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A Novel Role of Classical Swine Fever Virus E^{rns} Glycoprotein in Counteracting the Newcastle Disease Virus (NDV)-mediated IFN-β Induction

Yan-hua Xia[#], Liu Chen[#], Zi-shu Pan and Chu-yu Zhang*
State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, 430072, China

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E^{rns} is an envelope glycoprotein of classical swine fever virus (CSFV) and has an unusual feature of RNase activity. In the present study, we demonstrate that E^{rns} counteracts Newcastle disease virus (NDV)-mediated induction of IFN-β. For this purpose, E^{rns} fused to the enhanced green fluorescent protein (EGFP) was transiently expressed in porcine kidney 15 (PK15) cells. In luciferase activity assay, E^{rns}-EGFP was found to prevent IFN-β promoter-driven luciferase expression and block the induction of IFN-β promoter mediated by NDV in a dose-dependent manner. Through IFN-specific semi-quantitative RT-PCR detection, obvious decrease of IFN-β mRNA in NDV-infected PK15 cells was observed in the presence of E^{rns}-EGFP. In contrast, EGFP alone showed none of this block capacity. In addition, E^{rns}-EGFP mutations with RNase inactivation were also found to block NDV-mediated induction of IFN-β. These evidences establish a novel function for CSFV E^{rns} glycoprotein in counteraction of the IFN-β induction pathway.

Keywords: Classical swine fever virus, E^{rns}-EGFP, Interferon-beta, Newcastle disease virus

Introduction

Classical swine fever virus (CSFV), together with bovine viral diarrhea virus (BVDV) and border disease virus (BDV), belongs to the genus *Pestivirus* of the family *Flaviviridae* (Paton *et al*., 1995). CSFV is the causative agent of classical swine fever, an Office International des Epizooties (OIE) list A disease of pigs that leads to important economic losses worldwide (Paton and Greiser-Wilke, 2003). CSFV carries an RNA genome of positive polarity about 12.3 kb in length that is translated to form a single virus polyprotein, which, through cleavage by both host and virus proteases, gives rise to 12 mature viral proteins (NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) (Lindenbach and Rice, 2001).

E^{rns} is an envelope glycoprotein of CSFV which exists on the surface of the virion (Weiland *et al*., 1999) or is secreted from infected cells into the culture medium (Rumenapf *et al*., 1993). An unusual feature of E^{rns} is its RNase activity (Schneider *et al*., 1993; Hulst *et al*., 1994) which shows a strong preference for single-stranded RNA (ssRNA) (Windisch *et al*., 1996). The unexpected finding leads to several interesting studies regarding the function of E^{rns} in the life cycle of pestiviruses. Results of *in vitro* and *in vivo* studies indicate that E^{rns} (and its enzyme activity) plays a role in the regulation of RNA synthesis in infected cells and in weakening the immune defense of the host early in infection (Bruschke *et al*., 1997; Hulst *et al*., 1998; Meyers *et al*., 1999).

Pestiviruses are characterized by their ability to sustain a persistent infection after in utero infection of a fetus. The evasion of innate immunity in the fetus is at least partly due to the lack of alpha/beta interferon (IFN-α/β) production after pestivirus infection (Charleston *et al*., 2001). Evidences have shown that cultured cells infected with CSFV or noncytopathogenic (ncp) BVDV are immune to the IFN-inducing effect of infection with heterogenous virus or the addition of double-stranded RNA (dsRNA) to cells (Schweizer *et al*., 2001; Ruggli *et al*., 2003). Another well-established property of pestiviruses is that they show the exaltation of Newcastle disease virus (END) phenomenon (Kumagai *et al*., 1958; Inaba *et al*., 1968). The END phenotype is related to suppression of IFN-α/β production by pestiviruses. Thus, pestiviruses can take active measures to block the production of IFN-α/β.

[#]They have contributed equally to this paper.

Abbreviations: BVDV, bovine viral diarrhea virus; CSFV, classical swine fever virus; NDV, Newcastle disease virus; PK15, porcine kidney 15; IFN-β, interferon-beta; EGFP, enhanced green fluorescent protein.

*To whom correspondence should be addressed.
Tel: 86-27-68752833; Fax: 86-27-68754941
E-mail: zhangcy_1107@163.com

So far, two proteins of pestivirus have been identified to interfere with interferon system. Ruggli *et al.* (2003) proved that CSFV shows the capacity to interfere with cellular antiviral activity because of the presence of N^{pro} gene. N^{pro} protein could prevent both poly(IC)-and NDV-mediated IFN-α/β induction, independently of the CSFV context (Ruggli *et al.*, 2003; Ruggli *et al.*, 2005). The block by N^{pro} protein was thought to involve an inactivation of interferon regulatory transcription factor 3 (IRF-3) (La Rocca *et al.*, 2005). In addition, E^{rns} protein of BVDV was proved to specifically inactivate dsRNA-dependent signaling events (Iqbal *et al.*, 2004). In the present study, we demonstrate that CSFV E^{rns} protein can block NDV-mediated IFN-β induction and that this block operates at the transcriptional level of IFN-β synthesis.

Materials and Methods

Cells and viruses. The porcine kidney 15 (PK15) cell line was obtained from China Center for Type Culture Collection (CCTCC). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were maintained at 37ºC in a humidified atmosphere containing 5% of CO_2.

The CSFV standard highly virulent Shimen-strain, vaccine C-strain and NDV Mukteswar strain were provided by China Institute of Veterinary Drug Control.

Construction of plasmids containing E^{rns} The complete E^{rns} gene was cloned from the genome RNA of CSFV Shimen strain through RT-PCR and inserted into pGEM-T vector (Promega). After cut from the pGEM-T by EcoRI and BamHI, E^{rns} sequence was inserted into the pEGFP-N1 vector (Clontech) to produce a plasmid named $pE^{rns}_{(c)}$-EGFP. Plasmid $pE^{rns}_{(h)}$-EGFP was made in the same way as that of $pE^{rns}_{(c)}$-EGFP from the genome RNA of CSFV vaccine C-strain. The primers used to amplify 758 bp PCR product of E^{rns} gene were 5'-CGGAATTCTGATGGCCCTATTGGCATGG GCG-3' (sense) and 5'-CGGGATCCCGGGCATAGGCACCAAA CCA-3' (antisense). Based on $pE^{rns}_{(c)}$-EGFP, another two mutants $pE^{rns}_{(H297K)}$-EGFP and $pE^{rns}_{(H346K)}$-EGFP were constructed. In the two mutants, the histidine residue at position 297 or 346 was replaced by lysine residue. Both mutated plasmids mentioned above were sequenced to confirm that the desired amino acid changes had occurred.

Luciferase activity assay. Reporter plasmid pIFN-β-Luc expresses firefly luciferase under the control of IFN-β promoter element. Plasmid pRL-CMV (Promega) constitutively expressing *Renilla* luciferase was used for internal normalization of inducible firefly luciferase. The expression plasmid pEGFP-N1 (Clontech) was used to express E^{rns} gene as described elsewhere.

Confluent monolayer of PK15 cells, seeded in 24-well plate, were either mock treated or transfected with 1.0 μg of pEGFP-N1, $pE^{rns}_{(c)}$-EGFP, $pE^{rns}_{(h)}$-EGFP, $pE^{rns}_{(H297K)}$-EGFP or $pE^{rns}_{(H346K)}$-EGFP together with 2.0 μl of Lipofectamine 2000 (Invitrogen), respectively. All samples above were co-transfected with 100 ng of pIFN-β-Luc and 20 ng of pRL-CMV. At 12 h post-transfection, all of the samples were either mock treated or infected with NDV (10^5TCID_{50}). Cells were then incubated at 37ºC for 24 h prior to lysis and firefly luciferase and Renilla luciferase activity quantification using a Dual-Luciferase® Reporter Assay System (Promega). The detailed procedures performed according to the manufacturer's instructions.

IFN-specific semi-quantitative RT-PCR. Confluent monolayer of PK15 cells cultured in 24-well plates were either mock treated or transfected with 1.0 μg of pEGFP-N1 or $pE^{rns}_{(c)}$-EGFP together with 2.0 μl of Lipofectamine 2000 (Invitrogen). After incubation for 12 h at 37ºC, the samples were treated with NDV (10^5TCID_{50}) or mock treated. Then after incubation for 24 h at 37ºC, cells were washed once with PBS (pH 7.0-7.2) and then total RNA was extracted by using an RNeasy kit (Qiagen) including a DNase treatment step according to the manufacturer's protocol. Reverse transcription (RT) was performed in a reaction volume of 30 μl containing 0.70 μg of total RNA, 1 μl of oligonucleotide dT(10 μmol/L), and 30 U of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega) and incubated at 42ºC for 1 h, followed by 5 min at 95ºC. Then, 2 μl of cDNA was amplified for 20-35 cycles for PCR exponential phase determination. Each PCR regime included a 5 min initial denaturation step at 94ºC, followed by amplification cycles (28 cycles for β-actin and 30 cycles for IFN-β) consisting of 35 s at 94ºC, 35 s at 56ºC and 40 s at 72ºC, followed by 10 min at 72ºC. The primers used to amplify 571 bp product of porcine IFN-β gene were 5'-CCGAATTCGCTA ACAAGTGCATCCTCC-3' (sense) and 5'-GCGAAGCTTTCAGT TCCGGAGGTAATC-3' (antisense). 180 bp PCR product of β-actin was used as an internal normalization from all samples. Equal volumes of PCR mixtures were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. For normalization, we calculated the relative ratio of the various IFN-β mRNAs to the β-actin mRNA of each sample. Each sample was run in duplicates in three independent experiments and standard error of the mean (SEM) calculated.

Results

IFN-β promoter is induced by NDV but not by CSFV. NDV is a paramyxovirus which induces IFN in sensitive cells (Lomniczi, 1973). Here NDV acted as an inducer of IFN-β. A reporter construct pIFN-β-Luc which expresses firefly luciferase under the control of IFN-β promoter element was used to detect the activity of IFN-β promoter. As shown in Fig. 1A, when NDV fluid was performed 1000-, 200-, 100-, 50- and 20-flod dilution, IFN-β promoter was increasingly induced. However, 50-fold dilution of NDV mostly induced IFN-β promoter and therefore was the optimal dose which was about $10^5TCID_{50.}$ When PK15 cells were infected with optimal dose of NDV, the induction of IFN-β promoter increased with incubation time and reached peak level beginning at 18 h and then continuing through 48 h. (Fig. 1B). On the other hand, when cells were infected with CSFV, whatever dose and whatever incubation time was, IFN-β

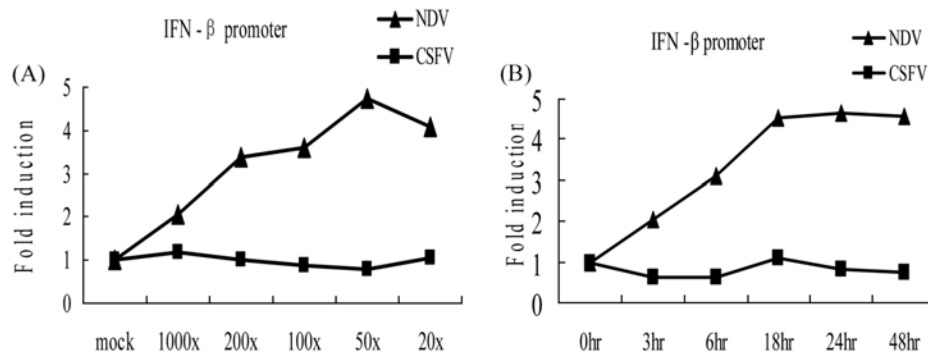

Fig. 1 Induction kinetics of IFN-β promoter by NDV and CSFV. (A) NDV and CSFV fluid were first titrated and then performed series dilutions from 1000-fold to 20-fold (MOI of 0.8 TCID50/cell) for optimum titer determination. (B) Cells were incubated with NDV or CSFV for 0, 3, 6, 18, 24 or 48 h before luciferase activity assay for optimum incubation time determination. The results are expressed as fold induction of IFN-β promoter relative to the level of cell mock.

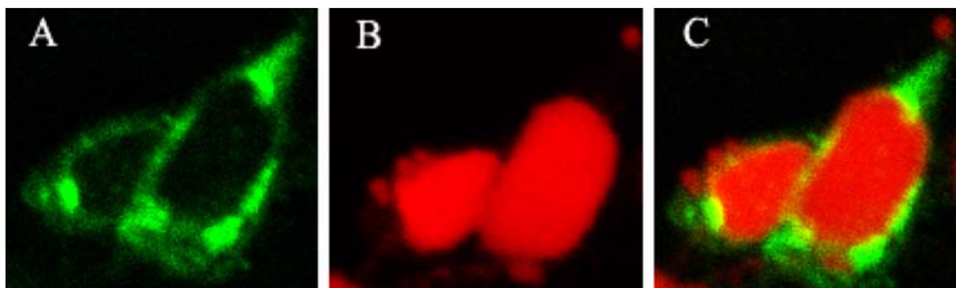

Fig. 2. The distribution of E^{rns}-EGFP in PK15 cells. PK15 cells transiently expressed E^{rns}-EGFP, while the nucleus were stained with PI (10 μg/ml), then observed under confocal microscope. (A) cell was observed under blue light; (B) cell was observed under green light; (C) a merge of (A) and (B).

promoter was not induced (Fig. 1). These results proved again that NDV can efficiently induce IFN-β in PK15 cells while CSFV can not.

E^{rns} blocks NDV-mediated IFN-β promoter induction. In order to see whether E^{rns} of CSFV affect IFN-β promoter activity, two expression plasmids $pE^{rns}_{(c)}$-EGFP and $pE^{rns}_{(h)}$-EGFP were constructed, in which E^{rns} gene fragments were from virulent and avirulent CSFV, respectively (see Materials and Methods). PK15 cells transiently expressed the three kinds of proteins EGFP, $E^{rns}_{(c)}$-EGFP and $E^{rns}_{(h)}$-EGFP, while the nucleus were stained with PI (10 μg/ml), then observed under confocal microscope. As observed, the density of E^{rns}-EGFP in cytoplasm was higher than in other parts of cells just as that of wild E^{rns} protein (Fig. 2), in contrast to the distribution of the EGFP, which was uniformly distributed over the cell (data not shown). Since obvious difference exists between the distribution of E^{rns}-EGFP and EGFP, E^{rns}-EGFP was considered to be expressed as a fusion protein.

pEGFP-N1, $pE^{rns}_{(c)}$-EGFP and $pE^{rns}_{(h)}$-EGFP were trasfected into PK15 cells while pIFN-β-Luc as a luciferase reporter construct and pRL-CMV as an internal normalization were cotransfected. At 12 h post transfection, optimum dose of NDV was added. After incubation for 24 h, cells were performed luciferase assay. As shown in Fig. 3, IFN-β promoter was not activated in any samples without NDV infection. When NDV existed, IFN-β promoter was activated by 4- to 5-fold in mock or EGFP-N1 expressing cells. However, when $E^{rns}_{(c)}$-EGFP or $E^{rns}_{(h)}$-EGFP was expressed, IFN-β promoter was activated by a basal level as that of mock cells. From these results, two points of view could be concluded. First, E^{rns} could block IFN-β promoter-driven luciferase expression in response to the addition of NDV. Second, no difference in block capacity exists between E^{rns} proteins of CSFV virulent and avirulent strain.

RNase activity is not important to the block capacity of E^{rns}. One of the most remarkable features of pestivirus is the virus-encoded glycoprotein E^{rns}, which has RNase activity. E^{rns} belongs to the family of extracellular RNase consisting of several fungal (e.g., RNase T2 and Rh) and plant (e.g., S glycoproteins of *Nicotiana alata*) RNases (Schneider *et al*., 1993; Hulst *et al*., 1994). These RNases contain two homologous regions of 8 amino acids each which are spaced by 38 nonhomologous amino acids and which form the RNase activate site. Histidine residues (297 and 346 sites in CSFV E^{rns}) in both regions appear to be crucial for RNase catalysis (Kawata *et al*., 1988), and this has been proved in subsequent studies (Hulst *et al*., 1998; Meyers *et al*., 1999). Here two plasmids $pE^{rns}_{(H297K)}$-EGFP and $pE^{rns}_{(H346K)}$-EGFP

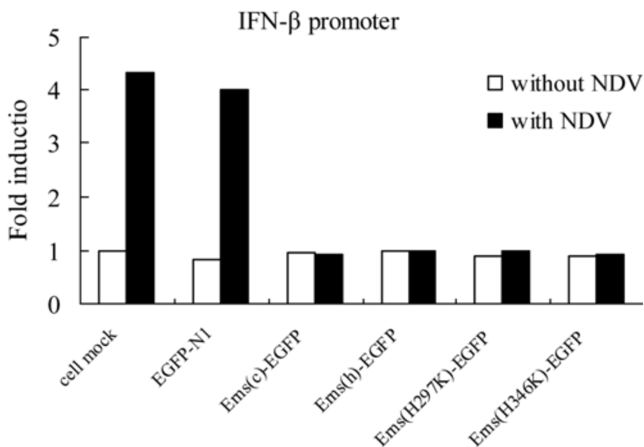

Fig. 3. Block of NDV-mediated IFN-β promoter induction by E^{rns}-EGFP. PK15 cells were transfected with pEGFP-N1, $pE^{rns}_{(c)}$-EGFP, $pE^{rns}_{(h)}$-EGFP, $pE^{rns}_{(H297K)}$-EGFP or $pE^{rns}_{(H346K)}$-EGFP alone or in the presence of NDV, after incubation of 24 h, all samples were performed luciferase activity assay (see Materials and Methods).

were constructed, in which the two histidine residues at positions 297 and 346 were mutated into lysine residue to make RNase inactivate. In luciferase assay, we found that $E^{rns}_{(H297K)}$-EGFP and $E^{rns}_{(H346K)}$ -EGFP could also block NDV-mediated IFN-β promoter induction (Fig. 3), which seems that RNase activity is not required for the block capacity of E^{rns}.

Block effect exhibits in an E^{rns} dose-dependent manner. In order to see whether the precedence order between E^{rns}-EGFP expression and NDV infection is crucial to the block effect, we set a serial of experiments. PK15 cells with only NDV infection was established as a positive mock while cells without NDV infection or E^{rns}-EGFP expression as a negative mock. As shown in Fig. 4A, IFN-β promoter was activated by 4.5-fold in positive mock while by a basical level in negative mock. When NDV infection is simultaneous with or 6 h ahead of pE^{rns}-EGFP transfection, IFN-β promoter was activated by 1.8- and 2.8-fold, respectively. However, when NDV infection is 6 h, 12 h or 24 h behind of pE^{rns}-EGFP transfection, the activation of IFN-β promoter was completely inhibited. Therefore, IFN-β promoter would be blocked so long as E^{rns}-EGFP exists and this block effect seemed more obvious when $E^{rns}_{(c)}$ -EGFP expressed prior to NDV infection.

In order to see whether block effect enhances with the expression quantity of E^{rns}-EGFP, 100 ng, 200 ng, 400 ng, 800 ng and 1 μg of pE^{rns}-EGFP and pEGFP-N1 were transfected into PK15 cells, respectively. As shown in Fig. 4B, the more transfection dose of E^{rns}-EGFP is the more obvious the block effect of IFN-β promoter activity shows. In contrast, this dose-dependent phenomenon was not observed in pEGFP-N1 transfection. These results evidence again that E^{rns} can inhibit NDV-mediated IFN-β promoter activation.

E^{rns} blocks IFN-β mRNA transcription. Since IFN-β promoter was inhibited by E^{rns}, IFN-β mRNA production was thought to be affected. To testify this, IFN-specific semi-quantitative RT-PCR was used to analyze IFN-β mRNA production. As shown in Fig. 5 upper panel, PK15 cells (lane 2) without any treatment showed none of IFN-β signal just as that of distilled water (lane 1). The DNase-treated mRNA, which was extracted from NDV-infected PK15 cells, directly processed PCR amplification without RT step and showed no IFN-β mRNA signal (lane 7). This excludes the possibility of IFN-β DNA jamming in PK15 cell genome. PK15 cells either stimulated with NDV alone (lane 8) or treated with both NDV and EGFP (lane 4) showed a clear IFN-β mRNA signal in contrast to that treated with NDV in the presence of E^{rns}-EGFP (lane 6) ($P < 0.05$). Cells solely transfected with pEGFP-N1 (lane 3) or $pE^{rns}_{(c)}$-EGFP (lane 5) without NDV infection also induced a little of IFN-β signal. This

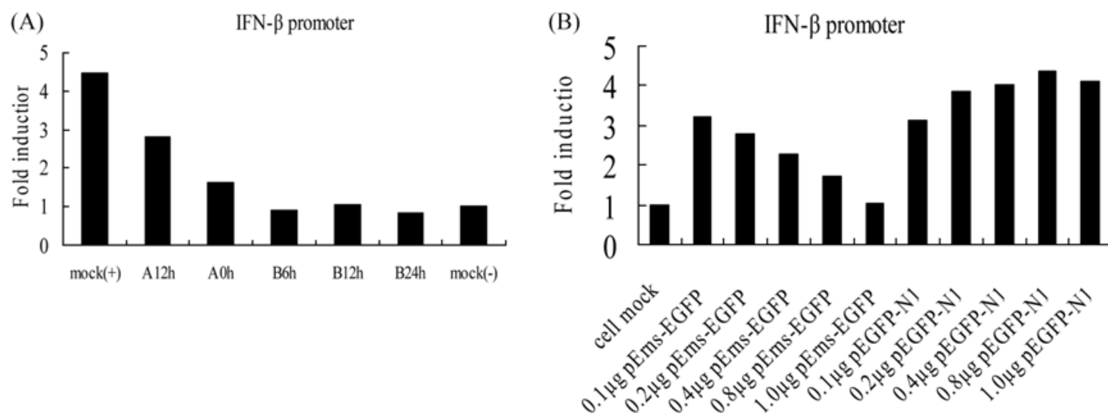

Fig. 4. Effect of E^{rns}(c)-EGFP expression on NDV mediated-IFN-β promoter induction. (A) mock (+): cells with NDV infection; mock (-): cells without NDV infection or $E^{rns}_{(c)}$-EGFP expression; A12 h and A0 h: NDV infection is 12 h and 0 h ahead of $pE^{rns}_{(c)}$-EGFP transfection; B6, B12 and B24 h: NDV infection is 6, 12 and 24 h behind of $pE^{rns}_{(c)}$-EGFP transfection. (B) 0.1, 0.2, 0.4, 0.8 and 1.0 μg of pE^{rns}-EGFP and pEGFP-N1 were transfected into PK15 cells, respectively. After incubation for 12 h at 37°C, NDV was added. Then at 24 h post infection all samples were performed luciferase activity assay.

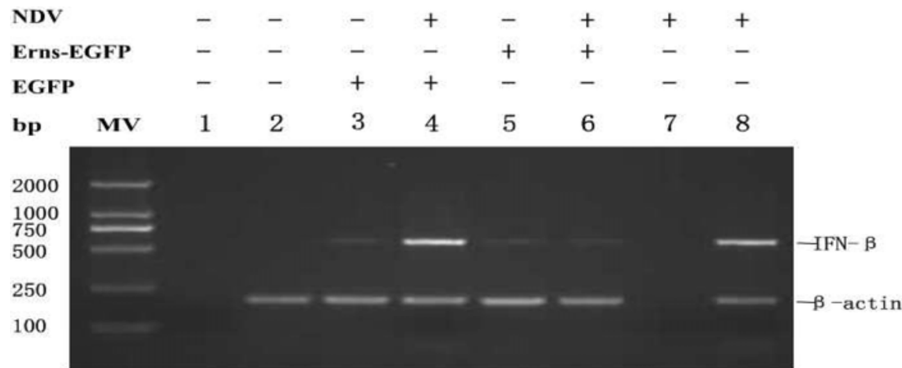

Fig. 5. Induction of IFN-β mRNA by NDV is inhibited by E^{rns}-EGFP fusion protein. PK15 cells were treated with 10^5TCID$_{50}$ of NDV (lanes 4, 6, 7 and 8) or mock treated (lanes 2, 3, and 5) in the presence (lanes 5and 6) or absence (lanes 2, 3, 4, 7, and 8) of E^{rns}-EGFP fusion protein. After 24 h of NDV infection, RNA was extracted from cells, reverse transcribed with oligonucleotide (dT) as a primer, and amplified by PCR with primers specific for porcine IFN-β mRNA (upper panel) or β-actin mRNA (lower panel). Lane 1 shows a PCR product of distilled water. Lane 7 shows the PCR product from NDV-infected cells without RT step. The DNA marker for IFN-β and β-actin is DL2000 and displayed on the left-hand sides of each panel.

phenomenon was ever observed by Park *et al*. (2003) who have got the conclusion that dsRNA produced by transcription from the transfected plasmids is the actual trigger of IFN production (Park *et al*., 2003). As shown in Fig. 5 lower panel, β-actin mRNA was detected in all RT reactions, indicating that all preparations contained similar amounts of RNA. Results above clearly demonstrate that NDV-mediated induction of IFN-β mRNA synthesis is inhibited by E^{rns}-EGFP fusion protein.

Discussion

CSFV can replicate efficiently in PK15 cells without inducing IFN production. In our study, this is proved again. Far more importantly, we demonstrate that CSFV E^{rns} has the capacity of blocking IFN-β induction mediated by NDV and that this block occurs at transcriptional level.

In our study, E^{rns}-EGFP fusion protein was used to analyze the function of E^{rns}. Fusion to EGFP brings some conveniences, such as intuitionistic detection of transfection efficiency and shortened experimental period. However, question may be taken up about whether fusion to EGFP affects the function of E^{rns}. In order to answer this question, we designed some experiments. We compared the localization of EGFP and E^{rns}-EGFP and found there was distinct difference between them (Fig. 2), which proved that E^{rns}-EGFP did express in a fusion form and fusion to EGFP did not affect the localization of E^{rns}. Although, transient expression is unable to detect the RNase activity of E^{rns}-EGFP, RNase activity was proved to be not important to the block effect in subsequent mutation experiment (Fig. 3). Added to these, a mock that expressed EGFP alone was established in every assay to exclude the effect of EGFP. All these strive prove that fusion to EGFP has no effect on the block capacity of E^{rns}.

E^{rns} protein of pestviruses is always being paid attention to because of its RNase activity. Though, E^{rns} protein was presumed to act in the regulation of RNA synthesis in infected cells and in weakening the immune defense of the host early in infection, so few of evidences exist. Here we provide a proof that CSFV E^{rns} protein can interfere with NDV-mediated IFN-β induction. Reporter construct that expresses luciferase under the control of IFN-β promoter element was used to reflect the IFN-β promoter activity. In NDV-infected cells, IFN-β promoter was mostly activated (Fig. 1). On the other hand, this activation by NDV was mostly blocked by E^{rns} protein (Fig. 3). Since CSFV E^{rns} protein can specially block IFN-β promoter activity, IFN-β mRNA production must be limited. Through RT-PCR, we did find that IFN-β mRNA decreased to a large extent (Fig. 5). Based on these evidence, we can deduce that CSFV E^{rns} protein interfere with IFN-β induction pathway at least at the transcription level, which is identical to that in BVDV E^{rns} protein research (Iqbal *et al*., 2004).

It is worth mentioning that RNase activity seems not to be important to the block effect in our study. Here histidine residues at position 297 and 346, which are crucial amino acids to RNase activity, were replaced by lysine residues to inactivate the RNase activity of E^{rns}. In luciferase assay, two mutations without RNase activity showed the same block capacity as that of wild E^{rns} protein. The result was different from that in BVDV. Though, the conformation of the mutated proteins was thought not severely affected by the inactivation (Hulst *et al*., 1998), we rather believe that this discrepancy is most probably due to different inducer of IFN-β, different kinetics of induction and different cells in our two studies. At the same time, we can also think that E^{rns} protein has more than one strategy to interfere with IFN system. If one is blocked, the other acts.

Type I interferons (IFN-α/β) are potent antiviral cytokines and modulators of the adaptive immune system. They are induced by viral infection or by double-stranded RNA

(dsRNA), a by-product of viral replication, and lead to the production of a broad range of antiviral proteins and immunoactive cytokines. IFN-I induction involves multi-factors including RIG-1/MDA5, IPS-1/MAVS, TBK-1/IKK-ε, IRF-3, IRF-7, TFH/D and RNAP. Every stage may be utilized by viruses to counter the IFN production (Otto Haller *et al*., 2006). According to the study of BVDV, this inhibition of IFN-β transcription by ncp BVDV is through an interferon regulatory factor 3-dependent mechamism (Susan *et al*., 2002). E^{rns} glycoprotein locates in cytoplasm and it can not directly participate in nucleus events. We speculate that E^{rns} glycoprotein mostly regulates some factors (eg.IRF3 and NF-κB) which involve in IFN induction. Whether CSFV adopts the same strategy as that of BVDV needs further research. However, it is exciting to find that E^{rns} has the capacity of blocking IFN-β induction, which partly explains the persistent infection of CSFV. We believe further study of E^{rns} will help to reveal the pathogenesis mechanism of CSFV.

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