Porcine reproductive and respiratory syndrome virus non-structural protein 1 suppresses tumor necrosis factor-alpha promoter activation by inhibiting NF-κB and Sp1

Sakthivel Subramaniam, Byungjoon Kwon, Lalit K. Beura, Charles A. Kuszynski, Asit K. Pattnaik, Fernando A. Osorio

Abstract

The objective of this study was to identify porcine reproductive and respiratory syndrome virus (PRRSV)-encoded proteins that are responsible for the inhibition of TNF-α expression and the mechanism(s) involved in this phenomenon. Using a TNF-α promoter reporter system, the non-structural protein 1 (Nsp1) was found to strongly suppress the TNF-α promoter activity. Such inhibition takes place especially at the promoter’s proximal region. Both Nsp1α and Nsp1β, the two proteolytic fragments of Nsp1, were shown to be involved in TNF-α promoter suppression. Furthermore, using reporter plasmids specific for transcription factors (TFs) that bind to TNF-α promoter, Nsp1α and Nsp1β were demonstrated to inhibit the activity of the TFs that bind CRE-κB3 and Sp1 elements respectively. Subsequent analyses showed that Nsp1α moderately inhibits NF-κB activation and that Nsp1β completely abrogates the Sp1 transactivation. These findings reveal one of the important mechanisms underlying the innate immune evasion by PRRSV during infection.

Introduction

Porcine reproductive and respiratory syndrome is an economically important disease causing losses to swine industry worldwide. The disease is characterized by late term reproductive failure in sows and respiratory distress in young growing pigs (Wensvoort, 1993). The severity of the disease in young pigs is related to the age and the multi-factorial nature of the disease. The disease is caused by an arterivirus, porcine reproductive and respiratory syndrome virus (PRRSV) that belongs to the order, Nidovirales. PRRSV is a single stranded, positive sense RNA virus having a genome of approximately 15 kb in size (Meulenberg et al., 1993). PRRSV predominantly replicates in differentiated cells of myeloid lineage such as macrophages and dendritic cells (Duan et al., 1997; Wang et al., 2007; Wensvoort, 1993). Since macrophages and dendritic cells are major producers of innate defensive cytokines such as interferon-alpha (IFN-α) and tumor necrosis factor-alpha (TNF-α) during viral infections, it seems plausible that PRRSV may actively suppress their induction in order to replicate in the host cells for multiple rounds and fulfill the pathogenic goal of establishing persistency in the host (Allende et al., 2000; Beura et al., 2009; Lopez-Fuertes et al., 2000). In addition, the defective induction of innate immune defense against PRRSV may contribute to the delayed and weak development of antigen specific adaptive immune responses later during PRRSV infection (Lopez and Osorio, 2004; Meier et al., 2003). PRRSV encodes 8 open reading frames that produce 14 non-structural proteins (Nsp)s and 7 structural proteins. Recent studies from our laboratory have shown that several proteins such as Nsp1, Nsp2, Nsp4, and Nsp11 are able to suppress IFN-α promoter activation upon dsRNA treatment (Beura et al., 2009). These proteins suppress the induction of Type I IFNs and signaling by inhibiting transcription factors such as interferon regulatory factor-3, nuclear factor-κB, CREB-binding protein (CBP) and signal transducers and activators of transcription-1 (STAT-1) (Beura et al., 2009; Chen et al., 2009; Kim et al., 2010). However, the molecular mechanisms underlying the TNF-α suppression by PRRSV are unknown.

TNF-α is an immediate-early cytokine, which is secreted in response to various viral infections by activated macrophages, dendritic cells and T cells. TNF-α has two biological functions: First, it induces apoptosis in virus infected cells. This effect of TNF-α on virus infected cells is synergistically augmented by the presence of IFN-γ (Wong and Goeddel, 1986). Second, TNF-α promotes inflammation at the site of infection by inducing the production of other proinflammatory cytokines at the vicinity of infection (Toews, 2001). TNF-α directly inhibits replication of both DNA and RNA viruses in vitro (Cirino et al., 1993; Heise and Virgin, 1995; Wong and Goeddel, 1986). Viruses such as African swine fever virus (ASFV) and herpes simplex virus-1 (HSV-1) directly inhibit induction of TNF-α during...
infection (Granja et al., 2006; Mogensen et al., 2004). Several studies have shown that PRRSV is also able to suppress the TNF-α production both in vitro and in vivo (Lopez-Fuentes et al., 2000; Thanawongnuwech et al., 2004; Van Gucht et al., 2003; Van Reeth et al., 1999).

The objective of the present study was to identify the PRRS viral proteins that inhibit TNF-α expression, especially at the promoter level, and determine the underlying mechanism(s) through which this virus inhibits TNF-α production. Among 11 PRRSV encoded proteins screened, only Nsp1 was able to strongly suppress the TNF-α promoter. Moreover, both Nsp1α and Nsp1β, products of Nsp1 derived by auto-cleavage process, were involved in the TNF-α promoter suppression. Further detailed analyses showed that Nsp1α and Nsp1β inhibit TNF-α promoter by inhibiting NF-κB activation and Sp1 transactivation, respectively. These findings provide a mechanistic understanding of how PRRSV utilizes two of its non-structural proteins to suppress the TNF-α promoter activation. This study also gives insights into how PRRSV evades the immune response by inhibiting innate immune defenses during infection.

Results

PRRSV inhibits TNF-α production in infected swine macrophages

Previous studies have shown that PRRSV induces a weak TNF-α response during in vitro and in vivo infection (Lopez-Fuentes et al., 2000; Van Reeth et al., 1999). In order to reconfirm the defective TNF-α response during PRRSV infection in our own experimental system, macrophages were derived in vitro from peripheral blood mononuclear cells (PBMCs) as described in Materials and methods. The phenotype of these macrophages was characterized by the expression of various surface markers and was found to be SWC3+ MHCII+ CD163+. These cells were found to be permissive for PRRSV to a level comparable to that exhibited by pulmonary alveolar macrophages, the natural host cells for PRRSV in vivo (data not shown). These PBMC-derived macrophages were infected with a highly virulent PRRSV strain derived from an infectious clone, FL12 (Kwon et al., 2006a,b; Truong et al., 2004) at a multiplicity of infection of 10. LPS-stimulated cells and mock infected cells were used as positive and background controls for TNF-α response, respectively. When TNF-α mRNA levels were measured by quantitative PCR, there were no detectable TNF-α mRNA levels during 2 and 6 h post PRRSV infection (PI), when compared to LPS stimulated cells (Fig. 1A). However, significant levels of induction of TNF-α mRNA were observed at 12 and 24 h PI. Even though there was a delayed TNF-α transcriptional response observed during PRRSV infection, there were no detectable levels of secreted TNF-α protein in the supernatant of infected macrophages as determined by ELISA (Fig. 1B). The kinetics of PRRSV infection in the infected macrophages was measured by titrating virus contained in the supernatant of infected cells, which showed peak viral titers at 12 h PI (Fig. 1C). Hence, PRRSV induces a delayed TNF-α transcriptional response, which coincides with peak viral replication but fails to elicit TNF-α protein secretion at any time points observed during in vitro infection in PBMC derived macrophages.

Fig. 1. PRRSV down modulates TNF-α response during in vitro infection in PBMC derived macrophages. (A) Quantitative PCR analysis of TNF-α mRNA levels in PRRSV infected and LPS stimulated cells. PBMC derived macrophages were either mock infected or infected with PRRSV (FL12 strain) at a multiplicity of infection of 10. LPS stimulated cells were used as positive control for TNF-α response in these cells. Cells were collected at indicated time points after infection. RNAs were isolated from these cells and cDNAs were prepared and used for quantitative PCR. The TNF-α mRNA copy numbers were normalized with that of endogenous β-actin mRNA copy numbers. Bars represent the average amount of TNF-α mRNA copy numbers relative to that in mock control ± standard error of mean (SEM) from three independent experiments done with cells collected from three different pigs. (B) Measurement of TNF-α protein levels in the supernatant of same treatments mentioned in panel A. Supernatants were collected and the TNF-α protein levels were measured by using a commercial swine TNF-α specific ELISA. Bars represent the average amount of TNF-α (in picograms (pg)/mL ± SEM). (C) Viral titers in the supernatants of same treatments mentioned in panel A, to check for kinetics of viral replication. Viral titers were measured in MARC-145 cells and expressed as tissue culture infectious doses50/mL. Error bars represent the SEM.

PRRSV Nsp1α and Nsp1β suppress activation of the proximal region of swine TNF-α promoter

ASFV A238L protein inhibits TNF-α production by modulating the activity of transcription factors at the promoter level (Granja et al., 2006). In the current study with PRRSV, we examined which PRRSV proteins are involved in the down-regulation of TNF-α expression at the promoter level. To that end, a swine TNF-α promoter-based luciferase reporter plasmid was constructed and used to screen various PRRSV non-structural proteins and the N protein for suppression of the reporter activity in transfected RAW 264.7 cells. We used lipopolysaccharide (LPS), a known inducer of TNF-α, to activate swine TNF-α promoter. Upon LPS stimulation, of all the PRRSV proteins tested, only Nsp 1 was able to strongly and significantly suppress the full length swine TNF-α promoter activation (Fig. 2A). Previous reports suggested that the proximal region of
TNF-α promoter (−192) was amply sufficient for activation of TNF-α promoter (Haudek et al., 1998; Yao et al., 1997). Hence, the effect of Nsp1 on the proximal region (−194) of TNF-α promoter was next examined. As shown in Fig. 2B, Nsp1 was able to strongly suppress the proximal region (−194) of swine TNF-α promoter in transfected cells. This indicates that Nsp1’s suppressive activity on TNF-α promoter resides on the proximal region of the promoter.

PRRSV Nsp1 is auto-cleaved into two separate proteins, Nsp1α and Nsp1β in infected cells (Chen et al., 2009; Den Boon et al., 1995). Recently, the exact cleavage site between Nsp1α and Nsp1β has been determined (Chen et al., 2009; Sun et al., 2009). In order to find the role of these cleaved products in TNF-α promoter suppression, their respective coding regions were cloned separately in frame with a N-terminal Flag tag under CMV promoter. As shown in Fig. 2C, both Nsp1α and Nsp1β equally suppress the TNF-α promoter but not to the levels of the whole Nsp1 protein. In summary, PRRSV Nsp1 and its cleaved products may target cellular transcriptional factors that bind to the proximal region of TNF-α promoter and consequently suppress the promoter activation. Both subunits of Nsp1 are collaboratively involved in this TNF-α promoter suppression.

Effect of PRRSV Nsp1α and Nsp1β on different transcription factors that bind to TNF-α promoter

Different transcription factors are known to bind TNF-α promoter especially at −192 proximal region of the promoter and form a
Fig. 3. Transcription factors affected by Nsp1α and Nsp1β at the proximal region (−194) of TNF-α promoter. (A) Pair wise sequence alignment of swine TNF-α promoter sequence (lower row) with that of human sequence (upper row). The nucleotides in the human sequence that are conserved with that in swine sequence are denoted by dots. Gaps in the sequences are denoted by dashes. Predicted functional elements that bind to transcription factors are in bold and underlined. The names of the cis-acting functional elements or binding transcription factors are indicated above the sequence. Number represents the position of nucleotide relative to transcription start site of human sequence. TBP: TATA binding protein. The transcription start sites of respective sequences were denoted by ts (+1 position). The ts of human was considered for all cases in this study. (B) Schematic diagram that shows the sequences and the repeats of cis-acting TFs specific elements that were cloned upstream of a basal TATA promoter in a luciferase construct. The sequences of functional elements are depicted above the schematic diagrams of the constructs and are denoted in bold as well as underlined. (C) Effect of Nsp1 and its autocleaved products on the activity of different transcription cis-acting functional elements at TNF-α promoter. RAW264.7 cells were transfected with each TF(s) specific reporter construct (0.2 μg) along with plasmids expressing Nsp1 or its autocleaved products (1 μg) and renilla luciferase vector (10 ng). After 24 h post-transfection, cells were stimulated with LPS and luciferase activities were measured as described earlier. For non-specific luciferase activities, a Gal4 specific reporter construct was employed. Data represent the average of relative luciferase units from three independent experiments ± SEM. A p-value of less than 0.05 was considered as statistically significant (unpaired t test). CV: Control vector.
specific transcriptional complex upon activation by various stimuli (Falvo et al., 2000; Tsai et al., 2000). The pair-wise sequence alignment comparison of human and swine TNF-\(\alpha\) proximal promoter sequences (Fig. 3A) shows that the transcription factor binding sites (except Sp1/Egr1 binding site) seem to be well conserved between human and swine. With the aim of finding the transcription factors affected by Nsp1\(\alpha\) and Nsp1\(\beta\), luciferase reporter plasmids specific for different functional transcription factors were constructed by utilizing specific sequences derived from swine TNF-\(\alpha\) promoter (Fig. 3B). In addition, Gal4, a yeast transcription factor specific reporter plasmid was constructed to use as non-specific response control in the assay. As shown in Fig. 3C, Nsp1\(\alpha\) was able to significantly suppress the activity of transcription factors that bind to the Egr1/Sp1 element. However, in contrast to Nsp1\(\alpha\), Nsp1\(\beta\) suppresses the activation of a different transcription factor (Sp1) especially at the proximal site of the TNF-\(\alpha\) promoter (Fig. 3B). Nsp1\(\beta\) also strongly suppresses the transcription factors that bind to Egr1/Sp1 element (Fig. 3B). In conclusion, Nsp1\(\alpha\) partially inhibits the transcription factors that bind to CRE-\(\kappa\)B, Nsp1\(\beta\) strongly inhibits the activity of Sp1 at both sites in the TNF-\(\alpha\) promoter.

**PRRSV Nsp1\(\alpha\) partially inhibits NF-\(\kappa\)B activation**

Nsp1\(\alpha\) is able to suppress the transcription factors that bind CRE-\(\kappa\)B element of TNF-\(\alpha\) promoter (Fig. 3C). ATF2/c-jun and p65/p50 complexes are known to directly interact with CRE and \(\kappa\)B sites, respectively (Tsai et al., 2000). Many viruses such as human immunodeficiency virus and poxviruses are known to encode proteins that modulate NF-\(\kappa\)B activation during infection (Bour et al., 2001; Nichols and Shisler, 2009). Hence, the effect of Nsp1\(\alpha\) on NF-\(\kappa\)B activation was next examined. To this end, using a reporter plasmid specific for NF-\(\kappa\)B activation, Nsp1\(\alpha\) was found to partially suppress the NF-\(\kappa\)B activation in transfected cells upon LPS stimulation (Fig. 4). Hence, the Nsp1\(\alpha\) mediates an inhibitory effect on NF-\(\kappa\)B activation to suppress the activation of TNF-\(\alpha\) promoter.

**PRRSV Nsp1\(\beta\) strongly inhibits transactivation of Sp1 especially through its B domain**

Nsp1\(\beta\) was found to inhibit the reporter activity specific to endogenous Sp1 upon LPS stimulation (Fig. 3C). Consistently, Sp1 is recruited to TNF-\(\alpha\) promoter in an inducible manner during virus infections in different cell types (Falvo et al., 2000). Since Sp1 is a transactivator at many inducible promoter activations (Wierstra, 2008), we examined whether Nsp1\(\beta\) affects the transactivation potential of Sp1. The n-terminal sequences (1–615 amino acids position) of Sp1 were found to mainly contribute to the transactivation potential of Sp1 (Wierstra, 2008). To that end, it was fused in frame with DNA binding domain of Gal4, a yeast transcription factor to construct Gal4-Sp1 fusion protein (Fig. 5A). This Gal4-Sp1 fusion protein can bind Gal4 responsive element containing promoter to stimulate the reporter activity in transfected cells upon LPS stimulation. This reporter activity is then directly proportional to the transactivation potential of Sp1 n-terminal region fused to Gal4 DNA binding domain. As shown in Fig. 5B, Nsp1\(\beta\) was able to completely suppress the transactivation of Sp1 upon LPS stimulation.

The transactivation potential of Sp1 is mainly carried out by A and B domains of Sp1, each of which contains a glutamine-rich domain (Wierstra, 2008). In addition, Sp1 has a negative inhibitory domain at its n-terminus which can repress the transactivation mediated through other domains, such as A and B domains, by recruiting repressors and co-repressors to Sp1 (Lee et al., 2005; Murata et al., 1994). During activation, this negative inhibitory domain is removed by ubiquitin-dependent proteasomal activity (Spengler and Brattain, 2006). Complete removal of this negative regulatory domain from Sp1 transactivation region did increase the transactivation ability of Sp1 but did not have any effect on the ability of Nsp1\(\beta\) to suppress the activity of Sp1 (Fig. 5C). Hence, n-terminal negative inhibitory domain of Sp1 has no role in Nsp1\(\beta\) mediated inhibition of Sp1 transactivation. LPS was found to activate transactivation of Sp1 mainly through its B domain rather than A domain (Brightbill et al., 2000). Gal4 fusion protein with only B domain of Sp1 has transactivation ability comparable to that of the whole transactivation region without N domain (Fig. 5C and D). Moreover, it was known that glutamine-rich c-terminal region of Sp1 B domain (Sp1BC) was found to be sufficient for activation of Sp1 dependent promoter (Gill et al., 1994). Hence, we investigated whether Nsp1\(\beta\) was able to suppress the B domain especially through its glutamine-rich c-terminal region. As shown in Fig. 5D, Nsp1\(\beta\) was able to strongly suppress the transactivation of Sp1 B domain as well as its glutamine rich c-terminal region. Overall, Nsp1\(\beta\) seems to inhibit the TNF-\(\alpha\) promoter by suppressing the transactivation of Sp1 probably by modulating the interactions of glutamine-rich region of Sp1 B domain.

**Discussion**

Porcine reproductive and respiratory syndrome virus replicates in macrophages and dendritic cells and suppresses innate cytokines such as Type I interferons and TNF-\(\alpha\) (Beura et al., 2009; Chen et al., 2009; Kim et al., 2010; Lopez-Fuertes et al., 2000). In this paper, we identified the PRRS viral protein-Nsp1 that mediates such suppression at the level of the promoter of TNF-\(\alpha\). Moreover, we found that both constitutive subunits of Nsp1 (Nsp1\(\alpha\) and Nsp1\(\beta\)) suppress the TNF-\(\alpha\) promoter by modulating activities of transcription factors NF-\(\kappa\)B and Sp1, respectively. Our data also suggest that by inhibiting transcription factors activated by two important pathways, NF-\(\kappa\)B pathway and MAPK pathway, PRRSV can effectively inhibit the TNF-\(\alpha\) promoter to suppress TNF-\(\alpha\) mediated apoptosis of infected cells.

In our experiments, PRRSV induced negligible levels of TNF-\(\alpha\) during in vitro infection of PBMC-derived macrophages. Particularly, there was a failure of induction of TNF-\(\alpha\) mRNA during PRRSV infection at early time points after infection, which would suggest that, at the MOI used, the genomic RNAs of input virus may not activate TNF-\(\alpha\) gene. However, there is an up regulation of TNF-\(\alpha\) mRNA levels observed at later time points of PRRSV infection. Moreover, there may be an involvement of a post-transcriptional mechanism used by PRRSV to suppress TNF-\(\alpha\) production either at
translation or at secretion levels. Even in the presence of TNF-α mRNAs at later phases of infection, the secreted levels of TNF-α were not detected. Our results were consistent with previous publications reporting that, apart from inducing anti-apoptotic transcripts, PRRSV does not up regulate TNF-α production during in vitro as well as in vivo infection (Genini et al., 2008; Thanawongnuwech et al., 2004; Van Gucht et al., 2003; Van Reeth et al., 1999). Lopez-Fuertes et al. (2000) reported that PRRSV elicits a defective transcriptional response during early hours of infection with no protein secreted at any time points after infection.

In order to find the proteins that may have inhibitory effect on TNF-α promoter, several non-structural proteins and N protein of PRRSV were screened using a swine TNF-α promoter based luciferase reporter assay. LPS was used as a stimulant for TNF-α transcription in the luciferase assay to mimic TLR mediated induction of TNF-α by virus (Takeda and Akira, 2005). Only Nsp1 was found to be actively suppressing the swine TNF-α promoter. Hence, in addition to anti-IFN activities (Beura et al., 2009), Nsp1 also has an anti-TNF activity. Moreover, regarding Nsp1 mediated suppression, there was no difference observed between a full length TNF-α promoter and a region of TNF-α promoter involving 194 nucleotides proximal to transcriptional start site. This proximal region of TNF-α promoter had been previously demonstrated to be sufficient for optimal stimulation of TNF-α promoter (Panne et al., 2007; Yao et al., 1997). Like in the case of anti-IFN activities, both of the auto-cleaved subunits of Nsp1, Nsp1α and Nsp1β, were involved in the suppression of the TNF-α production. Whole length Nsp1 suppresses TNF-α promoter stronger than Nsp1α or Nsp1β do. Hence, it is conceivable that a synergistic functional interaction may exist between these two Nsp1 subunits to strongly suppress TNF-α promoter by targeting activities at the

---

**Fig. 5.** Nsp1β suppresses the transactivation of Sp1. (A) Schematic diagram that shows the sequences of Sp1 (black box) cloned in frame with Gal4 DNA binding domain (DBD, 1–147 aminoacids position, grey box). The letters above Sp1 TAD (black box) indicate the position of Sp1 domains in the transactivation region. The number below each construct indicates the position of aminoacids in Sp1 sequence. (B) Effect of Nsp1β on transactivation potential of Sp1. RAW264.7 cells were transfected with pGal4RE-luc (1 μg), plasmids expressing Nsp1 or Nsp1β, pCDNA3.1 vector expressing Gal4-Sp1TAD fusion protein (1 μg), and renilla luciferase vector (10 ng). Cells were stimulated with LPS and luciferase activities were measured as described earlier. (C and D) Effect of Nsp1β on the activity of n-terminal negative inhibitory domain (N domain) or internal transactivating B domain (D) of Sp1. Transactivation assay was done as described earlier (5B) except that pCDNA3.1 vector expressing Gal4 fusion proteins that lack 1–90 aminoacids of Sp1 (N domain), that have either whole B domain (263–542 aminoacids position) or c-terminal of B domain (421–542 aminoacids position) of Sp1 were used to transactivate Gal4RE promoter of luciferase construct. Data in all of above experiments are the averages of values from three independent experiments ± SEM. A p-value of less than 0.05 was considered as statistically significant (unpaired t test). CV: Control vector.
proximal region of the promoter. It may also be plausible that these two Nsp1 subunits are synthesized at the earliest times following PRRSV infection as they may have been selected evolutionarily to inhibit innate cytokines such as TNF-α and type I interferons. Hence, in addition to its role in replication, transcription and virion biogenesis (Beura et al., 2009; Chen et al., 2009; Kim et al., 2010; Nedialkova et al., 2010; Tijms et al., 2007), the multi-functional protein Nsp1 has anti-innate immunity roles that could facilitate viral replication in antigen presenting cells, such as macrophages. This negative effect on innate immunity may play a key role in dampening subsequent adaptive immune responses during PRRSV infection.

Both Nsp1α and Nsp1β suppresses functional activities of different regions of the TNF-α promoter such as CRE-κB3 and Sp1 elements in the proximal region of the TNF-α promoter respectively (Fig. 6). Hence, both these proteins may target some transcription factors that bind to these elements, which are strictly essential for TNF-α transcription regardless of the type of stimuli or cell type (Falvo et al., 2000; Tsai et al., 2000). In that regard, Nsp1α was found to partially inhibit the activity of NF-κB in reporter assay which employs tandem NF-κB binding sites. Therefore, it is speculated that the Nsp1α-mediated inhibition of NF-κB at κB3 may prevent the assembly of ATF2-c-jun-NF-κB complex at CRE-κB3 site and further recruitment of CBP or p300 to TNF-α promoter (Gerritsen et al., 1997; Kawasaki et al., 1998). A previous study also shows that mutation in either CRE or κB element completely abrogates the functionality of CRE-κB3 site in the context of TNF-α promoter (Yao et al., 1997).

In contrast to Nsp1α, Nsp1β suppresses a different transcription factor (Sp1) both at proximal and distal Sp1 binding sites. Nsp1β does inhibit the transcription activity of Sp1, mediated through glutamine-rich region of its B domain that was demonstrated to be primarily involved in the LPS mediated Sp1 transcriptional activation (Brightbill et al., 2000). Consequently, Nsp1β may be involved in the inhibition of interactions of glutamine-rich region of Sp1 B domain with RNA polymerase II transactivation factor TFIID at proximal Sp1 binding site in TNF-α promoter and suppresses the transcription. The glutamine rich C-terminal region of Sp1 B domain (Sp1BC) is sufficiently able to interact with basal transcriptional machinery especially through direct interactions with transcription initiation factor TFIID subunit 4 (Taf4) and TATA binding protein (TBP) to recruit the RNA polymerase II transactivation complex to the promoter in a specific manner (Emili et al., 1994; Gill et al., 1994). In addition to the suppression of Sp1 at proximal binding site, Nsp1β also strongly suppresses the transcription factors that bind to distal Egr1/Sp1 element. This may be explained by the fact that Egr1 and Sp1, both of which bind to this element, would be reciprocally regulating the activity of each other (Ruo-Pan Huang et al., 1997). Consequently, by inhibiting Sp1, Nsp1β would also control the activity of Egr-1 at this element.

Moreover, based on our studies, Nsp1β of PRRSV would be one of the rare examples of viral proteins of a RNA virus capable of modulating the inducible activity of Sp1 and inhibiting a Sp1-dependent gene i.e. TNF-α. Only one other viral protein, HIV tat was shown to bind and modulate the activities of Sp1 (Howcroft et al., 1995). Interestingly, Sp1 is also involved in the expression of several immunomodulatory genes such as MHC Class I Chain-Related Protein A (MICA), interleukin-12 p35 subunit, etc. (Andresen et al., 2007; Gorely et al., 2003). Therefore, the possible role of Nsp1β in the regulation of other Sp1 dependent genes during PRRSV infection would be of further interest.

The logical extension of this research is to fine map Nsp1 sequences that are involved in TNF-α suppression. Using a reverse genetics approach, mutant viruses that relieve TNF-α suppression could be generated by mutating the sequences that normally suppress TNF-α in the context of PRRSV-FL12 full length infectious clone. Such enhanced TNF-α production could be anticipated to limit PRRSV viral replication in susceptible cells in vivo thereby reducing viremia and pathogenicity. In addition, the virus-induced TNF-α is expected to improve antiviral adaptive immune responses especially by inducing other immunoregulatory cytokines such as IL-8, IL-6, IL-1, thus potentially making such mutant PRRSV interesting as a putative live attenuated virus vaccine candidate.

Materials and methods

Cell lines and viruses

RAW264.7, a murine macrophage cell line (obtained from ATCC) was maintained in RPMI-1640 with 25 mM HEPES (Mediatech) supplemented with 10% fetal bovine serum (FBS). MARC-145 cells (obtained from Dr. Will Laeragrid, USMARC, and USDA/ARS) were maintained in DMEM supplemented with 10% FBS. PRRSV FL12 virus stocks, an infectious clone derived highly virulent North American strain (Kwon et al., 2006a,b; Truong et al., 2004), were prepared by propagating and titrating the virus in MARC145 cells.

Preparation and phenotypic characterization of monocyte derived macrophages

The procedure for the preparation of monocyte derived macrophages was followed as previously described with some modifications (Loving et al., 2007). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient...
centrifugation using lymphocyte separation medium (Mediatech). Monocytes were enriched from PBMCs by glass adherence overnight in DMEM supplemented with 10% FBS. Non-adherent lymphocyte populations were removed by rigorous washing with PBS. Subsequently, the enriched adherent monocytes were incubated with RPMI-1640 (Gibco) supplemented with 10% FBS, 25 mM HEPES, 1 mM sodium pyruvate, 1× non-essential amino acids, 50 μg/mL gentamicin (Sigma) and 5 ng/mL murine macrophage-colony stimulating factor (M-CSF) for 7 days. After 7 days, differentiated macrophages were harvested by using Hank’s salt solution (HSS) based cell dissociation buffer (Invitrogen). The cells were phenotypically characterized by indirect flow cytometry staining using the following mouse primary antibodies such as anti-SWC3 mAb, anti-MHCII mAb (VMRD), anti-CD163 mAb (Aberserotic) and a goat anti-mouse IgG secondary antibody conjugated with Alexafluor 488 (Invitrogen). Staining with secondary antibody only was used as negative control.

**Quantitative PCR and ELISA for swine TNF-α**

Monocyte derived macrophages were infected with PRRSV, FL12 strain, at MOI 10 or stimulated with LPS (1 μg/mL) for indicated time points. Total RNAs were isolated from cells using Trizol LS reagent (Invitrogen). 1–2 μg of total RNAs were reverse-transcribed using MMLV-RT (invitrogen) and oligo-dT (Invitrogen) as primer. The complementary DNAs (cDNAs) synthesized were used as template to quantify the mRNA copy numbers for TNF-α and β-actin in samples by quantitative PCR (qPCR) using specific primers and Taqman hydrolysis probes. The following specific primers and probes were used in qPCR reaction as previously described (Kwon et al., 2006b): For TNF-α: the forward primer is 5′ TCCAGTGTCCGACCACCC 3′ and the Taqman probe is 5′ ATTGCTCTGCGGCAACGGC 3′. For β-actin: the forward primer is 5′ CTCTTCTCCTGCGGATGGA 3′, the reverse primer is 5′ CACGCTTGGACATCTGAG 3′, the reverse probe is 5′ CACGCTTGGACATCTGAGC 3′, and the Taqman probe is 5′ TCGGCCATCGCCGAGACACTGACT 3′. All primers and probes were custom synthesized from Sigma-Genosys. The optimized concentrations of primers and probes were 300 nM each primer for TNF-α and 900 nM each primer for β-actin and 250 nM taqman probe for both TNF-α and β-actin. qPCR was performed using Fast start PCR master mix (Roche Applied sciences) and the reactions were run in Smartcycler (Cepheid). The thermal profile that was followed includes: 95 °C for 10 min (hot start) followed by 95 °C for 15 s (denaturation), 72 °C for 60 s (extension) for a total of 40 cycles. The mRNA copy numbers were calculated based on a standard curve prepared using respective PCR product with calculated copy numbers as template. The TNF-α mRNA copy numbers were normalized with β-actin mRNA copy numbers which served as internal control. The relative TNFα mRNA copy numbers (%) were calculated as previously described (Stordeur et al., 2002). TNF-α protein levels in supernatant were measured by using a commercial swine TNF-α specific ELISA (Pierce) as per manufacturer’s instructions.

**Plasmids construction**

pGL3-Basic and pGL4.32NFkB8RE-luc were purchased from Promega. pswTNF-α-luc plasmid was constructed by cloning swine TNF-α promoter sequences (−1348 to +58, position +1 refers to transcription start site of TNF-α gene, NCBI accession No: NC_010449) into pGL3 basic vector (Promega). pswTNF-α-194 luc was constructed by cloning −194 to +58 region of TNF-α promoter into pGL3-Basic vector. All plasmids expressing non-structural proteins of PRRSV were described previously (Beura et al., 2009). Plasmids expressing each TNF-α promoter sequences (−1348 to +58, position +1 refers to transcription start site of TNF-α gene, NCBI accession No: NC_010449) into pGL3 basic vector (Promega). pswTNF-194 luc was constructed by cloning −194 to +58 region of TNF-α promoter into pGL3-Basic vector. All plasmids expressing non-structural proteins of PRRSV were described previously (Beura et al., 2009). The expression of these constructs was confirmed by Western blot using rabbit polyclonal antibody custom developed against PRRSV-FL12 Nsp1 (Open Biosystems). TF-specific reporter plasmids were constructed by cloning sequences of 3X Sp1/EG1RE or 3X CRE-kB3 RE or 5X Sp1RE fragments (indicated in Fig. 3A, whose sequences were derived from swine TNF-α promoter) into pGL4.32 vector (Promega) by replacing 5× NF-κB RE fragment between Nhel and BfgI cloning sites. GAL4-Sp ITAD expressing plasmid was constructed by cloning the transactivation region (1–615 amino acids) of Sp1 (GenBank Accession No: MM_013672.2) fused in frame with DNA binding domain (1–147 amino acids) of Gal4 (GenBank Accession No: NC_001134) into a mammalian expression vector, pCDNA3.1 + (Invitrogen). Gal4-Sp1-N, Gal4-Sp1B and Gal4-Sp1Bc were constructed by cloning 90–615, 263–542, and 421–542 amino acid sequences of Sp1 respectively in frame with Gal4 DNA binding domain into pCDNA3.1 + vector. Sequences of all primers used for PCR amplification were indicated in Table 1. The 5× Gal4RE-luc was constructed by cloning five tandem repeats of Gal4 responsive element using the following sequence derived from natural Gal10 promoter sequence of Saccharomyces cerevisiae (GenBank accession no: NC_001134), 5′ ACCAGGAGAGTCTTCCGGGCGGAGGC GTTGCGCCCTCGCCGCTTCAATACGTG 3′ (underlined sequences correspond to Gal4 binding elements) into pGL4.32 vector by replacing NF-κB element as described above.

**RAW cell transfection and luciferase reporter assays**

RAW 264.7 cells were transfected with all plasmids using DEAE-dextran chemical transfection by a procedure that has been slightly modified from a previous report (Escher et al., 2005). Briefly, 2.0×105 cells/mL were plated in a 12 well plate one day before transfection. Just before transfection, cells were washed once with serum free RPMI medium. Then, cells were incubated for 2 h with 1 mL of transfection cocktail prepared with serum free RPMI medium that contains 50 mM Tris-Cl, pH 7.4, 1.6 mg of DEAE-dextran (Sigma) and required amount of plasmid DNAs. After 2 h incubation at 37 °C, cells were washed twice with serum free RPMI medium following 10 % DMSO (prepared in PBS) shock for 1 min. Then, cells were washed again two times with serum free RPMI medium before adding the growth medium. For western blotting, final growth medium was supplemented with 0.5 μM 5-azacytidine to increase the transfection efficiency as described previously (Escher et al., 2005). For luciferase reporter assays, cells were transfected with PRRSV protein expressing plasmids along with relevant firefly reporter plasmid (200 ng), pRL-TK (Promega) renilla vector (10 ng) and further incubated for 24 h at 37 °C to facilitate protein expression. After 24 h of incubation, transfected RAW cells were stimulated with LPS (Sigma) (final concentration, 1 μg/mL) for 6 h to stimulate the promoter. Cells were then lysed and luciferase activities measured using a commercially available enzyme detection system (Promega).

**Table 1**

Primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′ to 3′)</th>
<th>a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTNFa-F</td>
<td>ATATACCTAGGGCGGCTTTCTCTGAAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>pTNFa-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>pTNFα-194-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Nsp1-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Nsp1a-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>FL2Nsp1a-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Nsp1-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Nsp1-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Nsp1a-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Gal4BDI147-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Gal4BDI147-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Sp1TAD-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Sp1TAD-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Sp1N-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Sp1N-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Sp1B-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Sp1B-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Sp1C-F</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Sp1C-R</td>
<td>ATATATCATCCGTTCTCCTGGAATGCTGTC</td>
<td></td>
</tr>
</tbody>
</table>

* a Restriction enzyme sites employed are underlined.
* b Primer sequences for TNF-α, Nsp1, Sp1, Gal4 were designed utilizing the sequences with NCBI accession numbers, NC_010449, AV543985, NM_013672.2 and NC_001134 respectively.
were measured in Gmmax 20/20 luminometer (Promega) using dual luciferase assay kit (Promega) as per manufacturer’s instructions. Firefly luciferase activities in stimulated cells were expressed as luciferase units relative to unstimulated cells after normalizing the transfection efficiency with renilla luciferase values.

Acknowledgments

In order to prepare primary cells ex vivo, blood was collected from donor pigs by following protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska-Lincoln under the protocol IACUC No.07-10-048C. This research has been supported by a grant from the USDA NRCGP (project 2008-0903 USDA-NRCGP).

References


Ruo-Pan Huang, Y.F., Ni, Zhengyu, Mercola, Dan, Adamson, Eileen D., 1997. Reciprocal regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain. J. Biol. Chem. 269 (32), 20674–20681.


