Measles virus V protein blocks interferon (IFN)-α/β but not IFN-γ signaling by inhibiting STAT1 and STAT2 phosphorylation

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Abstract Measles virus (MV), a member of the family Paramyxoviridae, encodes C and V non-structural proteins. To clarify the functions of MV C and V proteins, HeLa cell lines constitutively expressing C or V protein were established. We found that expression of V protein inhibited interferon (IFN)-α/β signaling but not IFN-γ signaling. C protein had no inhibitory effect on IFN signaling in our experimental condition. Degradation of selective signal transducers and activators of transcription (STAT) proteins was not observed in HeLa cells expressing V protein. In contrast, tyrosine phosphorylation of both STAT1 and STAT2 was inhibited in these cells after IFN-β stimulation.

Key words: Paramyxovirus; Measles virus; Interferon; V protein; C protein; STAT

1. Introduction

Interferons (IFNs) are secreted from cells infected with viruses and play important roles in innate immunity. Both IFN-α/β and IFN-γ can induce an antiviral state in cells through the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway [1]. Following the binding of IFN-α/β to the type I IFN receptor, receptor-associated tyrosine kinases JAK1 and Tyk2 are activated, which in turn phosphorylate STATs, STAT1 and STAT2 [2–4]. According to the sequential activation model, STAT2 and STAT1 are activated in this order [5]. Phosphorylated STAT1 and STAT2 proteins form heterodimers, which migrate to the nucleus and associate with p48 to form the active IFN-stimulated gene factor 3 (ISGF3) complex. ISGF3 binds to IFN-stimulated response elements (ISREs) and thereby active transcription of IFN-α/β-responsive genes. On the other hand, IFN-γ uses a similar but distinct pathway. Following the binding of IFN-γ to its receptor, JAK1 and JAK2 are activated, which in turn phosphorylate STAT1. Phosphorylated STAT1 protein forms a homodimer, termed γ-activated factor, that migrates to the nucleus where it binds to specific cis-acting γ-activated sequences (GASs) to activate the transcription of IFN-γ-responsive genes. In both cases, IFN-inducible gene products such as dsRNA-dependent protein kinase (PKR) and the 2′-5′ oligoadenylate synthetases induce cells to an antiviral state.

Viruses have evolved to have a variety of different molecular mechanisms to block IFN signaling and thereby circumvent the host immune response [6–9]. For example, it has been shown that several paramyxoviruses in the genus Rubulavirus circumvent IFN signaling through the action of their V proteins [10–28]. The V proteins of simian virus 5 (SV5), mumps virus, and SV41 block IFN signaling by targeting STAT1 for degradation, while the V protein of human parainfluenza virus type 2 targets STAT2 for degradation. Other paramyxoviruses in the genus Respirovirus, such as Sendai virus and human parainfluenza virus type 3, block IFN signaling by a distinct mechanism [12,29–39]. The Sendai virus C protein blocks IFN-α/β and IFN-γ signaling by inhibiting phosphorylation of STAT1. Recently, it has been shown that the Sendai virus C protein completely inhibits phosphorylation of STAT2 [40]. In addition, the Nipah virus (the genus Henipavirus) V protein blocks IFN-α/β and IFN-γ signaling by preventing STAT1 and STAT2 activation and nuclear accumulation [41]. In other report, the Nipah virus V, W and C proteins and Newcastle disease virus V protein have been reported to have IFN-antagonist activity [42]. Recently, it has been shown that the mumps virus V protein associates with RACK1 resulting in dissociation of STAT1 from the IFN-α receptor complex [43]. The carboxy-terminal region of STAT1α is not necessary for its ubiquitination and degradation caused by the mumps virus V protein [44].

Measles virus (MV), a member of the family Paramyxoviridae, genus Morbillivirus, causes an acute exanthematous disease that kills about one million children per year. The MV genome encodes the non-structural C and V proteins in addition to the structural proteins [45]. Recently, Yokota et al. have reported that cells infected with MV displayed suppression of the IFN-α-induced antiviral state [46]. However, it was not known which MV protein blocks IFN signaling.

Here, we show that MV V protein blocks the IFN-α/β-induced antiviral state, but not the IFN-γ-induced state, and that MV V protein does not induce the degradation of STATs as was shown for paramyxoviruses in the genus Rubulavirus,

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Abbreviations: EMC, encephalomyocarditis; GAS, γ-activated sequence; IFN, interferon; ISGF3, IFN-stimulated gene factor 3; ISRE, interferon-stimulated response element; MV, measles virus; ORF, open reading frame; pS, serine-phosphorylated; pY, tyrosine-phosphorylated; SEAP, secreted alkaline phosphatase; STAT, signal transducers and activators of transcription; SV, simian virus

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but instead inhibits phosphorylation of both STAT1 and STAT2.

2. Materials and methods

2.1. Antibodies

A peptide designated C20-40 (NH2-CWPSRKPWQHGQKYQ-TTQDRTE-COOH) corresponding to the amino acids at positions 20–40 of MV C protein of the IC-B strain was synthesized and coupled to keyhole limpet hemocyanin (KLH). A cysteine residue found in the C20-40 peptide was used for conjugation to KLH. Another synthetic peptide designated V279-299 (NH2-CRTDTGVDTRI-WYHNLPEIPE-COOH) corresponding to the carboxy-terminus of MV V protein of the IC-B strain was synthesized and coupled to KLH. Rabbits (two rabbits for each peptide) were injected with the peptides and bled, and sera were tested for the presence of anti-peptide antibodies by enzyme-linked immunosorbent assay using the free peptides as antigens. Anti-STAT1α/β rabbit polyclonal antibody (no. sc-346; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-STAT2 rabbit polyclonal antibody (no. sc-476; Santa Cruz Biotechnology), anti-phospho-(Tyr701) STAT1 (anti-tyrosine-phosphorylated (pY)-STAT1) (no. 9171; Cell Signaling Technology, Beverly, MA, USA), anti-phospho-(Tyr689) STAT2 (anti-pY-STAT2) (no. 07-224; Upstate Biotechnology, Lake Placid, NY, USA), and anti-phospho-(Ser727) STAT1 (anti-serine-phosphorylated (pS)-STAT1) (no. 06-802; Upstate Biotechnology) were used in Western blotting analysis.

2.2. Plasmids

To synthesize cDNA encoding the C protein open reading frame (ORF), polymerase chain reaction (PCR) was carried out using CBam1 (GCGGATCCGGCACGCCATGTCAAAAACG) and CBam2 (GCGGATCCTCAGGAGCTCGTGGATCTCC) primers and the full-length cDNA clone of the IC-B strain of wild-type MV as a template [47,48]. Since the mRNA for the V protein contains an extra guanine (G) residue by the mechanism of RNA editing [49], two-step PCR was carried out to synthesize cDNA encoding the V protein ORF. Two fragments containing an extra G residue were synthesized using a set of VBam1 (GCGGATCCGGAGACGATGGCAGAA-GAGC) and VBam2 (AATCTCGCGTCTGTGCCCCTT) primers and a set of VBam3 (AAGGGGCACAGACGCGAGATT) and VBam4 (GCGAATTCGGATCCTCATTATTCTGGGATCTCGG) primers and the full-length cDNA clone of the IC-V strain of wild-type MV as a template [47,48]. Then, the two PCR fragments were mixed and a second PCR was carried out using VBam1 and VBam4 primers. Resulting PCR fragments containing the C and V ORF were digested with *Bam*HI and cloned into the *Bam*HI site of plasmid pKS336 (generous gift from K. Sakai, National Institute of Infectious Diseases, Tokyo, Japan), to make pKS-C (C protein), and pKS-V (V protein).

2.3. Establishment of stable transformants

Establishment of cells stably expressing V or C protein was done as described previously [37]. Briefly, 1×10^6 HeLa cells per 9-cm-diameter dish were transfected with 10 μg of pKS-C or pKS-V plasmids using a mammalian transfection kit (Stratagene, La Jolla, CA, USA.). Two days later, the medium was replaced with Dulbecco's modified Eagle's medium containing 2 μg/ml of blasticidin-S (Funakosi, Tokyo, Japan). Several colonies grown in the selection medium were picked up and used in this study.

2.4. Antiviral activity of IFNs

Antiviral activity of IFNs was measured as previously described [37] with minor modification. Briefly, cells plated at a density of 3×10^5/well in 24-well plates were treated with human IFN-β or IFN-γ at 0, 10, 100 or 1000 IU/well for 24 h. The cells were washed with phosphate-buffered saline and infected with encephalomyocarditis (EMC) virus at a multiplicity of infection of 0.04 for 60 min at 37°C, and further incubated in the same serum-free medium without IFN. For viral cytopathic effect assay, cells were fixed and stained with 0.5% amido black.

2.5. Reporter gene assay

For luciferase assays, 0.5 μg of plasmid pMx-luc [50] containing ISRE sequence or pGAS-TA-luc (Clontech, Palo Alto, CA, USA) containing GAS sequence along with pSEAP-Control (Clontech) were cotransfected into parental HeLa or HeLa-V cells grown in six-well plates (2×10^5cells/well) with standard calcium phosphate procedures. At 20–24 h post transfection, the cells were incubated with or without IFN-β or IFN-γ at 1000 IU/ml for 0, 2, 4 or 6 h. Portions of cell lysates were assayed for luciferase activities using the luciferase assay system (Promega, Madison, WI, USA) and a MiniLumat LB9506 luminometer (Berthold, Pforzheim, Germany). To monitor transfection efficiency, a portion of each cell supernatants was assayed for secreted alkaline phosphatase (SEAP) by using the SEAP assay kit (Toyobo, Osaka, Japan).

2.6. Western blotting

Cells were lysed in a lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.6% NP40, 1 mM Na_3VO_4, 4 mM PMSF, 1 mM EDTA, 1 mM NaF) and disrupted by sonication for 1 min. After centrifugation, lysates were electrophoresed by sodium dodecyl sulfate–polyacrylamide gels. Proteins in the gel were transferred to PVDF membrane (Millipore, Bedford, MA, USA). The protein was detected using horseradish peroxidase-conjugated goat anti-rabbit antibodies (Amersham, Piscataway, NJ, USA) and Western Blotting Luminol Reagent (Santa Cruz Biotechnology) or biotinylated anti-rabbit Ig (Amersham) and streptavidin–alkaline phosphatase (Amersham).

3. Results

3.1. Establishment of stable cell lines constitutively expressing MV C or V protein

To establish cell lines constitutively expressing C or V protein, either the C or V protein ORF was inserted into the plasmid pKS336 and transfected into HeLa cells. In the V-expressing plasmid, the nucleotide sequence preceding the ATG initiation codon was changed from CCGATG to AC-GATG according to Kozak's rule to favor exclusive synthesis of V protein and prevent leaky scanning for the synthesis of C protein. Several cell lines were established, and two cell lines were selected as described in Section 2 for further analysis. One of these (HeLa-C) expresses MV C protein, and the other (HeLa-V) expresses MV V protein. The expression of C or V protein was confirmed by immunoblotting analysis using antisera specific for C (Fig. 1A, lane 3) or V protein (Fig. 1A, lane 5). No C protein was detected in HeLa (Fig. 1A, lane 1) and HeLa-V (Fig. 1A, lane 2) cells. Likewise, no V protein was detected in HeLa (Fig. 1A, lane 4) and HeLa-C (Fig. 1A, lane 6) cells in our experimental condition.

3.2. MV V protein counteracts IFN-α/β-mediated induction of an antiviral state

To see the effect of C and V proteins on establishment of an antiviral state, cells were incubated with IFN-β or IFN-γ at 0, 10, 100 or 1000 IU/well for 24 h, and challenged with EMC virus. Parental HeLa cells incubated with IFN-β were protected from EMC virus challenge (Fig. 1B). HeLa-C cells incubated with IFN-β were also protected from EMC virus challenge (Fig. 1B), indicating that C protein did not counteract IFN-α/β-mediated induction of an antiviral state. In sharp contrast, HeLa-V cells incubated with IFN-β were completely sensitive to EMC virus challenge (Fig. 1B). IFN-α treatment gave the same results (data not shown). These results indicated clearly that MV V protein counteracted IFN-α/β-mediated induction of an antiviral state.

For IFN-γ-mediated induction, parental HeLa cells incubated with IFN-γ were protected from EMC virus challenge (Fig. 1C). Similarly, HeLa-C as well as HeLa-V cells incubated with IFN-γ were also protected from EMC virus challenge, indicating that neither C nor V protein of MV could counteract IFN-γ-mediated induction of an antiviral state

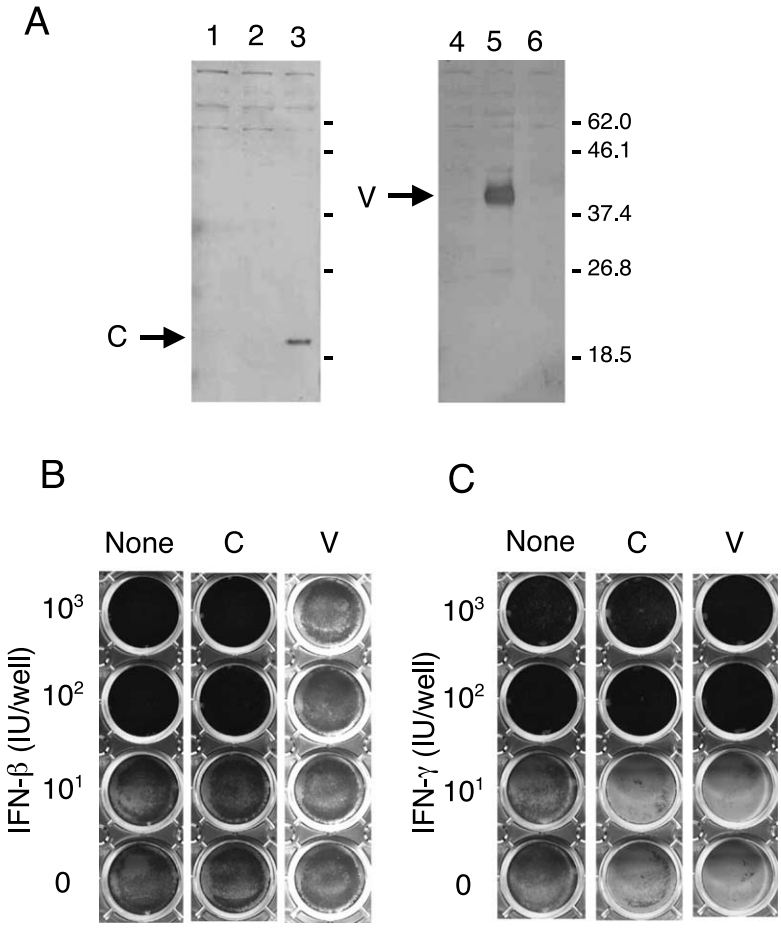

Fig. 1. IFN-induced antiviral states of parental, C-expressing, and V-expressing HeLa cells. A: Extract lysates (10 μg of protein) from parental HeLa (lanes 1 and 4), HeLa-V (lanes 2 and 5) and HeLa-C (lanes 3 and 6) cells were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by Western blotting analysis with C-specific antiserum (lanes 1–3) or with V-specific antiserum (lanes 4–6) using the alkaline phosphatase method and molecular weight standards (in thousand) are indicated. Cells in 24-well plates were incubated for 24 h with various concentrations of IFN-β (B) and IFN-γ (C), and were challenged with EMC virus. Cells that survived the challenge infection and remained attached to the plates were fixed and stained.

(Fig. 1C). We confirmed these results by using other HeLa cell clones expressing C or V proteins (data not shown).

3.3. MV V protein blocks IFN-α/β but not IFN-γ signaling

To test the effect of V protein on IFN-α/β-mediated signaling, an IFN-α/β-responsive plasmid pMx-luc and a control plasmid pSEAP-Control were cotransfected into parental HeLa and HeLa-V cells. The relative luciferase activities increased linearly with incubation time in IFN-β-treated parental HeLa cells (Fig. 2A). In contrast, no increase in relative luciferase activity was found in HeLa-V cells treated with IFN-β (Fig. 2A), indicating that V protein blocked IFN-α/β signaling. To test the effect of V protein on IFN-γ-mediated signaling, an IFN-γ-responsive plasmid pGAS-TA-luc and the control plasmid pSEAP-Control were cotransfected into parental HeLa and HeLa-V cells. IFN-γ treatment of parental HeLa and HeLa-V cells resulted in the induction of the GAS-luciferase reporter gene activity (Fig. 2B), indicating that V protein did not block the IFN-γ signaling pathway.

3.4. MV V protein does not induce degradation of STAT1 or STAT2 but instead inhibits phosphorylation of STAT2 and STAT1

It has been shown that V proteins of paramyxoviruses in the genus Rubulavirus induce degradation of STAT1 or STAT2 [10–28]. On the other hand, V protein of Nipah virus and C protein of Sendai virus have been shown to inhibit tyrosine or serine phosphorylation of STAT1 without degradation of STAT1 or STAT2 [33,37,41]. The degradation of STAT1 was also reported in mouse embryo fibroblast cells infected with Sendai virus [31]. The suppression of tyrosine phosphorylation of STAT1 is thought to contribute to the blockade of IFN-α/β signaling. The suppression of serine phosphorylation of STAT1 was reported for Sendai virus-infected cells [12,36], although serine phosphorylation of STAT1 is not important for transactivation function of ISGF3. Very recently, Sendai virus C protein has been shown to inhibit tyrosine phosphorylation of STAT2 completely [40].

To see whether V protein induces the selective degradation of STAT1 or STAT2 as reported for other paramyxoviruses, immunoblotting was performed using antibodies specific for STAT1 or STAT2. As shown in Fig. 3, total amounts of STAT1α/β at time 0 between parental HeLa and HeLa-V cells were almost the same (Fig. 3A,B). Similarly, total amounts of STAT2 at time 0 between parental HeLa and HeLa-V cells were almost the same (Fig. 3C). These results indicated that V protein of MV does not target STAT1 or STAT2 for proteasome degradation. Upon IFN-β treatment, total amounts

of STAT1α/β (Fig. 3A,B) and STAT2 (Fig. 3C) increased gradually in parental HeLa cells as previously reported [34,37]. On the other hand, the levels of STAT1α/β (Fig. 3A,B) and STAT2 (Fig. 3C) in HeLa-V cells remained constant on IFN-β treatment.

Next, the effect of V protein expression on tyrosine or serine phosphorylation of STAT1 was examined. Parental HeLa and HeLa-V cells were left unstimulated or treated with IFN-β and then lysed at the times indicated. As shown in Fig. 3A, pY-STAT1α/β increased within 5 min of IFN-β treatment in parental HeLa cells as previously reported [34,39]. In contrast, tyrosine phosphorylation of STAT1α/β was strongly inhibited in HeLa-V cells after IFN-β stimulation (Fig. 3A). The effect of V protein expression on serine phosphorylation of STAT1 was also examined. As shown in Fig. 3B, pS-STAT1α gradually increased up to 24 h after IFN-β treatment in parental HeLa cells. In contrast, serine phosphorylation of STAT1α was not detected in HeLa-V cells after IFN-β stimulation (Fig. 3B).

Finally, tyrosine phosphorylation of STAT2 in parental HeLa and HeLa-V cells was examined. As shown in Fig. 3C, pY-STAT2 was increased within 5 min of IFN-β treatment and was prolonged for up to 24 h in parental HeLa cells. In contrast, pY-STAT2 was below the detection level in HeLa-V cells at any incubation periods upon IFN-β stimulation as reported for cells expressing Sendai virus C protein

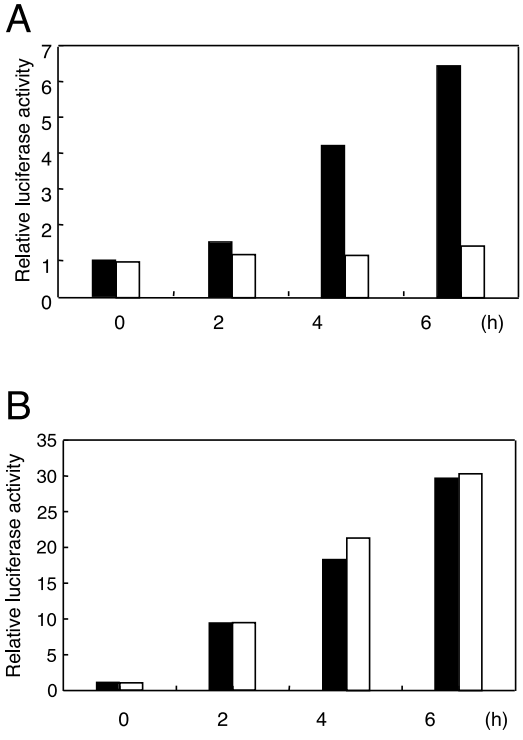

Fig. 2. Expression of the V protein inhibits IFN-α/β but not IFN-γ signaling. A: Parental (solid bar) and V-expressing HeLa cells (open bar) were transfected with an ISRE-luciferase reporter gene and control pSEAP plasmid. The cells were treated with 1000 U of IFN-β per ml for 0, 2, 4 or 6 h. Relative expression levels were normalized by SEAP activity and expressed as fold activation. B: Parental (solid bar) and V-expressing HeLa cells (open bar) were transfected with a GAS-luciferase reporter gene and control pSEAP plasmid. The cells were treated with 1000 U of IFN-γ per ml for 0, 2, 4 or 6 h. Relative expression levels were normalized by SEAP activity and expressed as fold activation.

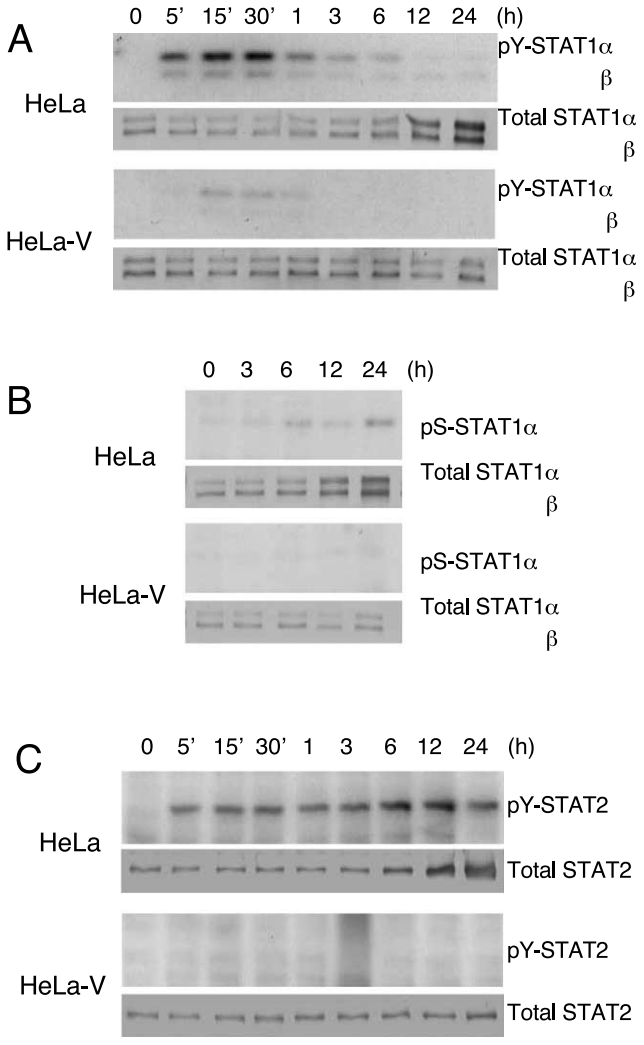

Fig. 3. Effects of the V protein on tyrosine or serine phosphorylation of STAT1 or STAT2. Parental and V-expressing HeLa cells treated with 1000 IU per ml of IFN-β were incubated for the indicated times and harvested. Total cell extracts were subjected to enhanced chemiluminescence–Western blot analysis with anti-phospho-(Tyr701)-STAT1 (A), anti-phospho-(Ser727)-STAT1 (B) or anti-phospho-(Tyr690)-STAT2 (C). Total amounts of STAT1 or STAT2 were estimated with anti-STAT1 (sc-346) or anti-STAT2 (sc-476) using the alkaline phosphatase method.

[40]. These results suggest crucial roles of STAT1 and STAT2 in the inhibitory effect of MV V protein on IFN-α/β signaling.

4. Discussion

By expressing C or V protein of MV in HeLa cells, we demonstrated that V protein but not C protein counteracts the IFN-α/β-mediated induction of an antiviral state by blocking IFN-α/β signaling. C proteins are encoded by viruses belonging to the genus Respirovirus (for example, Sendai virus), the genus Morbillivirus (for example, MV) and the genus Henipavirus (Nipah virus) among the family Paramyxoviridae. The C protein of Sendai virus was shown to counteract the IFN-mediated induction of an antiviral state by blocking IFN signaling [29,33,37]. These observations led us to examine whether MV C protein can block IFN signaling. Also, during the progress of our research, the C protein of Nipah virus was

reported to have IFN-antagonist activity [42]. However, MV C protein did not inhibit IFN signaling in our experimental condition, although we cannot rule out the possibility that high-level expression of MV C protein might inhibit IFN signaling to some extent. The C proteins of Sendai virus, Nipah virus and MV are all small in size and highly basic, but their amino acid sequences are divergent [42,51]. MV C protein might be a prototype, and the C proteins of Sendai virus and Nipah virus might have evolved to acquire an additional activity to block host IFN signaling. A recombinant MV without the expression of the C protein has been generated, and this virus showed alterations in growth and pathogenicity [52–54]. Thus, MV C protein could have yet unidentified function(s) different from blocking IFN signaling.

We found that MV V protein inhibits IFN-α/β but not IFN-γ signaling without inducing selective degradation of STAT1 or STAT2. Thus, MV V protein is different from those of the genus Rubulavirus, which inhibit IFN signaling by targeting STAT1 or STAT2 for degradation [10–28]. Since Nipah virus V protein blocks IFN signaling by preventing STAT activation without degradation of STATs [41], MV V protein is similar to the Nipah virus V protein in this respect. However, the Nipah virus V protein differs from MV V protein in that it can block IFN-γ signaling as well as IFN-α/β signaling [41]. It is interesting that the IFN-α/β-induced but not the IFN-γ-induced antiviral state was abrogated in HeLa-V cells, although phosphorylation of both STAT1 and STAT2 was inhibited by MV V protein. It should be noted that the phosphorylation of STAT2 was completely blocked, while the phosphorylation of STAT1 was strongly but not completely blocked. This result suggests that inhibition of tyrosine phosphorylation of STAT2 accounts for the blockade of IFN-α/β signaling by MV V protein. Although pY-STAT1 is the common component of both IFN-α/β and IFN-γ signaling, IFN receptor complexes responsible for phosphorylation of STAT1 are different in IFN-α/β and IFN-γ signaling pathways. Therefore, it is not surprising that MV V protein inhibits IFN-α/β signaling but not IFN-γ signaling. We are currently examining IFN-γ signaling in parental HeLa and HeLa-V cells in detail. We do not know at present why different paramyxovirus V proteins use different strategies to counteract IFN signaling in spite of structural similarities among V proteins of MV, Nipah virus and those of the genus Rubulavirus, which all have a well-conserved cysteine-rich domain at their carboxy-terminal ends [41,49,51]. The N-terminal domain of the V proteins might be responsible for determining a pathway to inactivate STAT [13]. Making chimeric V proteins among these V proteins would give us some clues to identify the domain or amino acid sequence important for these differences.

It has been reported that a recombinant MV deficient in the expression of the V protein shows restricted growth phenotype and pathogenicity in infected rodents [52,54,55]. These differences might be attributable to the anti-IFN signaling activity of the MV V protein. Naniche et al. reported that particular strains of MV induced less IFN and were sensitive to the action of IFN [56]. It is interesting to compare the anti-IFN signaling activity of the V proteins of different MV strains.

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