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Identification of amino-acid residues in the V protein of peste des petits ruminants essential for interference and suppression of STAT-mediated interferon signaling

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ABSTRACT

Peste des petits ruminants virus (PPRV) causes a fatal disease in small ruminants. V protein of PPRV plays a pivotal role in interfering with host innate immunity by blocking IFNs signaling through interacting with STAT1 and STAT2. In the present study, the results demonstrated that PPRV V protein blocks IFN actions in a dose dependent manner and restrains the translocation of STAT1/2 proteins. We speculate that the translocation inhibition might be caused by the interfering of the downstream of STAT protein. Mutagenesis defines that Cys cluster and Trp motif of PPRV V protein are essential for STAT-mediated IFN signaling. These findings give a new sight for the further studies to understand the delicate mechanism of PPRV to escape the IFN signaling.

Introduction

Peste des petits ruminants virus (PPRV) causes a fatal disease in small ruminants, especially in goats and sheep, resulting in serious economic loss (Wang et al., 2009; Peste des petits ruminants). PPRV belongs to *Morbillivirus* of the *Paramyxoviridae* family and contains a non-segmented negative strand RNA genome, which encodes six structural proteins (N, P, M, F, H, L) and two non-structural proteins (C, V) (Diallo et al., 1994; Gibbs et al., 1979; Siddappa et al., 2014; Mahapatra et al., 2003; Bailey et al., 2005). In Paramyxovirus, non-structural protein V shares an approximately 50% identical conserved C-terminal domain (CTD), CTD includes one of an important zinc finger fold structure which contains a zinc binding domains constituted by one histidine and seven cysteine residues (Liston and Briedis, 1994; Paterson et al., 1995). The previous studies showed that V protein played an important role in interfering with an interferon (IFN)-mediated immune system (He et al., 2002; Wansley and Parks, 2002; Poole et al., 2002; Sun et al., 2004; Fontana et al., 2008; Ramachandran et al., 2008; Chinnakannan et al., 2013; Takaki et al., 2011; Nakatsu et al., 2008; Caignard et al., 2007; Devaux et al., 2007; Rodriguez and Horvath, 2013).

IFNs, especially type I IFN (IFN-α/β) mediate the innate antivirus responses (Cardenas, 2010; Wang et al., 2010; Takaki et al., 2013). IFNs recognize the receptor leading to the activation and signal transduction of the receptor associated JAK-Tyk2 kinase, which phosphorylates signal transducer and activator of transcription 1 (STAT1) and STAT2. Both STAT1 and STAT2 interact with each other and are then translocated into the nucleus, and assemble with IFN regulatory factor 9 (IRF9) to form a transcription factor complex, ISGF3, which is responsible for binding the IFN stimulated response element, leading to an antiviral status. It has been demonstrated that Morbilliviruses develop three different strategies to evade IFN antiviral effects: suppression of IFNs induction (Huang et al., 2014; Ning et al., 2014; Komatsu et al., 2004; Ikegame et al., 2010); inhibiting IFN-induced antiviral proteins (Zhao et al., 2013; Metz et al., 2012); interfering IFN signal transduction (Sun et al., 2004; Ramachandran et al., 2008; Chinnakannan et al., 2013; Nakatsu et al., 2008; Devaux et al., 2007). In most cases, these activities are caused by V protein, although viral P protein and C protein have been reported to be involved (Devaux et al., 2007; Yamaguchi et al., 2014).

Recent study revealed that the V proteins of Rinderpest virus (RPV), Measles virus (MV), PPRV and Canine distemper virus (CDV) could all interfere with phosphorylation of the interferon-receptor-associated

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kinase Tyk2 and efficiently block IFNs-induced STAT1/2 phosphorylation. Morbillivirus V proteins showed variable capabilities of binding STAT1/2 and exhibit multiple mechanisms to block IFNs signaling pathways. MV V protein contributes to the inhibition of the IFN antiviral state through binding with STAT1/2 to prevent nuclear translocation of STAT proteins (Chinnakannan et al., 2013). It has been demonstrated that MV V protein inhibits JAK1-mediated phosphorylation of STAT1 to antagonize signaling transduction of type I IFN (I-IFN) (Caignard et al., 2007). However, another report has showed that MV V protein targets STAT2 to block IFN signaling (Ramachandran et al., 2008). The zinc finger domain in MV V protein is necessary to bind STAT2 and disrupts IFN signaling transduction. Several specific residues within the CTD domain of V protein are found to be critical to inhibit the ability to associate with STAT2.

Despite an important role of PPRV proteins interacting with host proteins in evasion of IFN-induced antivirus effects, little is known about the mechanism for interaction of PPRV V protein with STATs. To test the hypothesis that PPRV V protein might have evolved to use specific function sites to inhibit STAT signaling, V protein-dependent inhibition of IFN actions were investigated. In the present study, PPRV V protein was found to influence the IFN signaling transduction by interacting with STAT1/2. In addition, PPRV V protein was also able to disturb the distribution of STAT protein. The STAT1 and STAT2 contact site was mapped to N terminal and/or C terminal. It was found that conserved Cys cluster and Trp motif in V protein were involved in interacting with STAT2, and amino acid 275 and 277 of V protein may play a critical role in blocking IFN actions. These data suggest that these important sites may participate in innate immune evasion.

Results

PPRV V protein inhibits type I IFN production and blocks its activation

In order to understand capability of PPRV V protein to inhibit induction of type I IFN induction, Cos7, HEK293T and A549 cells were used because of their susceptibility to PPRV infection. (Cos7, Fig. 1A, A549 cells and HEK293T cells, data not shown). Cos7 cells are unable to produce IFN but respond to exogenous IFN-β stimulation. Therefore, to investigate if PPRV V protein was able to inhibit induction of type I IFN induction, HEK293T cells were used. The cells were initially treated with Sendai virus (SeV) which is able to induce a large number of IFNs to induce IFNs and then infected with PPRV. The CPE was monitored for seven days. As shown in Fig. 1B, HEK293T cells treated without SeV appeared to detach earlier than those cell treated with SeV. Interestingly, PPRV significantly decreased the production of IFN-α/β induced by SeV (Fig. 1C). These data suggested that PPRV is able to interfere productions of IFN-α/β. To identify which PPVR protein is involved in inhibition of IFN production, HEK293T cells were transfected with the IFN-β luciferase reporter plasmid (pRL-TK-luc, 0.1 μg) either alone or together with plasmids expressing PPRV individual protein (N, F, H, M, V and P) and control plasmid. At 24 h post-transfections, cells were infected with SeV. The luciferase activity was measured at 24 h after infection. As shown in Fig. 1D, PPRV V protein was able to significantly inhibit IFN-α/β production induced by SeV. Similar results were observed in A549 cells (data not shown). The results are correlated with previous studies with MV that type I IFN production was interfered by MV V protein (Takaki et al., 2011). Furthermore, to investigate whether the proteins of PPRV was able to antagonize type I and type II IFNs actions, H293K cells were transfected with the pISRE-luc or pGAS-Luc plasmid alone or in the presence of plasmids expressing PPRV individual protein (N, F, H, M, V and P) and control plasmid. At 24 h post-infection, cells were treated with 1000 U/ml of IFN-β or IFN-γ for 24 h and then the luciferase activity was measured. It was found that stimulation of cells with IFN-β effectively activated the transcription of ISGF3 responsive ISRE-luciferase reporter gene (Fig. 1E) while IFN-γ effectively activated a STAT1-dependent γ-IFN activation promoter sequence (GAS)-luciferase reporter gene (Fig. 1F). However, PPRV V can significantly inhibit IFN actions although PPRV P, N proteins can also interfere IFN actions (Fig. 1E and F). Taken together, the results demonstrated that PPRV is able to use their own proteins to inhibit IFN production and block IFN activations in infected cells.

PPRV V protein blocks IFNs signaling transduction by inhibition of STAT1/2 translocation in a dose-dependent manner not a degradation way

It is known that IFNs bind to the receptors when they are secreted from the infected cells and signals to adjacent cells. Induction of antiviral effects by IFNs requires coordinated and cooperative action of the STAT1 and STAT2 (STAT1 and STAT2: STAT1/2). The results above indicated that PPRV proteins are sufficient to suppress STAT-dependent signaling. Overexpression of PPRV V protein can inhibit both IFNs-triggered activation of ISRE and GAS promoter. As shown in Fig. 2A, overexpression of PPRV V protein strongly inhibits IFN-α/β-induced activation of the ISRE promoter in a dose-dependent manner in HEK293 cells (Fig. 2A). Meanwhile, overexpression of PPRV V protein also inhibits IFN-γ-induced activation of the GAS promoter (data not shown). Previous study demonstrated that PPRV blocks IFN-induced phosphorylation of STAT1/2 for its nuclear translocation (Chinnakannan et al., 2013). To determine the abilities of different domains of PPRV V protein to block IFN-induced translocation of STAT1/2, HA-tagged PPRV V protein and Flag-tagged STAT1/2 proteins from Cos7 cells was used. The subcellular localization of both V protein and STAT protein were detected by immunofluorescence assay as described above. As shown in Fig. 2B and C, type I IFN-induced STAT protein translocation were blocked by V protein and also suppressed by PNT and VCT (both are also called VNT and CTD, respectively) domains of V protein. Intact V protein and its PNT and VCT were detected in both nucleus and cytoplasm without induction of type I IFN. In the context of confocal data, the results demonstrated that STAT1 and STAT2 shuttle between cytoplasm and nucleus. In response to stimulation with type I IFN in control cells, the distribution of STAT1 and STAT2 did not apparently change. In cells expressing PPRV V protein or PNT or VCT, STAT protein failed to translocate into nucleus after stimulation with type I IFN. These results revealed that PPRV V protein was involved in redistribution of the STAT protein. PNT blocks translocation of STAT1 nucleus while VCT changes distribution of STAT2. STAT1/2 were phosphorylated under condition of IFN treatment, then together with IRF9, assemble into a complex known as ISGF3. ISGF3 rapidly translocates into nucleus and binds to ISRE sequences to increase transcription rates of the type I IFN stimulated gene. These results above indirectly demonstrated that PPRV V protein blocks the type I IFN-induced phosphorylation of STAT proteins which was found by Senthil et al. (Chinnakannan et al., 2013). Quantitatively, based on 60 cells expressing the V protein and STAT protein, PPRV V, PNT and VCT showed 70%, 42% and 75% of cells with a complete change in type I IFN induced redistribution of STAT protein. It has been reported STAT1/2 proteins were targeted by V protein of *Paramyxoviridae*. Viruses adopt V proteins to degrade STAT protein by an ubiquitin system as one of the strategies to change translocation and accumulation of STAT protein within the nucleus (Randall and Goodbourn, 2008; Palosaari et al., 2003; Takeuchi et al., 2003; Didcock et al., 1999). To verify whether PPRV V protein changes STAT protein distribution via degradation, a proteasomal activity inhibitor MG132 was used to investigate degradation of the STAT

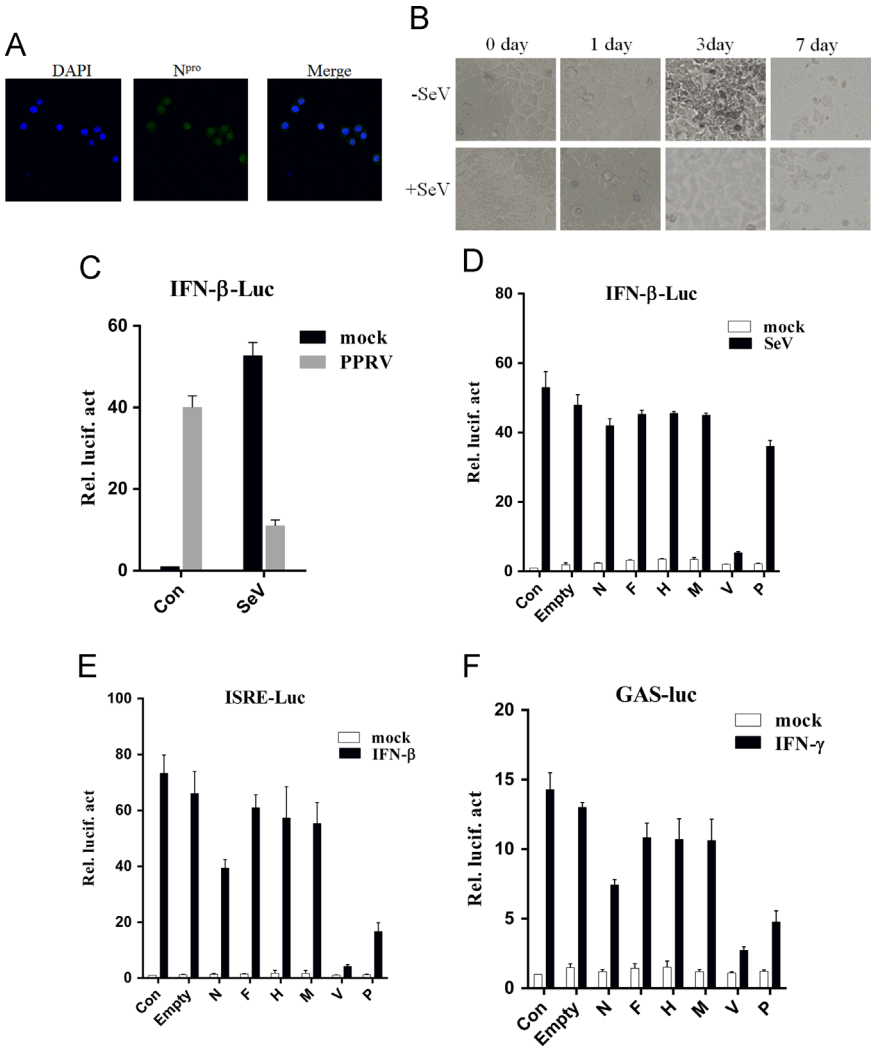

Fig. 1. PPRV V protein inhibits type I IFN production and blocks type I IFN activation. (A) Cos7 cells were infected with PPRV. Cells were stained with antisera to PPRV nucleoprotein N (green) and stained nuclei with DAPI (blue). Merge of the two staining is shown on the right panel. (B) HEK293T cells morphology infected by SeV in prior to PPRV became irregular rapidly. (C) PPRV interferes the production of IFN-α/β induced by SeV. HEK293T cells were transfected with IFN-β luciferase reporter plasmid, at 18 h post-transfection, cells were either infected with PPRV or left uninfected. At 36 h post-infection, cells were infected with SeV for 12 h and then luciferase activity was measured. Graph shows mean ± SD, $n=3$. (D) Screening proteins of PPRV which interfere the production of IFN-β induced by SeV. (E and F) Identification of proteins of PPRV which interfere the actions of IFNs. The results represent mean ± SD for triplicate samples.

protein (Fig. 2D). The PPRV V protein and STAT1 or STAT2 were co-expressed in Cos7 cells, HA-IP was carried out to detect Flag tagged-STAT proteins. As Fig. 2D shown, PPRV V protein was able to block accumulation of STAT protein within nucleus but not by degradation. Taken together, these data suggest that PPRV V protein negatively regulates IFNs-triggered activation of ISRE and GAS promoter and inhibits nuclear translocation of STAT protein by non-degradation.

Contribution of both the PNT and VCT domains of PPRV V protein to inhibit IFN-α/β signaling transduction through association with STAT protein

It was found that PNT domain and VCT domain in MV V protein contribute to the inhibition of type I IFN signal transduction (Andrejeva et al., 2002). Therefore, a luciferase assay was used to test whether PNT and VCT domains of PPRV V protein was able to inhibit the type I IFN signaling pathway. As shown in Fig. 3A, both PNT and VCT of PPRV V protein antagonize type I IFN signaling transduction. It also showed that PNT domain only partial inhibited type I IFN signaling transduction. Comparatively, VCT domain appears to be more effective in term of inhibition of IFN-α/β signaling transduction. In analysis of association of PNT and VCT domains with STAT protein, it was found that PNT domain interacted with STAT1 but did not with STAT2 (Fig. 3B). Because of failure to detect the molecular weight of VCT, the GST pull down experiment was then used. The result showed that interaction of VCT domain with STAT2 protein occurred (Fig. 3C). Meanwhile, results as shown in Fig. 2B and C demonstrate that PNT blocks nucleus translocation of STAT1 and VCT inhibits accumulation of STAT2 within cell nucleus. These results support that PNT inhibits type I IFN actions through association with STAT1 and blocks STAT1 translocation into nucleus. Unlike PNT, VCT to inhibit type I IFN actions through association with STAT2 and changes distribution of the STAT2 protein. These results suggest that PNT and VCT domains inhibit IFN transduction and redistribution of STAT protein in different ways.

PPRV V protein binds with STAT1 protein via a conserved Tyr amino acid residue, while a conserved Trp motif and Cys cluster are essential for STAT2 association

To examine if PPRV V protein Cys cluster contributes to binding STAT protein, Cys/Ala mutation plasmids were generated.

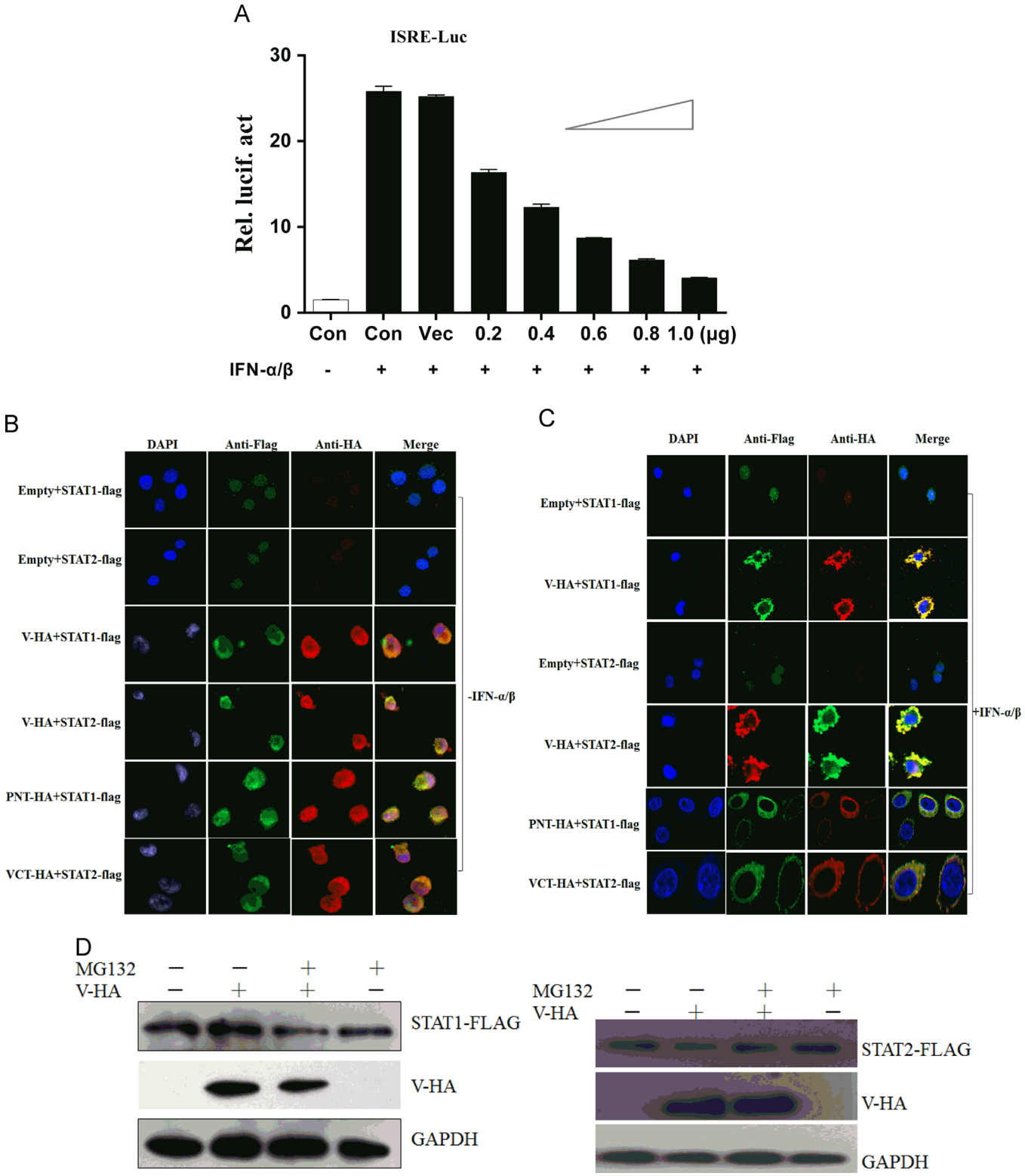

Fig. 2. PPRV V protein blocks IFNs signaling transduction in a dose-dependent manner by inhibition of STAT1/2 translocation not in a degradation way. (A) PPRV V protein inhibits the activation of the ISRE promoter induced by type I IFN in a dose-dependent manner. (+) treated and (−) left untreated. Graph shows mean ± SD, $n=3$. (B) PPRV V protein cannot inhibit the nuclear translocation of STAT1/2 without type I IFN treated. (C) PPRV V protein inhibits nuclear translocation of STAT1/2 proteins induced by IFN-α/β. (D) PPRV V protein combines with STAT protein but not promotes STAT protein degradation. Immunobloting of STAT1, GAPDH and V protein. ± V-HA DNA transfection of Cos7 cells, and all the cell sample transfected with STAT1-FLAG, treated ± MG132 for 4 h.

Alignment of the members of *Paramyxoviridae* showed conserved amino acid residues in V proteins and some conserved motifs were found to significantly interact with STAT protein (Ramachandran et al., 2008; Nishio et al., 2005). For instance, human parainfluenza virus type 2 (hPIV2) V protein can interacts with STAT1/2 via Trp motif. Meanwhile, Y110H acid residue of hPIV2 V protein is an important residue for STAT1 interaction (Nishio et al., 2005). MV V protein inhibits STAT1/2 proteins shuttle between cytoplasm and nucleus and interacts with STAT1/2 proteins with different ways (Ramachandran et al., 2008; Palosaari et al., 2003). So we

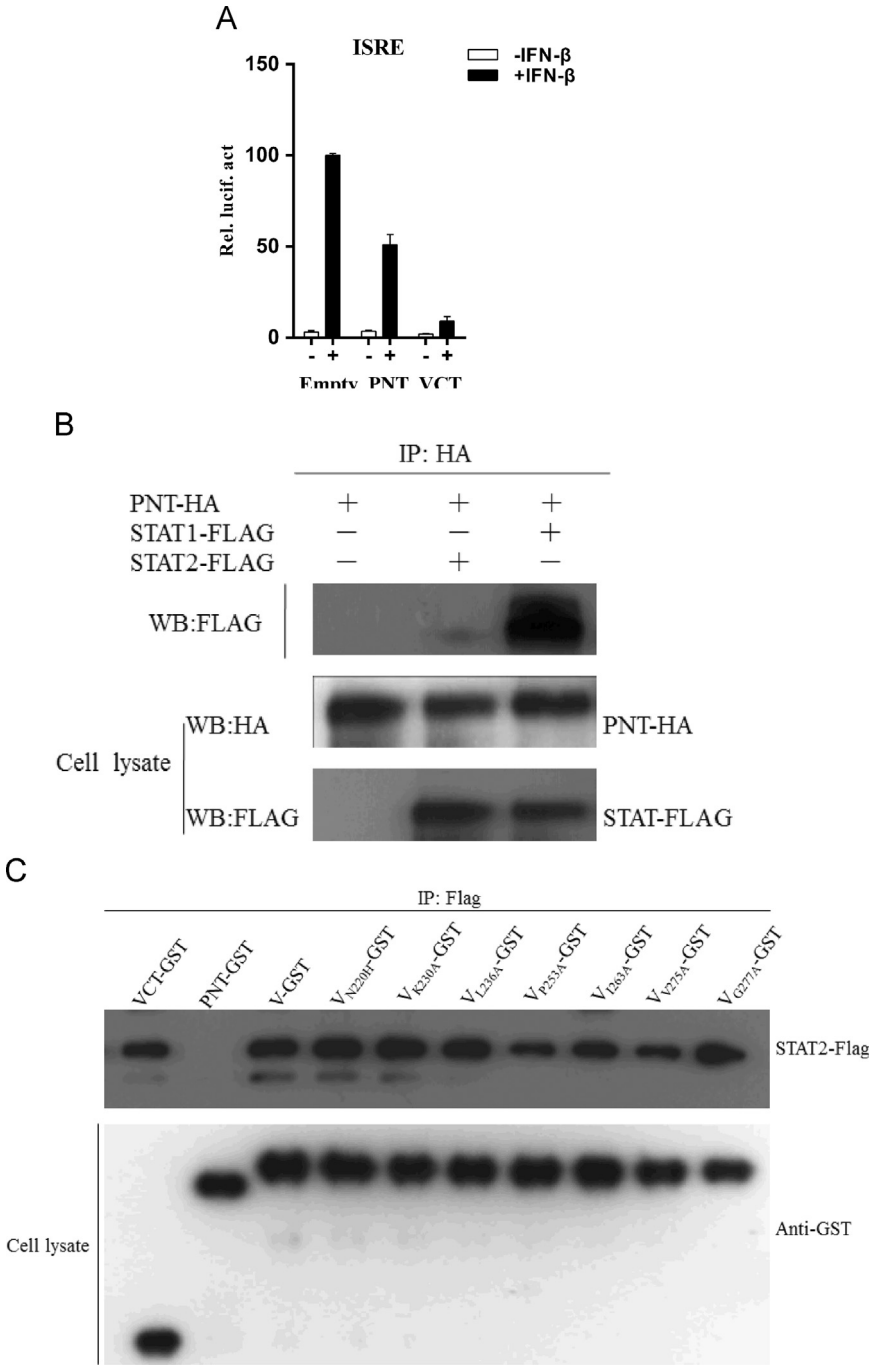

Fig. 3. Contribution of both the PNT and VCT domains of PPRV V protein to inhibit IFN-α/β signaling transduction through association with STAT protein. (A) Luciferase assay was used to test whether PNT and VCT domains of PPRV V protein are able to inhibit the type I IFN signaling pathway. (B) Use co-immuniprecipitate to verify PNT associate with STAT1 protein but not STAT2. (C) Use pull-down experiment to verify VCT associates with STAT2 and PNT does not interact with STAT2. All of the other mutations interact with STAT2.

speculate that similar amino acid residues might be essential for PPRV V protein to bind with STAT protein. To address it, after generation of deletion and substitution mutants, V-HA mutation was co-expressed with Flag-tagged STAT1 or STAT2 in Cos7 cells. Co-IP and pull-down were used to test which motif or amino-acid residues in PPRV V protein contributes to the binding ability to STAT protein. As shown in Fig. 4, when Trp amino-acid residues of V protein (W240A/W250A) was mutated, no V protein-STAT complex was observed, indicating that Trp motif of C terminal domain of PPRV V protein was essential to form V protein-STAT complex. The conserved Trp motif upstream of the Cys cluster contains two residues, W240 and W250, but some of *Paramyxoviruses* V proteins have another conserved Trp amino-acid residue W178. In PPRV V protein, alignment with other *Paramyxoviruses*, this conserved site residue is substituted by L236. Substitution mutants in which Trp residue were replaced by Ala acid residue, was then constructed in order to examine the role of Trp residues in binding STAT1/2. The results revealed that single Trp substitution resulted in loss of the ability of PPRV V protein to associate with STAT2 but do not affect association with STAT1. Interestingly, W250A mutant decreased the binding affinity to STAT1 protein. When the D248 site of PPRV V protein was replaced for Ala, PPRV V protein with replacement of the D248F lost binding affinity to STAT2. H232 of PPRV V protein could form a zinc structure with Cys cluster, therefore we replaced some of

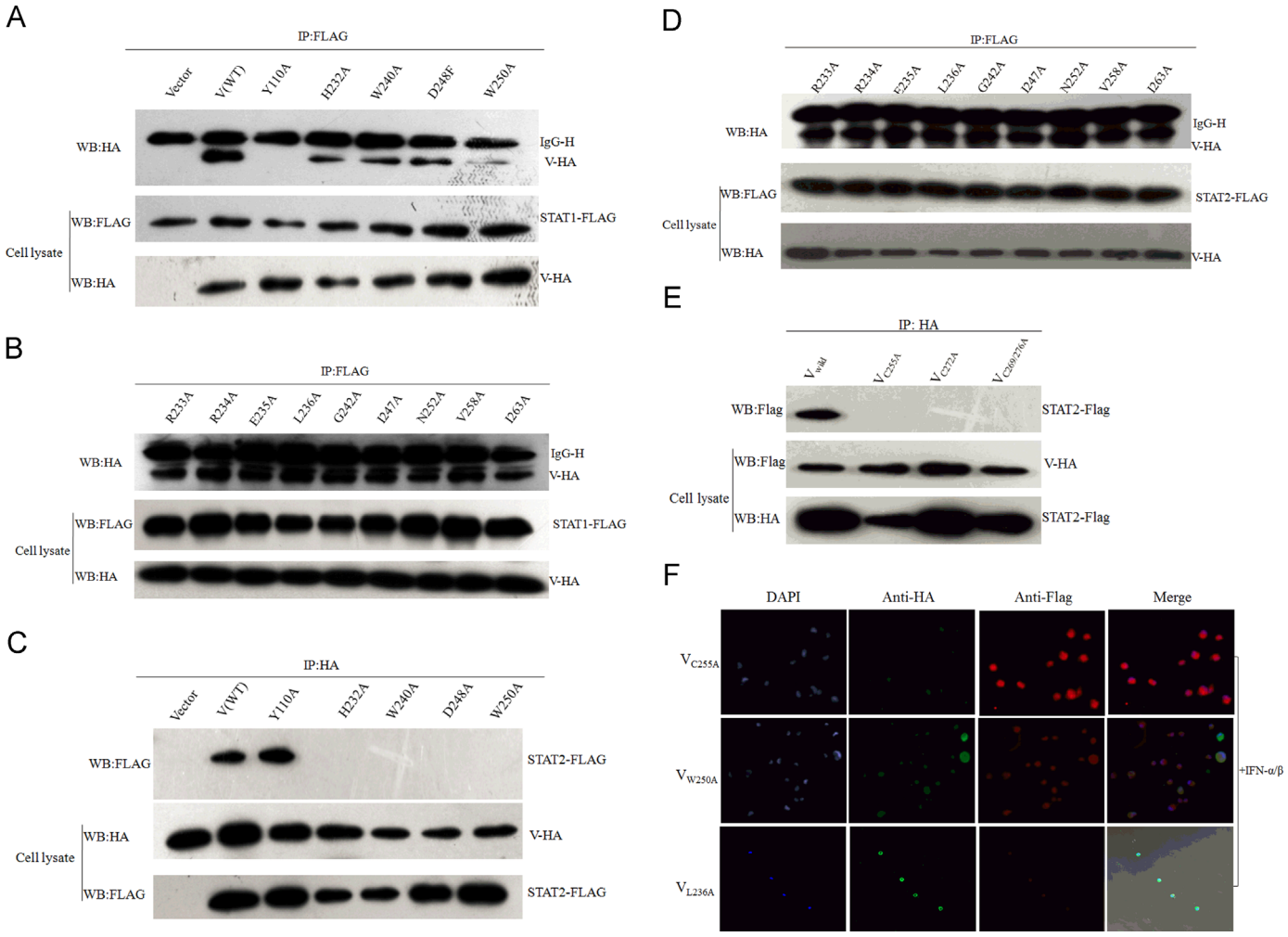

Fig. 4. Zinc structure of PPRV V protein is essential for formation of V protein-STAT2 complex formation. (A) Mutations were generated by site substitution to test the function of Trp motif and Y110 amino acid residue for binding STAT1 protein. (B) Site mutations were generated to test the role of finger domain in binding STAT1 protein process. (C) Like (A) but test the ability of V mutations combines with STAT2 protein. (D) Same site mutations from (B), using Co-IP assay to test the abilities of mutations to bind with STAT2 protein. (E) Mutations of C255/C272/C269-276 of V proteins do not have abilities to block the association with STAT2. (F) Mutations of V proteins have different abilities to block the translocation of STAT2. The 255/250 mutants do not interfere translocation of STAT2, but L236 mutant still has the ability to block the STAT2 translocation.

the Cys acid residues to Ala in order to destroy the zinc structure. As show in Fig. 4A and C, H232A and Trp-replacement mutations could result in losing the ability to bind with STAT2 but not with STAT1. Furthermore, replacement of Cys of C255/C272/C269-C276 to Ala, lead to lose the ability to interact with STAT2 (Fig. 4E). These results indicate that formation of PPRV V and STAT2 complex requires an intact Cys residues. When other acid residues was replaced to Ala between the first and second zinc fingers through alignment viruses of *Paramyxoviridae* abilities of binding with STAT1/2 proteins did not changed, indicating that these amino acid residues were not involved in the association with STAT1/2 (Fig. 4B and D).

The results above (Fig. 2B and C) suggested that PPRV V protein was able to interfere distribution of STAT proteins. Therefore, the abilities of mutations were further tested using immunofluorescence assay to explore if type I IFN-induced nuclear translocation of STAT1 and STAT2 was blocked. In Fig. 4E, mutations at 255/250/236 in PPRV V protein were applied as examples for the phenomenon. Results were coincident with the results above that Try motif and Cys amino acid residues are required for translocation of STAT2. Taken together, these results suggest that Cys cluster/Trp motif and D248 in zinc structure play important roles in STAT2 binding affinity and distribution but did not interfere the function of STAT1. However, Y110 is the specific binding site for V protein-STAT1complex.

Mutations of V protein result in lost the abilities to block type I IFN action

As previous reports, the conserved regions in MV V protein showed different inhibitions of the IFN-α/β signaling. To verify the activity of the conserved regions of PPRV V protein on IFN-α/β signaling, abilities of PPRV V protein and its mutants to block IFN action were compared. For this purpose, reporter gene assays was used for analysis of type I IFN induced transcription and a more biologically relevant assay was employed to test their abilities to block the induction of the antiviral status mediated by type I IFN using VSV-GFP reproduction experiment.

Cos7 cells expressing the indicated PPRV mutant V proteins were plated in a 96-well plate. At 24 h cells were treated with 10, 100, 1000 IU/ml of IFN-β for 12 h and then challenged with VSV-GFP at a MOI of 0.1 for 16 h. The results showed that V protein and its mutants were expressed at similar levels (Fig. 5A). Based on type I IFN-induced gene transcription, it was found that wild type V protein, D248F, Trp and Cys mutations inhibit the IFN-α/β signaling. In contrast, other

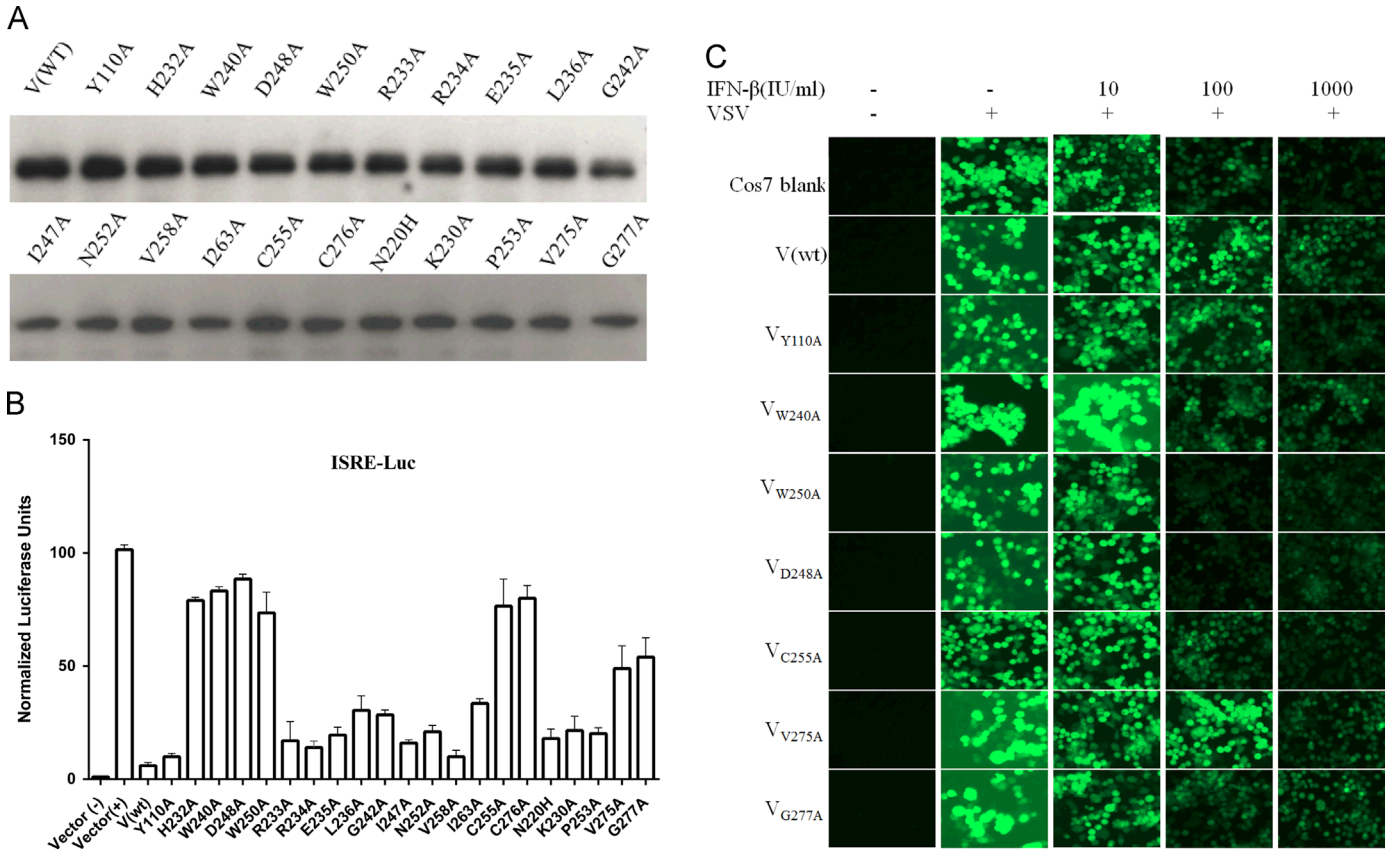

Fig. 5. Mutations of PPRV V protein result in loss of the abilities to block type I IFN action. (A) PPRV V protein and its mutants with HA tag were expressed at similar levels; (B) the same experiment process like above for ISRE-luc luciferase assay but use mutation V proteins; (C) VSV-GFP infection assay for the type I IFN-induced antiviral status.

mutations had a little impact on their ability to inhibit the IFN-α/β signaling (Fig. 5B). This result is as uniformly as GFP expression (Fig. 5C). In further analysis of some of the mutations, it was found that site of mutations of Trp motif and zinc fingers are essential for establishment of the IFN-induced antiviral state. Moreover, Y110 and D248 also affect VSV replication.

Discussion

PPRV infection leads to serious consequences for the host, including suppression of the immunity system, especially innate immunity. Many pathogenic viruses have evolved various strategies to evade the innate immune system. It is known that most of the sub-family *Paramyxovirinae* viruses have adopted effective ways to antagonize the host immune system. The first approach used is to inhibit the function of IFN-induced host antivirus proteins by viral protein (Takaki et al., 2011; Rodriguez and Horvath, 2013); as well as to antagonize the IFN production (Poole et al., 2002; Takaki et al., 2011; Rodriguez and Horvath, 2013). Most of RPV, MV, CDV and PPRV antagonize the production of type I IFN. The result in the present study shown PPRV can block the IFN-β production that induced by SeV (Fig. 1B). The third strategy is to target the IFN signaling transduction by interacting with the components of IFN signal transduction pathway or inducing ubiquitination of proteins within IFN pathway. In this case, V proteins of the *Paramyxovirinae* sub-family can directly combine with the cellular STAT protein that is responsible for IFN transduction inhibition.

PPRV V protein is a potent inhibitor of IFN transduction pathway that hijack cellular proteins (Chinnakannan et al., 2013), however, different viruses or the different strains of same genus virus or the same virus studied by different laboratory may varied (Ulane and Horvath, 2002). Experiments with luciferase assay in the present study demonstrated that only PPRV V protein interferes with the production of IFN-β. PPRV N, P, V proteins can restrict the ISRE and GAS promoter expression to limit the IFN signaling transduction, but in comparison with N, P proteins, V protein has the strongest ability to block the IFN signaling, especially, type I IFN-dependent pathway. The results also showed that N and P proteins were able to inhibit ISRE more effectively than GAS promoter, maybe due to the fact that STAT2 protein is the primary target for N and P proteins-mediated type I IFN signaling inhibition. This hypothesis needs to be confirmed. Finally, these results suggest that PPRV might have different ways to ensure effective inhibition of IFN activation.

In Paramyxoviridae viruses, the strategy of V protein inhibits antiviral state at least in part by targeting at both STAT1 and STAT2. For instance, transient expression of the V protein of simian virus 5 (SV5) can inhibit activation of IFN-β response and SV5V protein degrade the STAT1 protein to block IFN signaling transduction. Mumps virus (MuV) and simian virus 41 (SV41) also decrease the STAT1 expression level. Remarkably, hPIV2 V protein dose not target STAT1 but STAT2 to antagonize IFN signaling. As previous studies demonstrated that MV V protein targets STAT2 precede over STAT1 to inhibit IFN-α/β signaling (Poole et al., 2002; Ramachandran et al., 2008; Martens and Howard, 2006; Cardenas, 2010; Wang et al., 2010; Takaki et al., 2013). Some viruses of *Paramyxoviriuses* interfere IFN actions by disturbing STAT protein distribution. MV V protein contributes to the inhibition of the IFN signaling through binding with STAT1/2 to prevent nuclear translocation of STAT proteins (Chinnakannan et al., 2013), and the PNT and VCT of MV V protein bind with STAT1 and STAT2 respectively (Ramachandran et al., 2008). The subcellular distribution of PPRV V protein is similar with MV, but

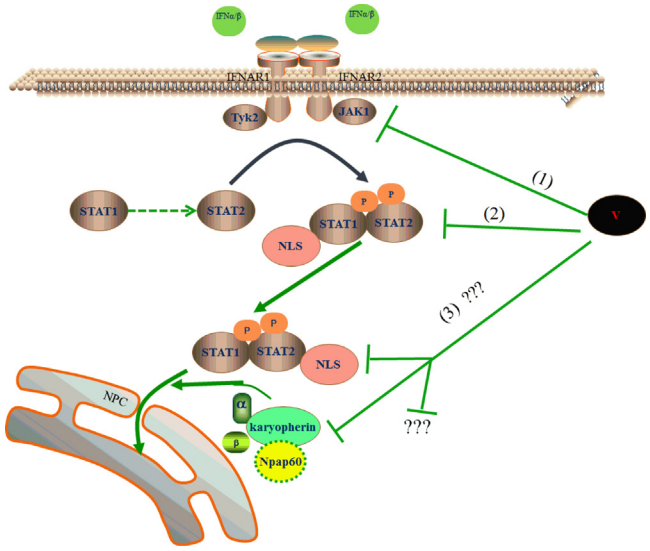

Fig. 6. The way of PPRV V protein blocks IFN signaling. (1) V protein blocks JAK-mediated STAT1 phosphorylation to inhibit IFN signaling. (2) V protein interacts with STAT1/2 to block IFN signaling or interacts with DDB1 to facilitate STAT2 degradation. (3) PPRV V protein blocks STAT proteins to translocate into nuclear and recently studies showed V protein could inhibit JAK-mediated STAT1 phosphorylation, but another report suggested STAT2 is the primary target for the interaction by V protein, we speculate that V protein inhibits STAT protein translocation may have another way through interfering the function of karyopherin or other importin proteins.

distinct from that of *Rubulavirus* genus and *Henipavirus* genus. PPRV V protein has no effect on nuclear distribution of STAT protein without IFN induction. In other *Paramyxovirus*, V proteins function to redistribute STAT protein which can be interfered by leptomycin B (LMB), but for MV V protein interferes with STAT protein nuclear shuttling being independent on a chromosomal region maintenance 1 (Crm1) nuclear export system (Palosaari et al., 2003). V protein is conserved between PPRV and MV, especially VCT domain. So we speculated that PPRV V protein may have the same mechanism as MV in STAT protein redistribution. V protein of *Paramyxovirus* also antagonize IFN actions through interfering phosphorylation of STAT protein or accelerate degradation of STAT protein to actively interfere its anti-IFN activity (Ramachandran et al., 2008; Caignard et al., 2007; Devaux et al., 2007; Martens and Howard, 2006; Precious et al., 2005). For the hPIV2, V protein is associated with STAT1/2 proteins and the VCT domain strongly affects the degradation of STAT2 protein (Ulane and Horvath, 2002). Our results (Fig. 2B and C) lead to a concept whether V protein may degrade STAT1 and STAT2 protein by the ubiquitin system. However, PPRV V protein does not inhibit IFN signaling by degradation of STAT. Recent studies revealed that MV, RPV and *Henipavirus* (HeV) V proteins sequester STAT protein to block them from translocating into nuclear without degradation. It has been demonstrated that MV V protein inhibits Jak1-mediated phosphorylation of STAT1 to antagonize type I IFN signaling transduction (Caignard et al., 2007). However, another report has verified that MV V protein is the primary target of STAT2 to block IFN signaling (Ramachandran et al., 2008). Meanwhile, VCT of PPRV V protein antagonizes STAT2 protein nuclear translocation. Previous study shows that VCT does not interact with JAK kinase (Caignard et al., 2007). So this means that VCT does not induce the STAT2 ubiquitination and VCT also does not inhibit the activation of JAK kinase. From results we know that PPRV V protein may interfere with the upstream or the downstream of STAT protein to block STAT protein nuclear translocation. Here we conjecture that PPRV may regulate the downstream of STAT protein within the host. PPRV may also regulate the nuclear import proteins. As previous studies revealed that Nuclear localization signal (NLS) or Npap60/Nup50 plays a critical role in disassembly of nuclear protein import complex (Nishio et al., 2005; Matsuura and Stewart, 2005), we speculate that failure of STAT1 and STAT2 proteins to be imported to nuclear may be caused by V protein which interferes with the function of NLS or NSL binding protein karyopherin-α or cofactor protein Npap60/Nup50. Further studies are required to dissect the principle of V protein to block the translocation (Fig. 6). This study may give a new insight into better understanding the intricate and delicate mechanism of PPRV to escape the IFN signaling.

Previous reports demonstrated that both the C- and N-terminal domains of *Morbillivirus* V protein contribute to inhibition of the IFN signaling (Ramachandran et al., 2008). Like MV, the results showed that PNT and VCT of PPRV V protein play a critical role in interfering with STAT1/2. PNT domain combines with STAT1 rather than STAT2, while the VCT domain interacts with STAT2 instead of STAT1. A mutation that deleted the Y110H of the PNT of MV V protein impaired its ability to bind with STAT1 and decreased the inhibition of the IFN signaling. In the present study, we demonstrated that the PPRV V protein mutation which substitute the Y110 to Ala acid residue impaired its ability to inhibit the IFN signaling, which is in agreement with previous studies which showed that the specific domain of V protein is important for its anti-IFN activity and required for interacting with STAT proteins (Ramachandran et al., 2008; Devaux et al., 2007; Matsuura and Stewart, 2005; Nishio et al., 2002; Nishio et al., 2001).

The finger structure of V protein is essential for interacting with STAT protein. The Cys cluster is the most notable feature of the V proteins of *Paramyxovirus*. This cluster binds two zinc ions to establish a Ring fingers structure. In *Paramyovirus*, the VCT also contains a Trp motif in the upstream of the Cys cluster, but in PRRV, one of the Trp residue was substituted by leucine acid residue and formed a new Trp motif (L-X_3-W_9-W) (Andrejeva et al., 2002). Here we constructed some of V protein mutations that are conserved in finger structure to verify the critical role of these binding sites. The results demonstrated that any of the Trp residues of Trp motif is sufficient for PPRV V protein interacting with STAT2 protein and blocking the IFN signaling. However, the Leu residue within Trp motif is not required for IFN-induced signal transduction. Meanwhile, the conserved acid residues was selected to confirm which one is critical for the STAT protein binding, indicating that Cys cluster as well as Trp motif are required in PPRV-induced anti-IFN activity. But interestingly, 275 and 277 acid residues site within V protein are more prone to interfere with the IFN signaling. Finally, both PNT and VCT of PPRV V protein were identified to play a synergistic role in innate immune evasion. Moreover and Trp motif and Cys cluster in zinc finger structure of PPRV V protein are essential for this IFNs evasion.

Materials and methods

Cell culture

Cos7, A549 and HEK293T were used in experiments. Cells were maintained in 1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Gibco-BRL) and incubated with 5% CO2. Growth of viruses was performed on Cos7 cells.

Recombinant constructs

N, F, H, M and P genes of PPRV also were cloned into pCAGGs. PPRV Tibet strain V gene (gb|FJ905304.1) and N terminal domain of V gene (PNT, 1-231aa) and C terminal domain of V gene (VCT, 232-299aa), STAT1 and STAT2 gene fragments were all cloned into pCAGGs plasmid with HA or Flag tag. Meanwhile, intact V gene recombinant construct (V-HA-pCAGGs) was used as template to

make the different point mutants. All of the point mutations from V gene were performed to substitute an Ala acid residue. V, PNT, VCT genes and point mutation of V gene were cloned into a prokaryotic expression vector (pGEX4T-1). All the recombinant plasmids were verified by DNA sequencing (Genewiz).

Transfection

Transient transfection of cells was carried out using lipofectamine 2000 (Invitrogen) according to manufacture's instruction.

Luciferase assay

Luciferase assay was performed as previously described (Caignard et al., 2007). Briefly, Cells (A549 or HEK293T) were plated in 24-well culture plates. After 24 h, cells were co-transfected with 1 μg per well of a recombinant plasmid, 0.01 μg of pRL-TK and 0.1 μg of either pIFN-β-luc or pISRE-luc or pGAS-luc. At 24 h post transfection, culture medium was replaced with fresh medium supplemented with 1000 U/ml of IFN-(α/β). After 18 h treatment with IFNs, the luciferase activity was analyzed using a Dual-Glo luciferase assay system (Promega).

Immunoprecipitation (IP)

Cos7 cells were seeded in 6-well culture plates. After 24 h, cells were co-transfected with (per well) 2 μg of plasmid containing of either wild-type V gene or mutation V gene, and 2 μg of Flag tagged STAT1/STAT2 constructs or empty vector as indicated. At 24 h post transfection, culture medium was replaced with fresh medium containing 1000 U of IFN-(α/β)/ml. After 18 h, cell extracts were pre-cleared with protein A/G Sepharose beads (Abmart), supernatant incubated with HA antibody (Sigma) and fresh protein A/G Sepharose beads for 2 h. After incubation and wash steps, precipitates were used for western blot analysis.

GST pull-down

GST pull-down was performed as previously described (Sambrook and Russell, 2006). Briefly, the E. coli-expressed V-GST or point mutation GST fusion protein was purified and co-mixed with Cos7 expressing STAT1 or STAT2 protein, Flag antibody and fresh protein A/G. Precipitates were analyzed by western blot.

Western blot

Proteins and beads complex with sodium dodecyl sulfate loading buffer, IP samples were separated with Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected using immunoblotting. The results of immunoblotting visualized by chemiluminescence. Anti-HA, anti-Flag and anti-GST of antibodies were purchased from Sigma.

Indirect immunofluorescence and laser scanning confocal microscope

Cos7 cells were seeded on glass circle cover slip (Fisher) and infected with PPRV at a MOI of 0.1 or co-transfected with plasmid expressing V or V mutation proteins and either of STAT1 or STAT2 as described above. 24 h after transfection, cells were washed with PBS, fixed, permeabilized and probed with HA or FLAG and DAPI (Sigma). Images were obtained using a Leica TCSSP confocal microscope for indirect immunofluorescence and ZEISS LSM 710 for confocal.

Antiviral assays

Antiviral responses were measured by vesicular stomatitis virus containing green fluorescent gene (VSV-GFP). Briefly, Cos7 cells were transfected with empty vector, wild-type (WT) V protein or mutant V for 24 h. Then cells were pretreated with IFN for 8 h. Cells were washed with IPTG-MEM medium and incubated with 9×10^5 PFU/well recombination VSV-GFP for 1 h at 37 °C and then cultured in IPTG-MEM medium. At 18 h post-infection, the cells were observed and photographed.

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