, mediating membrane fusion between viral envelope and cell plasma membrane to initiate infection [32, 33]. However, alphavirus glycoproteins have not been reported as IFN antagonists. Previously, there were just a handful of studies showing that viral envelop proteins are involved in the antagonism of type I IFN signaling: 1) co-expression of Andes virus nucleocapsid protein with a glycoprotein precursor was found to suppress IFN-β signaling [34], 2) CHIKV E1 and E2 strongly inhibited MDA5/RIG-I-mediated induction of promoter activation of IFN-β [1] 3) Zika virus-encoded envelop protein (E) seems to suppress TBK1-mediated induction of promoter activation of IFN-β [35] as well as NF-κB [36]. Our results showed that CHIKV-encoded E1, E2 and E1/E2 dimer mediate strong antagonism of MDA5/RIG-I-mediated induction of NF-κB promoter activities (Figs. 1-3). Thus, it is tempting to hypothesize that CHIKV-E1 and -E2 proteins add to the list of virus envelop proteins that inhibit type I IFN induction.

Taken together, the data presented in the current study provide evidence that nsP2, E1, and E2 exhibit inhibitory activity that may result in the dampening of NF-κB activation (summarized in Fig. 6). Further studies are warranted to elucidate the potential mechanisms of nsP2 and E1/E2-mediated inhibition of type I IFN signaling. Detailed description of the functions of those CHIKV-encoded proteins will provide insight into the development of effective therapeutics and preventive vaccines against CHIKV.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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