Activation of NF-κB by nucleocapsid protein of the porcine reproductive and respiratory syndrome virus

Rui Luo · Liurong Fang · Yunbo Jiang · Hui Jin · Yanwei Wang · Dang Wang · Huanchun Chen · Shaobo Xiao

Abstract Nuclear factor kappa B (NF-κB) is a critical transcription factor in innate and adaptive immune response as well as cell proliferation and survival. Previous studies have demonstrated that porcine reproductive and respiratory syndrome virus (PRRSV) infection activated NF-κB pathways through IκB degradation in MARC-145 cells and alveolar macrophages. To evaluate the mechanisms behind this, we investigated the role of PRRSV structural proteins in the regulation of NF-κB. In this study, we screened the structural proteins of PRRSV by NF-κB DNA-binding assay and luciferase activity assay and demonstrated that PRRSV nucleocapsid (N) protein could activate NF-κB in MARC-145 cells. Furthermore, we revealed that the region between aa 30 and 73 of N protein was essential for its function in the activation of NF-κB. These results presented here provide a basis for understanding molecular mechanism of PRRSV infection and inflammation response.

Keywords Porcine reproductive and respiratory syndrome virus (PRRSV) · NF-κB · N protein

R. Luo · L. Fang · Y. Jiang · H. Jin · Y. Wang · D. Wang ·
H. Chen · S. Xiao
State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China

R. Luo · L. Fang · Y. Jiang · H. Jin · Y. Wang · D. Wang ·
H. Chen · S. Xiao (✉)
Laboratory of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China
e-mail: vet@mail.hzau.edu.cn

Introduction

Porcine reproductive and respiratory syndrome (PRRS), which is characterized by severe reproductive failure in sows, and respiratory distress in piglets and growing pigs, is a major cause of economic loss for the swine industry worldwide [1]. The causative agent, PRRS virus (PRRSV), is an enveloped, positive-strand RNA virus belonging to the family *Arteriviridae*, along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV) [2]. The genome of PRRSV is approximately 15 kb in length and contains nine open reading frames. Two large open reading frames (ORFs) (ORF1a and ORF1b), occupying the 5′ end nearly 75% of the viral genome, encode the viral protease and replicase polyprotein, which are predicted to be cleaved into 13 nonstructural protein products. While the seven ORFs located at the 3′ end of the genome encode four membrane-associated glycoproteins (GP2, GP3, GP4, and GP5), two unglycosylated membrane proteins (E and M), and a nucleocapsid (N) protein [3, 4].

NF-κB is a family of inducible transcription factors involving pathogen- or cytokine-induced immune and inflammation responses as well as cell proliferation and survival [5, 6]. Classical NF-κB exists as heterodimers consisting of a 50-kDa subunit (p50) and a 65-kDa subunit (p65). Under normal physiological conditions, NF-κB is present in an inactive cytoplasmic complex in association with a family of inhibitors, the IκBs, and is maintained in the cytosol in this inactive state. When stimulated with a wide variety of stimuli, the IκB proteins is phosphorylated by IκB kinase (IKK) and degraded in proteasomes, thus allowing the release and translocation of NF-κB into the nucleus to activate the transcription of genes involved in innate and adaptive immunity [7, 8].

Many viruses have evolved to use the host innate responses including activation of the transcription factor NF-κB to their own advantages. For example, Epstein–Barr virus, through its latency membrane protein 1, activates NF-κB signaling pathways to facilitate the growth of B lymphoblastoid cells as its latent reservoir [9, 10]. Similarly, hepatitis C virus core protein enhances NF-κB signal pathway allowing persistence of HCV in a long-lived cell compartment, thus establishing a chronic, activated state of HCV infection [11]. Previous studies showed that PRRSV infection modulates NF-κB activation in MARC-145 cells and alveolar macrophages [12, 13]. However, the molecular mechanisms behind this event remain to be determined. In this study, we investigated the role of PRRSV structural proteins in the regulation of NF-κB activation in MARC-145 cells, providing a basis for understanding the molecular mechanisms involved in the functions of PRRSV proteome and pathogenesis of PRRSV.

Materials and methods

Virus and cells

PRRSV strain CH-1a was kindly provided by Dr. Guangzhi Tong (Harbin Veterinary Research Institute, Harbin, China). MARC-145 cell line was cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.25 μg/ml fungizone, 100 U/ml penicillin, 10 μg/ml streptomycin sulfate, and 5 μg/ml gentamicin.

Plasmids

Expression plasmids of PRRSV structural proteins used in this report were constructed by RT-PCR amplification from PRRSV strain CH-1a genomic RNA and insertion into the pCMV-Tag2B (Stratagene) vector. All plasmid inserts were sequenced and PRRSV proteins expression was verified by transfection and detection by western blot analysis using anti-FLAG antibody. Strategies for the construction of the N protein deletion mutants were according to the Wootton's reports [14, 15]. These genes fragments were subcloned into pCMV-HA (Clontech) to generate a series of N protein deletion mutants.

Transfection and luciferase reporter assay

Transient transfection was performed by using Lipofectamine 2000 (Invitrogen). Luciferase reporter assay were described previously [13].

Cellular extracts and ELISA-based NF-κB DNA-binding assay

Cytoplasmic and nuclear protein extracts from MARC-145 cells after transfection with indicated plasmids were prepared with the nuclear extraction kit (Active Motif, Inc., Carlsbad, CA) according to the manufacturer's protocol. Protein concentration was determined by Micro BCA™ Protein Assay (Pierce) with bovine serum albumin as a standard. The ability of NF-κB binding to κB sites was assessed by a Trans-AM NF-κB p65 transcription factor assay kit (Active Motif, Inc., Carlsbad, CA) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) without interaction terms followed by Dunnett's or Duncan's test for multiple comparisons. P values less than 0.05 were considered statistically significant.

Result

Nucleocapsid protein of PRRSV stimulates DNA-binding activity of NF-κB

To screen the PRRSV proteins for the capacity to activate NF-κB, their ability to increase NF-κB DNA-binding activity was detected by NF-κB p65 transcription factor assay. As shown in Fig. 1, the NF-κB p65 DNA-binding activity in N protein-transfected cells showed an approximately 2.5-fold higher binding activity than that in vector-transfected cells ($P < 0.01$), whereas the increased DNA-binding activity was not found in other transfected cells. These results demonstrated that expression of N protein of PRRSV induces NF-κB DNA-binding activity in MARC-145 cells.

Nucleocapsid protein of PRRSV enhances NF-κB-regulated gene expression, and such activation is in a dosage-dependent manner

We next assessed whether this enhancement of NF-κB DNA-binding activity by N protein is also reflected on the NF-κB-dependent transcriptional activity. To this end, an NF-κB reporter assay was used to determine if N protein enhanced NF-κB-regulated gene expression. As shown in Fig. 2a, compared with the level in the vector-transfected MARC-145 cells, about threefold enhancement of luciferase activity in N protein expressed cells was noted ($P < 0.01$), which correlated with an increased level of NF-κB
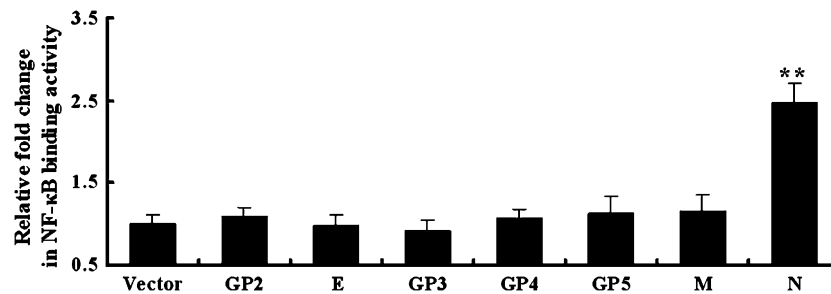
Fig. 1 NF-κB binding activity is increased by N protein. NF-κB p65 binding to DNA was determined using the TransAM NF-κB p65 transcription factor assay kit. Expression plasmids of PRRSV proteins were transfected to MARC-145 cells, then nuclear extracts of MARC-145 cells were prepared at 24 h post-transfection. Results are expressed as the fold increase of binding activity compared to empty vector as a control. Values are mean ± SEM of three independent tests. ** $P < 0.01$ compared with vector group. Significant differences between groups were determined by one-way ANOVA followed by Dunnett's multiple comparisons test
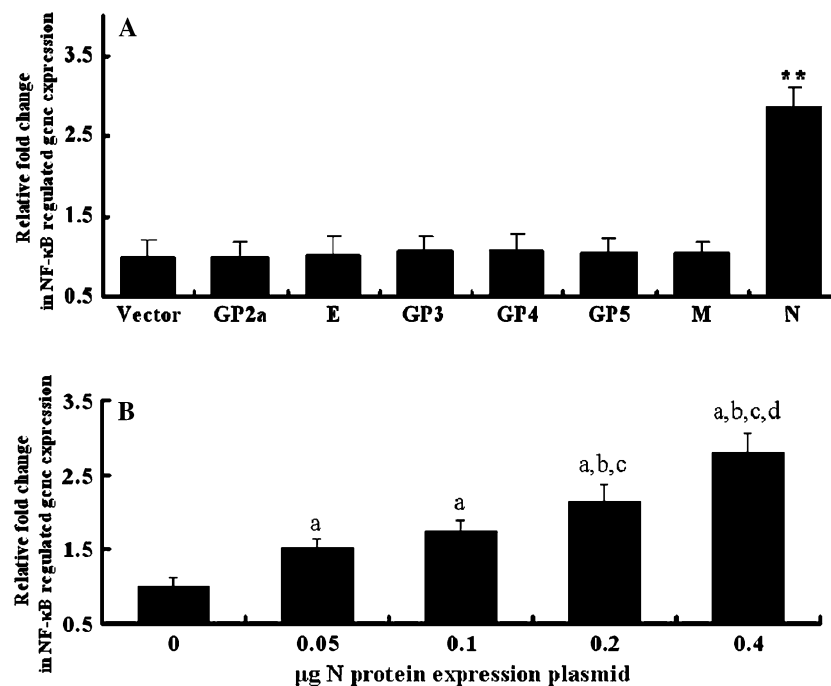
Fig. 2 N protein enhances NF-κB-driven transcription. **a** MARC-145 cells were cotransfected with 0.4 μg of pNF-κB-LUC, 0.04 μg pRL-TK, and 0.4 μg PRRSV protein expression plasmids or empty vector as negative control. Cells were harvested 24 h after transfection and assayed for luciferase activity. Values are mean ± SEM of three independent tests. ** $P < 0.01$ compared with vector group. Significant differences between groups were determined by one-way ANOVA followed by Dunnett's multiple comparisons test. **b** MARC-145 cells were cotransfected with 0.4 μg of pNF-κB-LUC, 0.04 μg pRL-TK, and 0, 0.05, 0.1, 0.2, 0.4 μg N protein expression plasmid separately and empty vector as negative control. Cells were harvested 24 h after transfection and assayed for luciferase activity. Values are mean ± SEM of three independent tests. (a) $P < 0.05$ compared with the vector group, (b) $P < 0.05$ compared with 0.05 μg N protein transfection group, (c) $P < 0.05$ compared with 0.1 μg N protein transfection group, (d) $P < 0.05$ compared with 0.2 μg N protein transfection group. Significant differences between groups were determined by one-way ANOVA followed by Duncan's multiple comparisons test

DNA-binding activity (Fig. 2a). In addition, enhancement of luciferase activity was not observed in cells transfected with constructs expressing other structural proteins. To further determine if there was a relationship between PRRSV N protein and NF-κB activation, NF-κB activity was monitored in MARC-145 cells which expressed increasing amounts of N protein. As shown in Fig. 2b, a dose-dependent increase in the luciferase reporter activity was observed ($P < 0.05$). These data demonstrated that only N protein can activate NF-κB in PRRSV structural proteins and this activation is dose-dependent.

Functional domain of N protein for NF-κB activation

To determine the functional domain of the N protein for NF-κB activation, serial deletion mutants of N protein were

Fig. 3 MARC-145 cells were cotransfected with 0.4 μg of pNF-κB-LUC, 0.04 μg pRL-TK, and 0.4 μg deletion mutants of N protein or empty vector as negative control. Cells were harvested 24 h after transfection and assayed for luciferase activity. Values are mean ± SEM of three independent tests. ** $P < 0.01$ compared with wide type N protein (N1-123) group. Significant differences between groups were determined by one-way ANOVA followed by Dunnett's multiple comparisons test
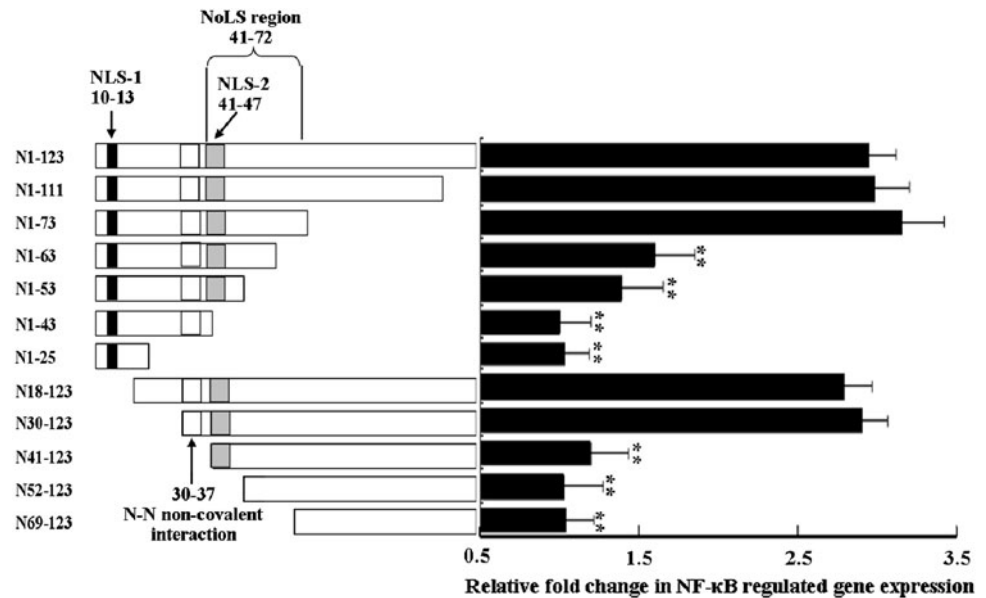
constructed in this study (Fig. 3). As shown in Fig. 3, several mutants, including N1-111, N1-73, N18-123, and N30-123, which all contain the stretch of basic amino acids at positions 30–73, had an increase in NF-κB-regulated gene expression. In addition, luciferase activity was significantly reduced in other mutants without this region compared with wide type N protein (N1-123) ($P < 0.01$). This suggests that the functional domain related to NF-κB activation might be located in the middle of the N protein.

Discussion

PRRS is an infectious disease characterized by abortions in sows and respiratory disorders. PRRSV replicates in porcine macrophages and induces several cytokines including IL-6, IL-8, IL-10, and TNF-α involving pulmonary inflammation response [16, 17]. NF-κB is a critical transcription factor regulating the transcription of many pro-inflammatory molecules that are thought to be important in the generation of inflammation, including certain adhesion molecules (ICAM-1), most cytokines (TNF-α, IL-6), and chemokines (IL-8) [18]. For example, Respiratory syncytial virus (RSV) has been shown to activate NF-κB and result in IL-8 and RANTES gene expression [19, 20]. The activation of NF-κB has been found in PRRSV infected MARC-145 cells and alveolar macrophages [12, 13], and involve pulmonary inflammation and innate immune responses. Interestingly, as a result of treatment with NF-κB inhibitor BAY-117082, PRRSV failed to induce IL-6, IL-8, and RANTES expression in MARC-145 cells (date not shown). To our knowledge, NF-κB activation seemly plays an important role in proinflammatory

molecules expression induced by PRRSV. In this study, we sought to identify the PRRSV proteins that are responsible for mediating the activation of NF-κB. Screening PRRSV structural proteins for the capacity to activate NF-κB, we observed that only the N protein significantly increased p65 DNA-binding activity (Fig. 1) and induced the activation of NF-κB in MARC-145 cells (Fig. 2a) and such activation was in a dosage-dependent manner (Fig. 2b). These results demonstrated that N protein is, at least partially, responsible for the induction of NF-κB activation.

PRRSV N protein, the principal component of the viral nucleocapsid, is the most abundant structural protein of the virus. During PRRSV infection, a proportion of the N protein has been found to specifically localize to the nucleus and nucleolus [21, 22]. Recently, dynamic trafficking of PRRSV N between cytoplasm and nucleolus was also observed [23]. In this study, we constructed a series of deletion mutations of N protein and observed that the middle region (from amino acids 30 to 73) of N protein was essential for the viral protein to activate NF-κB, since deletion of this region resulted in the loss of function in the activation of NF-κB (Fig. 3). Two classical types of nuclear localization signal (NLS) in the N protein have been identified at aa positions 10–13 (NLS-1) and 41–47 (NLS-2), respectively. Deletion of NLS-1 did not affect N protein localization to the nucleolus while NLS-2 mutagenesis blocked nuclear localization, indicating that NLS-2 was functional for translocation and retention of N protein in the nucleolus [21]. As shown in our results, NLS-2 as well as nucleolar localization signal sequence (NoLS) was essential for NF-κB activation induced by N protein. The failure of N protein mutants lacking NLS-2 or NoLS to activate NF-κB might be due to the loss of its ability to

target to the nucleus or nucleole. Previous studies demonstrate that PRRSV N protein nuclear localization is associated with viral pathogenesis and host response to PRRS [24]. It is postulated that PRRSV N protein, acting as a non-structural protein function, interact with a cellular transcription factor to modulates host cell gene expression favoring its replication and evasion of innate immune response. Compared with PRRSV N protein mutants N30-123 and N41-123 in inducing NF-κB activity, we also observed that aa 30–41 were essential for NF-κB activation induced by N protein (Fig. 3). Non-covalent N–N interaction domain (aa 30–37) [15], which mediates N protein homodimerization, is embedded in this region, implying that N protein homodimerization is probably essential in NF-κB activation induced by N protein.

NF-κB is a critical regulator of the immediate early pathogen response, playing an important role in promoting inflammation and regulating cell proliferation and survival [25]. So genes encoded by many viruses have evolved different strategies to activate NF-κB to facilitate their replication, host cell survival, and evasion of immune responses. For example, U$_L$37 tegument protein encoded by herpes simplex virus (HSV)-1 interacts with TNF receptor-associated factor 6 (TRAF6) resulting in NF-κB activation required for efficient viral replication [26]. NF-κB activation induced by Hepatitis C virus (HCV) core protein and Epstein–Barr virus LMP1 is important for infected cell survival and viral persistent infection [27, 28]. In this study, we first demonstrated that among PRRSV structural proteins only N protein was involved in NF-κB activation and such activation is dose-dependent. This study not only helps clarify the molecular mechanisms of NF-κB activation during PRRSV infection but also helps explain the ability of PRRSV to modulate the immune response by regulating NF-κB activation.

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