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The nucleocapsid protein of measles virus blocks host interferon response

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ABSTRACT

Measles virus (MV) belongs to the genus *Morbillivirus* of the family *Paramyxoviridae*. A number of paramyxoviruses inhibit host interferon (IFN) signaling pathways in host immune systems by various mechanisms. Inhibition mechanisms have been described for many paramyxoviruses. Although there are inconsistencies among previous reports concerning MV, it appears that P/V/C proteins interfere with the pathways. In this study, we confirmed the effects of MV P gene products of a wild MV strain on IFN pathways and examined that of other viral proteins on it. Interestingly, we found that N protein acts as an IFN-α/β and γ-antagonist as strong as P gene products. We further investigated the mechanisms of MV-N inhibition, and revealed that MV-N blocks the nuclear import of activated STAT without preventing STAT and Jak activation or STAT degradation, and that the nuclear translocation of MV-N is important for the inhibition. The inhibitory effect of the N protein was observed as a common feature of other morbilliviruses. The results presented in this report suggest that N protein of MV as well as P/V/C proteins is involved in the inhibition of host IFN signaling pathways.

Introduction

Measles virus (MV) is a leading cause of death among young children despite the availability of a safe and effective vaccine for the past 40 years (Moss and Griffin, 2006). Vaccination has greatly limited the spread of measles virus, and yet sufficient vaccine coverage has been difficult to achieve in developing countries. Factors such as immigration and public distrust of vaccine safety have contributed to local measles outbreaks even in developed countries that include the United States (Centers, 2008a,b; Mulholland, 2006) and Japan (Nagai et al., 2009). A greater understanding of the molecular mechanisms underlying host evasion by this pathogen would facilitate the design of new therapeutic strategies by identifying targets for pharmacological inhibition that could augment or replace vaccinations in some situations.

MV belongs to the genus *Morbillivirus* of the family *Paramyxoviridae*. Canine distemper virus (CDV), which has re-emerged in wild animals, and rinderpest virus (RPV), which most closely resembles MV, also belong to the genus *Morbillivirus*. Morbilliviruses are single-strand, negative-sense RNA viruses and consist of six tandemly linked genes that encode, from the 3′ to the 5′ end, the nucleocapsid (N), phospho (P), matrix (M), fusion (F), hemagglutinin (H) and large (L) proteins (Griffin, 2001). In addition to coding for the P protein, the P gene of morbillivirus encodes accessory proteins, C and V. The V protein is translated from the same start codon as the P protein; however, an additional nucleotide at the RNA editing site causes a frame sift (Cattaneo et al., 1989). Hence, V protein has a unique carboxy-terminal domain. In contrast, the open reading frame (ORF) of C protein begins downstream of the P protein start codon and is embedded in the first half of the P gene (Bellini et al., 1985).

A number of paramyxoviruses inhibit host interferon (IFN) signaling pathways by various mechanisms involving one or more of their accessory proteins. IFN-α/β (type I IFN) signals induce the phosphorylation and activation of signal transducer and activator of transcription 1 (STAT1) and STAT2 via Janus kinase 1 (Jak1) and Tyrosine kinase 2 (Tyk2). Upon phosphorylation, STAT1 and STAT2 form heterodimers which translocate to the nucleus, where they form IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds IFN-stimulated response elements (ISREs) to drive the expression of IFN-α/β regulated genes. Similarly, IFN-γ signals induce the phosphorylation and activation of STAT1 but not STAT2 via Jak1 and Jak2. STAT1 forms homodimers and they lend structure to the active transcription complex γ-activated factor (GAF), which binds to γ-activated sequence (GAS) elements in regulatory regions of IFN-γ-inducible genes (Stark et al., 1998). STAT3 is activated by many cytokine systems, including interleukin 6 (IL-6). In these signal transduction pathways, the V protein of the *Rubulavirus* encodes STAT-specific E3 ubiquitin ligase activities that function in combination with cellular proteins to target STAT1 and STAT2 (and STAT3 in mumps virus) for proteasomal degradation (Didcock et al., 1999; Parisien et al., 2001, 2002; Ulane and Horvath, 2002; Ulane et al., 2003). The P/V/W proteins of nipah virus (NiV), a member of the genus *Henipavirus*, block STAT1 phosphorylation (Park et al., 2003; Rodriguez et al., 2002; Shaw et al., 2004). The C protein of Sendai virus (SeV), a member of the genus *Respirovirus*, blocks IFN-γ signaling after the STAT1 nuclear translocation event (Gotoh et al., 2004).

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Although P/V/C proteins contribute to viral evasion of the host IFN response in MV, various molecular mechanisms of inhibition have been reported. V and P proteins were both found to inhibit IFN-α/β signaling (Ohno et al., 2004), and it has been suggested that V protein blocks the transport of IFN-α/β and IFN-γ-activated STAT molecules to the nucleus without interfering with tyrosine phosphorylation (Palosaari et al., 2003). Other studies found that V protein blocked only IFN-α/β-stimulated STAT1 and STAT2 phosphorylation, without an effect on IFN-γ action (Takeuchi et al., 2003). This finding is supported by the observation that V and C proteins both suppress Jak1 phosphorylation by association with the IFN-α receptor complex (Yokota et al., 2003). Shaffer et al. demonstrated the inhibition of an IFN-α/β and IFN-γ-responsive reporter gene by C protein (Shaffer et al., 2003). Additionally, another study also reported weak inhibition by C protein (Fontana et al., 2008). The reasons for these discrepancies remain unclear, although they might be due to differences in the virus strains and cell types. To date, no studies on the effects of other viral protein(s) on the IFN signaling pathway have been reported.

In MV and RPV infected cells, STAT1 is known to colocalize with N, P and L proteins in cytoplasm, a pattern that has been observed for paramyxoviruses (Nanda and Baron, 2006; Palosaari et al., 2003; Sweetman et al., 2001). Since P protein forms a ribonucleoprotein (RNP) complex with N and L proteins in the infected cells, and C protein colocalizes with them (Nakatsu et al., 2008), implication of other viral proteins on the inhibition of IFN signaling has not been studied.

Previous studies indicated that wild-type and attenuated strains of MV cannot be distinguished based on their sensitivity to IFN treatment (Fontana et al., 2008). In this study, we used the HL strain, which is a wild-type strain that was isolated from a patient and found to induce typical symptoms of measles, including rashes and Koplik's spots, in cynomolgus and squirrel monkeys (Kobune et al., 1996). Moreover, the HL strain also induces inhibition of host protein synthesis (shut-off) (Inoue et al., 2009), unlike that of the attenuated Edmonston strain (Graves, 1981; Wechsler and Fields, 1978). Thus, it appears that the HL strain maintains MV pathogenicity.

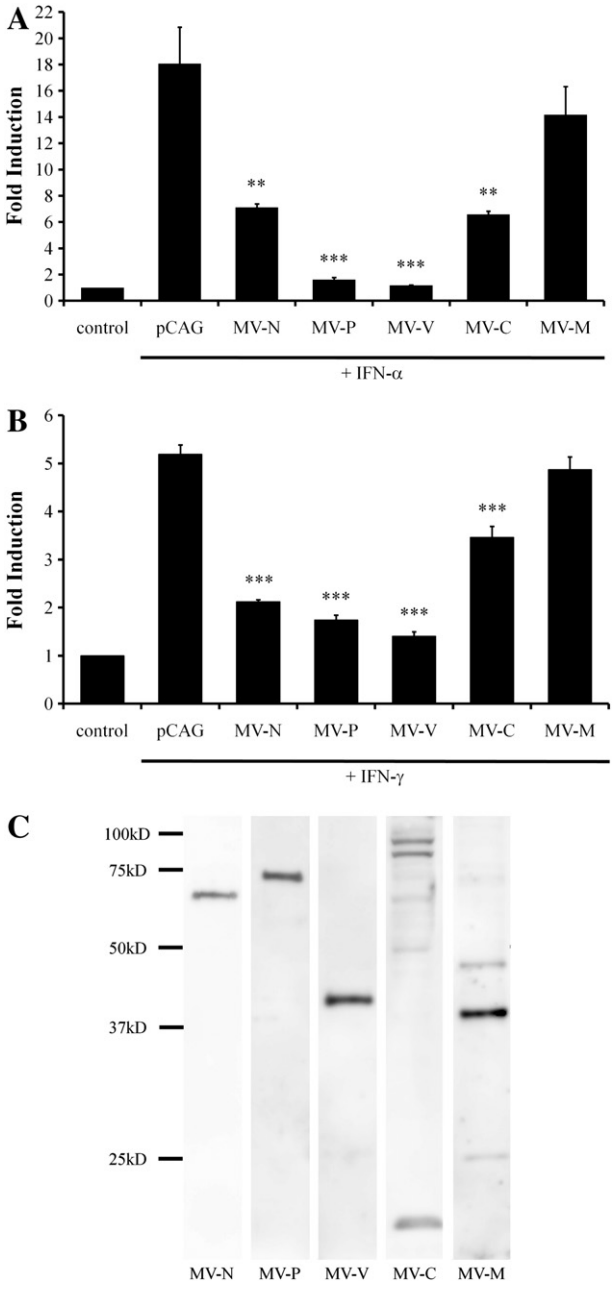

Fig. 1. MV-N as well as MV-P/V/C is involved in the inhibition of host IFN signaling pathways. (A) HEK-293T cells were transfected with pISRE-Luc, phRL-TK(Int−), and either empty vector (pCAG) or an appropriate protein (MV-N, P, V, C and M) expression plasmid. Cells were treated with 1000 U/ml IFN-α for 24 h prior to lysis and luciferase assays. (B) The same experiment was conducted using pGAS-Luc with 1000 U/ml IFN-γ. The results are expressed as induction (*n*-fold) relative to the value obtained from experiments without treatment. The data represent means ± SD for triplicate samples. Statistical analysis was performed using the Student's *t*-test; $^{**}P<0.01$, $^{***}P<0.001$ compared to pCAG. (C) Western blotting for MV proteins using A lysates is shown.

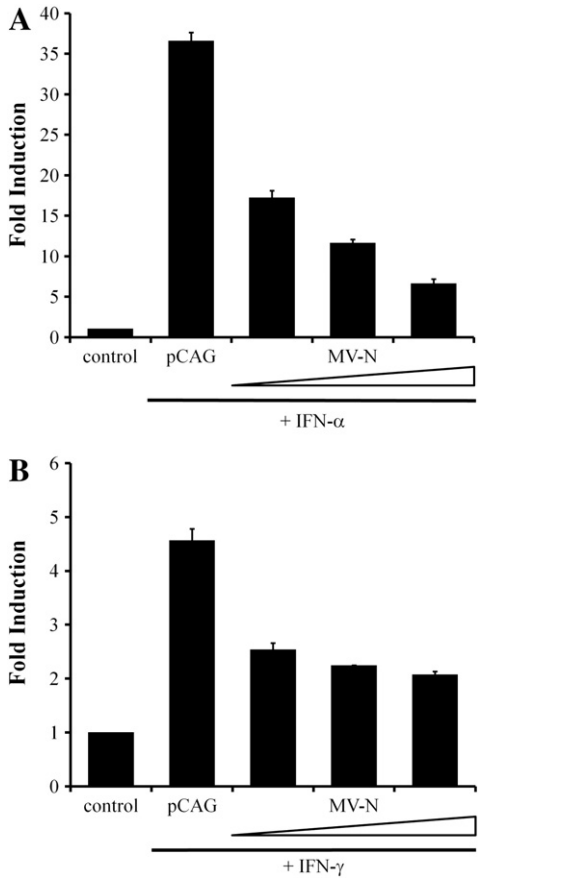

Fig. 2. MV-N inhibits IFN-α/β and γ signaling. (A) HEK-293T cells were transfected with pISRE-Luc, phRL-TK(Int−), and either empty vector (pCAG) or pCAG-MV-N (0.3, 0.6 and 1.2 μg/well transfected). Cells were treated with 1000 U/ml IFN-α for 24 h prior to lysis and luciferase assays. (B) The same experiment was conducted using pGAS-Luc with 1000 U/ml IFN-γ. The results are expressed as induction (*n*-fold) relative to the value obtained from experiments without treatment. The data represent means ± SD for triplicate samples.

In the present study, we confirmed the effects of the P gene products of MV-HL strain on host IFN signaling pathways and examined that of other viral proteins on these pathways. We found that the N protein of MV has an inhibitory function both on IFN-α/β and IFN-γ transcriptional responses for the first time; therefore, we investigated the molecular mechanisms of these inhibitory pathways.

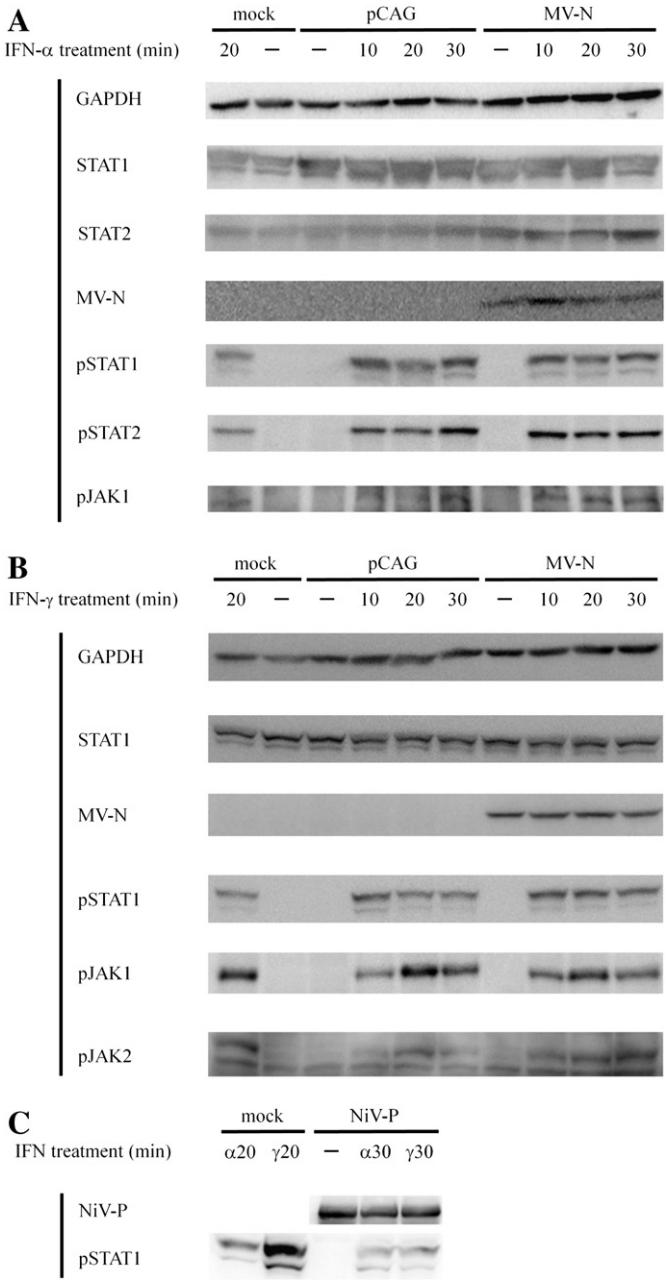

Fig. 3. Effect on IFN-induced phosphorylation of STATs and Jaks by MV-N. (A) HEK-293T cells were mock transfected or transfected with pCAG-MV-N expression plasmid or empty vector. The cells were treated 24 h later with 1000 U/ml of IFN-α at 37 °C for 10, 20 or 30 min prior to lysis. (B) HEK-293 cells were mock transfected or transfected with pCAG-MV-N expression plasmid or empty vector. The cells were treated 24 h later with 1000 U/ml of IFN-γ at 37 °C for 10, 20, or 30 min prior to lysis. (C) To show that STAT phosphorylation is blocked as positive control of this experiment, cells transfected with pCAG-NiV-P were used. For STAT1, 25 μg/lane lysates were applied to SDS-PAGE on a 7% polyacrylamide gel. For STAT2, 25 μg/lane lysates were applied to SDS-PAGE on a 5% polyacrylamide gel. For Jak1 and Jak2, 30 μg/lane lysates were applied to SDS-PAGE on a 5% polyacrylamide gel. For GAPDH, MV-N and NiV-P, 25 μg/lane lysates were separated by SDS-PAGE on a 10% polyacrylamide gel, and then analyzed by Western blotting.

Results

MV-N inhibits IFN-α/β and IFN-γ transcriptional responses

To confirm the effect of MV P gene products on the IFN signaling pathway in the HL strain, we investigated that of the P gene products together with other viral proteins of the MV-HL strain. We performed luciferase reporter gene assays to detect signal transduction in response to IFN-α/β and IFN-γ. ISRE was used as a promoter element of the firefly luciferase gene to examine the response to IFN-α/β. HEK-293T cells were transfected with the plasmid (pISRE-Luc) along with an expression plasmid encoding various viral proteins or with empty vector, and phRL-TK(Int−) to normalize transfection efficiency. At 24 h after transfection, IFN-α was added to the cells and luciferase activity was measured 24 h later. We checked the expression levels of each MV protein by Western blot. The result showed that all cell lysates contain enough viral protein (Fig. 1C). Expression of MV V and P proteins almost completely blocked the induction of luciferase activity in response to IFN-α as previously reported (Fig. 1A). In the C protein of the HL strain, the IFN-α/β reporter gene expression level was inhibited (36%), compared with transfection with the empty vector. Surprisingly, we found that MV-N similarly interfered with IFN-α/β signaling (40%). On the other hand, MV-M, which does not colocalize with STAT1 or form RNP, has minor effect on this signaling pathway.

Similarly, the response to IFN-γ was tested using a GAS-luciferase reporter gene (pGAS-Luc). MV V and P proteins induced significant inhibition of the transcriptional response to IFN-γ (about 30%) in the HL strain (Fig. 1B). MV-N also blocked the induction of luciferase activity in response to IFN-γ (60% inhibition) more than that of MV-C. MV-M

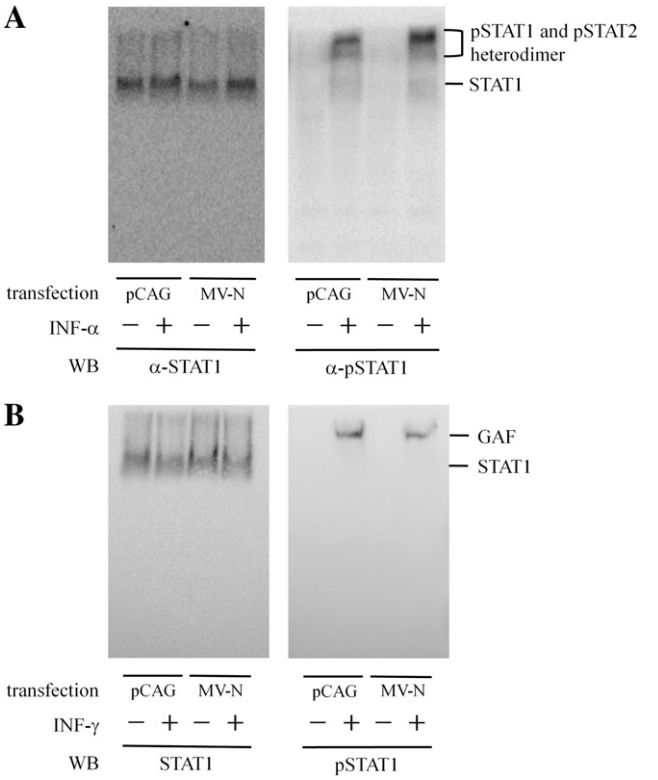

Fig. 4. MV-N has no effect on the pSTAT dimer formation. (A) HEK-293T cells were transfected with an empty vector (pCAG), or with MV-N expression plasmid, and then treated with 1000 U/ml IFN-α for 6 h prior to lysis. (B) HEK-293 cells were transfected with an empty vector, or with MV-N expression plasmid and then treated with 1000 U/ml IFN-γ for 6 h prior to lysis. Equal amounts of lysates were subjected to native PAGE on an 8% polyacrylamide gel. They were analyzed by Western blotting with anti-STAT1 or anti-pSTAT1 antibody.

showed no effect on IFN-γ signaling. To further understand the role of MV-N, we performed luciferase reporter gene assay in cells expressing variable concentrations of MV-N. Both IFN-α and γ signaling were inhibited in a dose-dependent manner in cells expressing MV-N (Figs. 2A and B). These results show that MV-N is an effective inhibitor of IFN-α/β and IFN-γ signaling.

MV-N has no effect on STAT and Jak phosphorylation and does not induce STAT degradation

To clarify the mechanism by which MV-N suppresses IFN signaling, we first investigated the early events of the IFN-α/β and IFN-γ signaling pathways. First, we examined whether MV-N can prevent the phosphorylation of STATs and Jaks. Control cells transfected with empty vector or cells expressing MV-N were left unstimulated or treated with IFN-α for 10 to 30 min prior to lysate preparation. We detected the phosphorylation levels of STAT1, STAT2 and Jak1 in the cells, with or without MV-N, by Western blot. Fig. 3A shows that the expression of MV-N did not detectably reduce IFN-α-induced phosphorylation of STAT1, STAT2 and Jak1. Furthermore, we monitored the phosphorylation of STAT1, Jak1 and Jak2 in cells with or without MV-N treated with IFN-γ. The results showed that MV-N also did not affect the IFN-γ-induced phosphorylation of STAT1, Jak1 and Jak2 (Fig. 3B). We also examined the phosphorylation level of STAT1 until 24 h after IFN-γ stimulation, but no significant changes were detected (data not shown). Figs. 3A and B showed no changes of all

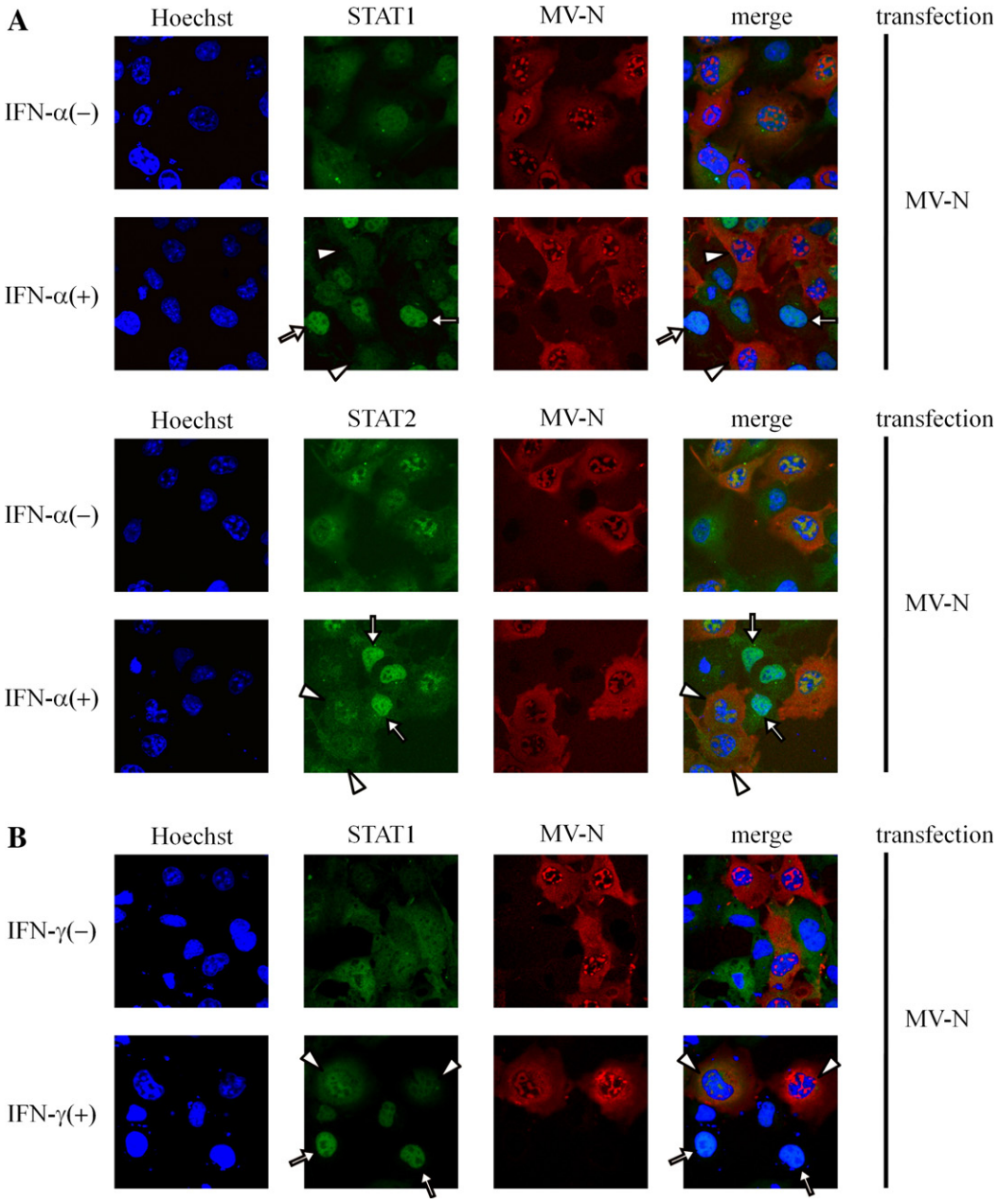

Fig. 5. MV-N prevents IFN-induced STAT nuclear accumulation. (A) COS-7 cells were transfected with pCAG-MV-N expression plasmid. 24 h later, the cells were treated with IFN-α at 2000 U/ml for 30 min, or not. (B) COS-7 cells were transfected with pCAG-MV-N expression plasmid. 24 h later, the cells were treated with IFN-γ at 1000 U/ml for 30 min, or not. They were fixed, permeabilized, and stained with monoclonal antibody against MV-N, and polyclonal antibody against STAT1 or STAT2. MV-N was visualized using Alexa Fluor 568 (red) and STAT using Alexa Fluor 488 (green) secondary antibodies. For clarity, arrowheads point to MV-N-expressing cells and arrows point to non-expressing cells. (C) The number of cells displaying mainly nuclear or homogenous (nuclear + cytosolic) protein distribution was determined by counting about 50 cells each in random microscopic fields.

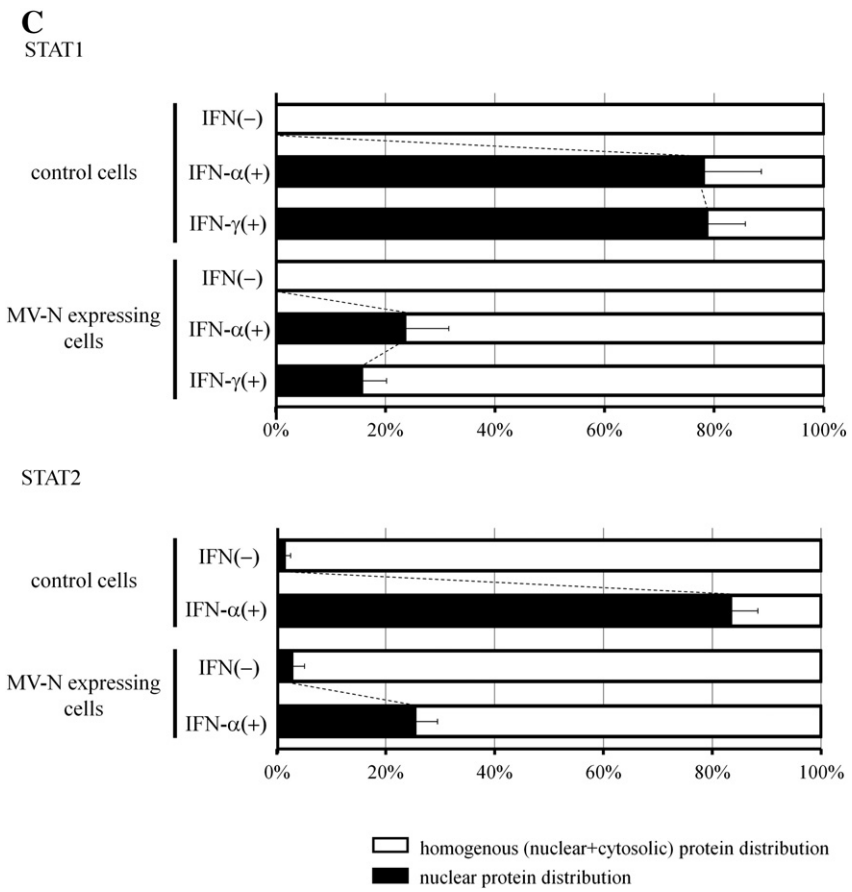

Fig. 5 (*continued*).

phosphorylation levels of detected proteins in Western blot by MV-N. Then we confirmed the experimental system using NiV-P expression cell. In the result, NiV-P blocked STAT1 phosphorylation in this experiment as previously reported (Shaw et al., 2004) (Fig. 3C). These results mean that the IFN-α/β or IFN-γ-dependent activations of STATs and Jaks' proteins are certainly not targeted by MV-N.

In the rubulavirus, it is known that V protein prevents IFN signaling by inducing STAT degradation (Didcock et al., 1999; Parisien et al., 2001, 2002; Ulane and Horvath, 2002; Ulane et al., 2003). Therefore, to determine if MV-N induces STAT degradation, total cell lysates were analyzed by Western blot probed with antibodies specific for STAT1 or STAT2. No reduction was observed in the levels of STAT1 and STAT2 proteins by MV-N expression (Figs. 3A and B). This result verifies that MV-N does not induce STAT protein degradation.

MV-N has no effect on the formation of pSTAT dimer

In SeV infected cells, both STAT1 and phospho-STAT1 (pSTAT1) are present in the form of aberrant high molecular weight complexes (HMWCs), not as a pSTAT1 homodimer or STAT1 monomer. HMWC formation is suggested to be linked to the blockade of IFN signaling to STAT1.

To determine whether the dimerization of pSTAT takes place normally in MV-N expression cells, native PAGE was performed, which would allow separation of the monomeric and dimeric forms (Shuai et al., 1994). STAT and pSTAT were visualized by Western blot analysis (Fig. 4A). The pSTAT1 and pSTAT2 heterodimers were detected in cells transfected with both empty vector and the MV-N expression plasmid after IFN-α stimulation. The other higher molecular weight complex was not detected. The pSTAT1 homodimer, known as GAF, was also detected in response to IFN-γ stimulation, and the other higher molecular weight complex was not detected (Fig. 4B). These results suggest that MV-N has no effect on the formation of STAT dimers.

MV-N prevents IFN-induced nuclear accumulation of STAT

To investigate the effects of MV-N on STAT intracellular distribution, indirect immunofluorescence was used to visualize the subcellular localization of MV-N and STATs. STAT1 or STAT2 was found in both the nucleus and cytoplasm in unstimulated cells, a result of balanced signal-independent basal nuclear shuttling (Marg et al., 2004; Meyer et al., 2002; Schindler et al., 1992) (Fig. 5A). MV-N was found in both the nucleus and the cytoplasm, as previously reported (Sato et al., 2006), and the MV-N expression did not alter the basal distribution of STATs (Fig. 5A). In response to IFN-α stimulation, STAT1 and STAT2 rapidly translocate to the nucleus and cells exhibit strong nuclear staining (Rodriguez et al., 2002; Schindler et al., 1992), as observed in control cells not expressing MV-N (Fig. 5A, arrow). However, in cells expressing MV-N, STAT1 and STAT2 failed to accumulate in nucleus after IFN-α treatment (Fig. 5A, arrowhead). Similarly, in the IFN-γ stimulated cells, STAT1 translocates to the nucleus (Fig. 5B, arrow), and MV-N also inhibits nuclear translocation of STAT1 in almost all cells (Fig. 5B, arrowhead). The subcellular locations of STAT1 and STAT2 in MV-N transfected and/or IFN stimulated cells were scored according to the cell distribution pattern as mainly in nuclear or homogenous (nuclear + cytosolic) (Fig. 5C). All cells without IFN stimulation showed a homogenous distribution pattern of STAT1 and almost all cells (95.8–98.0%) showed a homogenous distribution pattern of STAT2. After IFN-α stimulation, STAT1 translocated to the nucleus in 86.0% cells and STAT2 translocated to the nucleus in 78.0% cells. In MV-N

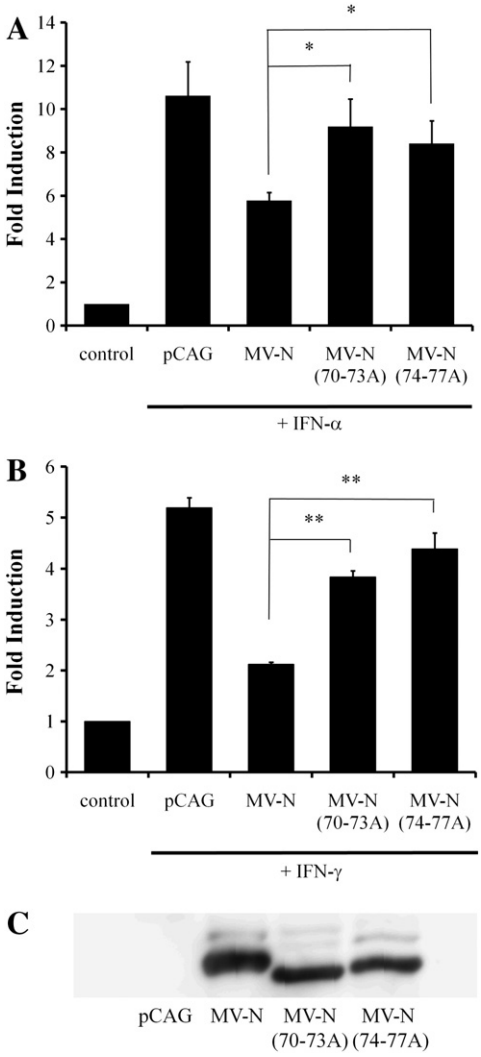

Fig. 6. The nucleocytoplasmic transport of MV-N is essential for IFN signaling inhibition. (A) HEK-293T cells were transfected with pISRE-Luc, phRL-TK(Int−) and either empty vector or an appropriate protein (MV-N(70–73A) and MV-N(74–77A)) expression plasmid. Cells were treated with 1000 U/ml IFN-α for 24 h prior to lysis and luciferase assays. (B) The same experiment was conducted using pGAS-Luc with 1000 U/ml IFN-γ. The results are expressed as induction (*n*-fold) relative to the value obtained from experiments without treatment. The data represent means ± SD for triplicate samples. Statistics were performed using the Student's *t*-test; $^{*}P<0.05$, $^{**}P<0.01$ compared to MV-N. (C) Western blotting for MV-N mutants using with A lysates is shown.

expressing cells, on the contrary, STAT1 translocated to the nucleus only in 10.9% cells and STAT2 translocated in 22.0%. After IFN-γ stimulation, STAT1 translocated to the nucleus in 90.0% cells. On the other hand, STAT1 translocated to the nucleus in 14.6% cells expressing MV-N (Fig. 5C). Previous reports have been indicated that STAT1–STAT2 heterodimer and STAT1 homodimer induced by IFN-α and IFN-γ, respectively, bind to importin α5, and are transported into nuclei (Fagerlund et al., 2002; McBride et al., 2002; Sekimoto et al., 1997). To analyze whether MV-N affects on the importin α5-mediated nuclear transport other than STATs, a cellular protein, Kir/GemW269E, which has been demonstrated to be transported into nucleus by importin α5 specifically (Mahalakshmi et al., 2007), was expressed together with or without MV-N. As a result, MV-N overexpression did not alter the intracellular localization of Kir/GemW269E (data not shown). These results suggested that MV-N does not interfere with general transport by importin α5, but inhibits IFN-α/β or IFN-γ signaling at the level of STAT nuclear translocation specifically.

The nucleocytoplasmic transport of MV-N is essential for IFN signaling inhibition

MV-V, a major IFN-antagonist, interacts with STAT1, and the interaction is considered to inhibit IFN signaling (Palosaari et al., 2003). A co-immunoprecipitation assay was performed to determine if MV-N interferes with the nucleocytoplasmic transport of STAT1 by direct binding with STAT1. We did not find any direct binding in this assay (data not shown).

Previous reports have indicated that morbillivirus N protein has an intrinsic ability to migrate into the nucleus when expressed in mammalian cells (Huber et al., 1991; Shin et al., 1997). Moreover, morbilliviruses are unique and are distinct from other paramyxoviruses in that intranuclear inclusion bodies are formed in infected tissues of patients (Griffin, 2001). These are accumulation of viral nucleocapsid particles, which are composed of N protein and the viral RNA genome, and are characteristic of late-stage morbillivirus infection (Huber et al., 1991; Koestner and Long, 1970; Oyanagi et al., 1971; Raine et al., 1969, 1971).

The nuclear localization signals (NLS) of MV-N were previously characterized at amino acid residues 70–77 (Sato et al., 2006). Nuclear transport of two mutants, MV-N(70–73A) and MV-N(74–77A), introduced by alanine substitution mutagenesis at positions 70–73 and 74–77, respectively, was markedly disrupted. To verify whether the nucleocytoplasmic transport of MV-N prevents activated-STAT nuclear accumulation, we performed IFN-α/β and IFN-γ luciferase reporter gene assays with these two mutants. The assay was performed as described in Fig. 1 with transfection of these two mutants instead of the MV-N expression plasmid. In the IFN-α/β reporter gene assay, the two mutants showed 79–87% luciferase activity, compared with transfection with an empty vector, and lost their IFN-α/β-antagonistic activity (Fig. 6A). Similarly, the two mutants lacked almost all abilities to inhibit IFN-γ-responsive reporter gene expression (74–85%) (Fig. 6B).

We confirmed this result by indirect immunofluorescence. MV-N(70–73A) and MV-N(74–77A) were found only in the cytoplasm, as previously reported (Sato et al., 2006). In cells treated with IFN-α the nuclear import of STAT1 and STAT2 was not disturbed by expression of MV-N(70–73A) (Fig. 7A, arrowhead). The same results were indicated in cells expressing MV-N(74–77A) (data not shown). In cells treated with IFN-γ, MV-N(70–73A) also did not disturb the nuclear import of STAT1 (Fig. 7B). MV-N(74–77A) showed a similar result (data not shown).

MV-P is known to possess ability to retain MV-N in cytoplasm in infected cells in vitro (Huber et al., 1991). Recent report demonstrated that MV-P mutant (Y110F, V112S, H115A) loses STAT1 antagonistic activity but maintains N–P interaction (Devaux et al., 2007). We confirmed that co-expression of the MV-P mutant retained MV-N in cytoplasm, and restored the interference of IFN-α/β and -γ responses induced by MV-N, as shown in Fig. 1, to 89% and 91%, respectively.

These data suggest that the nuclear transport of MV-N plays an important role in IFN signaling inhibition.

The N protein of other morbilliviruses also blocks IFN signaling

The N protein is the most conserved protein among viral proteins in morbilliviruses. In particular, the N-terminal regions of N protein (residues 1–420) demonstrate approximately 80% amino acid sequence similarity (Parks et al., 1992). A previous report has identified that the NLS of MV-N, CDV-N and RPV-N are located at the same position (Sato et al., 2006). We examined IFN-α/β and IFN-γ luciferase reporter assays with CDV-N and RPV-N. The assays were performed as described in Fig. 1. IFN-α/β and IFN-γ signaling were blocked to an equal level or even stronger than that of MV-N by the expression of CDV-N and RPV-N (Figs. 8A, B). Furthermore, we confirmed that CDV-N and RPV-N interfered with nuclear transport of STAT1 and STAT2, same as MV-N (data not shown). These results suggest that N protein of morbilliviruses has an intrinsic ability for the inhibition of IFN signaling.

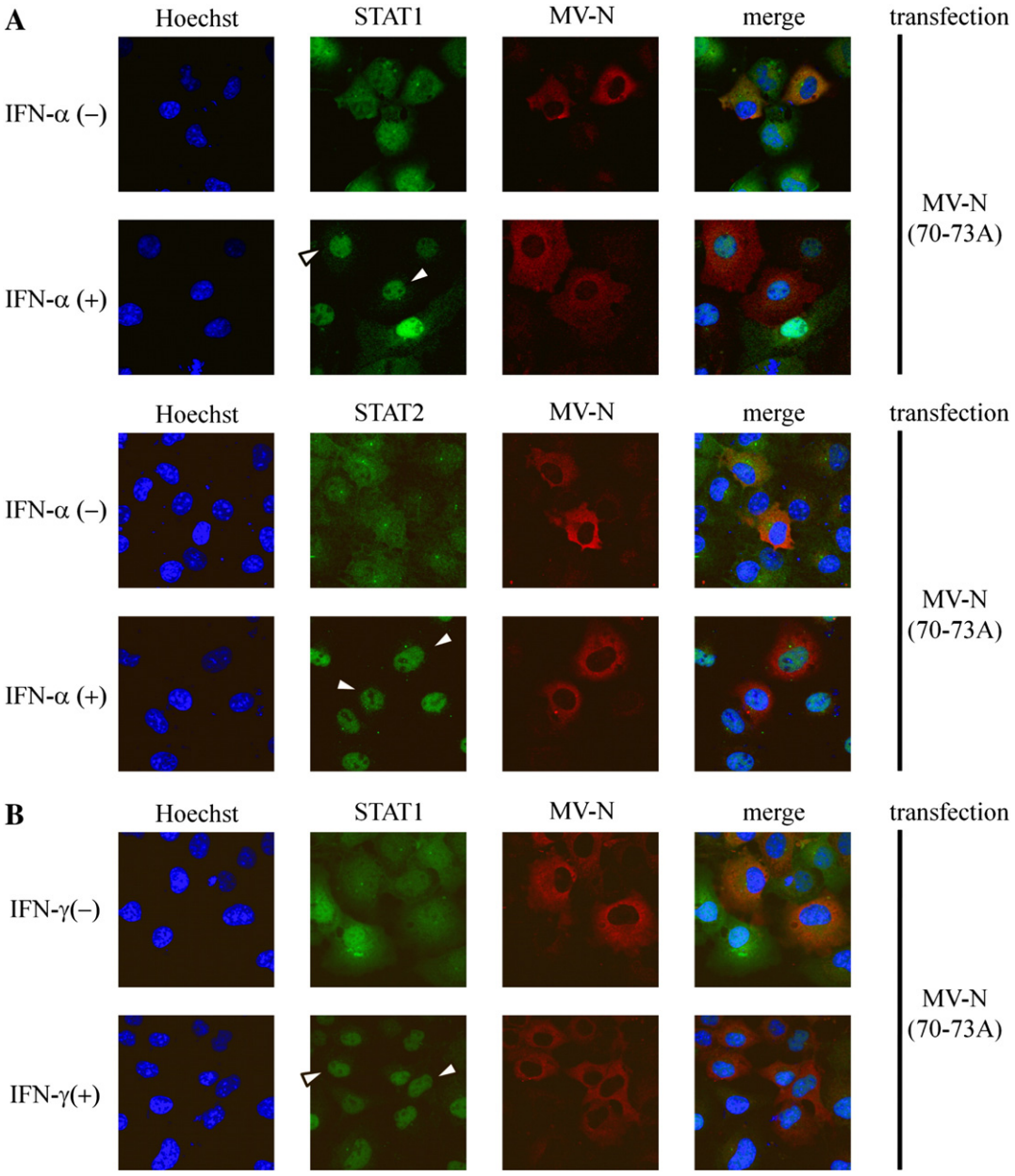

Fig. 7. MV-N mutants within NLS cannot inhibit the activated STAT nuclear accumulation. (A) COS-7 cells were transfected with pCAG-MV-N(70–73A) expression plasmid. 24 h later, the cells were treated with IFN-α at 2000 U/ml for 30 min, or not. (B) COS-7 cells were transfected with pCAG-MV-N(70–73A) expression plasmid. 24 h later, the cells were treated with IFN-γ at 1000 U/ml for 30 min or not. Cells were fixed, permeabilized, and stained with monoclonal antibody against MV-N and polyclonal antibody against STAT1. MV-N(70–73A) was visualized using Alexa Fluor 568 (red) and STAT using Alexa Fluor 488 (green) secondary antibodies. For clarity, arrowheads point to MV-N(70–73A)-expressing cells.

Discussion

Previous research relating to MV has revealed that the three proteins (V, P, and C) mediate evasion of the host immune response (Devaux et al., 2007; Fontana et al., 2008). It is agreed that V protein inhibits IFN-α/β signaling, but there are conflicting reports about the suppression of IFN-γ signaling by this protein (Palosaari et al., 2003; Takeuchi et al., 2003). In addition, Shaffer et al. demonstrated the inhibition of an IFN-α/β-responsive reporter gene by C protein (Shaffer et al., 2003); however, other studies reported that C protein has a minor role in the inhibition of IFN signaling (Nakatsu et al., 2006; Ohno et al., 2004; Takeuchi et al., 2005). In the present study, we indicated that the C protein of HL strain could inhibit IFN signaling. Interestingly, by an IFN-promoter activation assay, we found that N protein also has inhibitory activity on signal transduction in response to IFN, and to the same extent as that of C protein. Moreover, it inhibits not only IFN-α/β signaling but also IFN-γ signaling.

N protein is well conserved among the *Morbillivirus*, and MV has similar features and pathogenicity to CDV and RPV. Accordingly, we further confirmed IFN inhibition by CDV-N and RPV-N. Based on our experimental observations with CDV-N and RPV-N, it is possible to conclude that their inhibition of the IFN signaling pathway is characteristic of morbilliviruses (Fig. 8).

Upon infection, viral RNA rapidly induces the production of IFN-α/β and a response to IFN in infected cells. The MV V and C proteins are not associated with viral particles and expressed lower levels in the virus

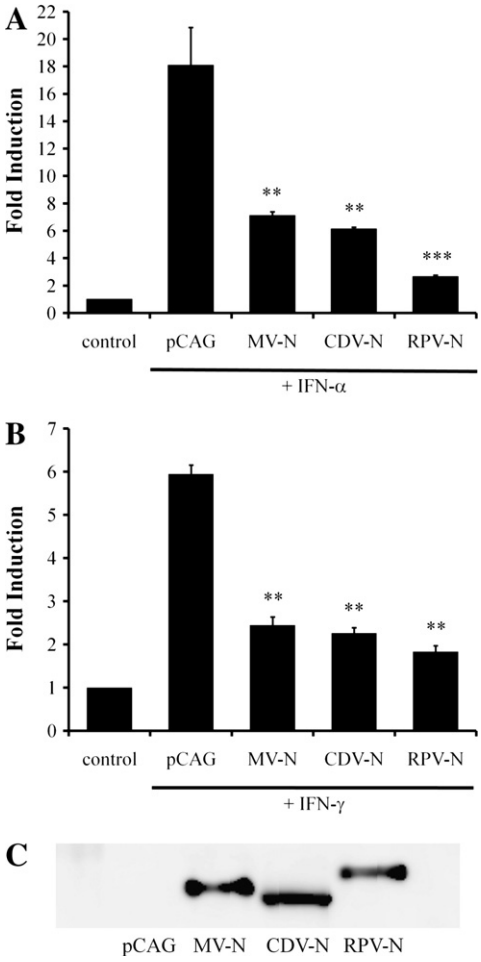

Fig. 8. The N proteins of other morbilliviruses also block IFN signaling. (A) HEK-293T cells were transfected with pISRE-Luc, phRL-TK(Int−), and either empty vector or an appropriate protein (MV-N, CDV-N and RPV-N) expression plasmid. Cells were treated with 1000 U/ml IFN-α for 24 h prior to lysis and luciferase assays. (B) The same experiment was conducted using pGAS-Luc with 1000 U/ml IFN-γ. The results are expressed as induction (*n*-fold) relative to the value obtained from experiments without treatment. The data represent means ± SD for triplicate samples. Statistics were performed using the Student's *t*-test; $^{**}P<0.01$, $^{***}P<0.001$ compared to pCAG. (C) Western blotting for viral proteins using with Fig. 8A lysates is shown.

infected cells. Meanwhile, N protein is the most abundant of the viral proteins and synthesized soon after infection. It is predicted that MV-N could disturb IFN response at an early-phase of infection.

We examined the MV-N inhibition mechanism step by step. MV-N does not prevent the activation of STATs and Jaks and does not induce the degradation of STATs (Figs. 3 and 4). In contrast, it blocks the nuclear import of activated STATs (Fig. 5). MV-N is known to possess NLS (Sato et al., 2006) and translocates to nucleus that has been often observed as intranuclear inclusion bodies in infected tissues of patients (Griffin, 2001). Mutations within NLS in N protein in the present study abrogated inhibition of IFN-α/β and IFN-γ signaling (Fig. 6) and the effect of MV-N mutants on IFN signaling was also confirmed using indirect immunofluorescence (Fig. 7). In addition, MV-P mutant devoid of STAT antagonist activity (Devaux et al., 2007) interfered with the inhibitory activity of MV-N on IFN-α/β and IFN-γ signaling (data not shown). Therefore, it is suggested that the nuclear translocation of MV-N disturbs the nuclear import of activated STATs. The mechanism of the inhibitory effect of MV-N on the nuclear import is unknown. Our investigation (data not shown) and previous report of RPV (Nanda and Baron, 2006) have demonstrated that N protein showed no interaction with STAT1. MV-N inhibits STAT1 nuclear import without direct interaction with STAT1. Thus, some host factor should be implicated in the inhibitory effect of MV-N on STAT-translocation into nuclei. The NLS of STAT1 was identified, and the sequence is nonclassical NLS. An importin α5 mediates nuclear transport of STATs (Fagerlund et al., 2002; McBride et al., 2002; Sekimoto et al., 1997). We analyzed whether MV-N affects on the other importin α5-mediated nuclear transport. As a result, MV-N overexpression did not alter the intracellular localization of Kir/GemW269E, which is transported by importin α5 (data not shown). Furthermore, we confirmed whether MV-N interacts with importin family by co-immunoprecipitation assay, but specific interaction has not been observed (data not shown). These results suggest that MV-N does not interfere with general transport by importin α5, but targets nuclear translocation of pSTAT specifically. We are investigating how MV-N inhibits pSTAT nuclear transport. Further experiments will determine the mechanisms of the inhibition.

Previous studies indicate that MV-N binds to various host proteins and is concerned in MV pathogenicity. It has been demonstrated that MV-N binds to the receptor for the Fc protein of IgG, Fc-γ receptor type II (FcγRII, CD32). This interaction inhibits human B cell antibody production in vitro (Ravanel et al., 1997) and induces apoptosis in cell lines (Laine et al., 2005). In addition, this interaction reduces the production of IL-12 by dendritic cells and induces a loss of antigen-specific T-cell proliferation ex vivo (Marie et al., 2001). MV-N is also reported to interact with interferon regulatory factor (IRF)-3. This interaction was described as leading to the phosphorylation-dependent activation of IRF-3, and the ensuing activation of the pro-immune cytokine RANTES gene (tenOever et al., 2002). Thus, it has been suggested that MV-N plays various roles in the virus-induced immune response.

Furthermore, the C-terminal part of MV-N binds to the 70-kDa heat-shock protein (HSP70), which is a cytosolic protein inducible by heat shock (Zhang et al., 2002). In vitro studies have shown that elevated cellular levels of HSP70 are associated with increased gene expression of MV and CDV via binding to N protein (Carsillo et al., 2006; Oglesbee et al., 1990, 1993; Vasconcelos et al., 1998a,b). A recent study showed that MV-N binds to the p40 subunit of eukaryotic initiation factor 3 (eIF3-p40). MV-N contributes to shut off host translation in MV-infected cells through protein–protein interaction with eIF3 (Sato et al., 2007). These studies indicate that MV-N has multiple functions in various host systems.

In conclusion, our present data indicates that the N protein has a crucial role in interfering with the host immune system. In the context of viral infection, evasion of the IFN response is complicated, and may involve interplay between multiple viral and host proteins. The results presented here give insight into understanding how the viral proteins of MV function as inhibitors of host IFN signaling.

Materials and methods

Cells

HEK-293T, HEK-293 (human embryonic kidney) and COS-7 (African green monkey kidney) cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin G and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

Plasmids

Construction of the mammalian expression plasmids encoding the MV-HL N gene (pCAG-MV-N), P (Hagiwara et al., 2008) and V (Sato et al., 2008) was described previously. To construct expression plasmids encoding the MV C and M proteins, cDNAs were amplified by PCR (Phusion; Finnzymes, Espoo, Finland) from pMV-HL (7+) encoding the full-length genome cDNA of MV-HL (Terao-Muto et al., 2008), with specific primers matching the ORF extremities. The cDNA fragments were blunted with T4 DNA polymerase (Takara, Shiga, Japan) and ligated into the pCAGGS mammalian expression vector (Niwa et

al., 1991). Construction of the two mutant MV-N plasmids (pCAG-MV-N(70-73A) and pCAG-MV-N(74-77A)) was described previously (Sato et al., 2006). To generate the N protein of the CDV (CDV-N) expression plasmid, cDNA was amplified by PCR from pCDV encoding the full-length genome cDNA of CDV-Yanaka (Fujita et al., 2007) with specific primers matching the ORF extremities, and the blunted cDNA fragment was subcloned in the pCAGGS. To generate the N protein of RPV (RPV-N) expression plasmid, the cDNA coding for the protein was obtained by PCR amplification of the full-length genome cDNA of RPV-L (Yoneda et al., 2004) with specific primers matching the ORF extremities, and the blunted cDNA fragment was ligated into the pCAGGS. To generate the P protein of the NiV (NiV-P) expression plasmid, the cDNA coding for the protein was ligated into the pCAGGS (Yoneda et al., 2010).

The IFN-α-inducible reporter plasmid pISRE-Luc, containing ISRE, was obtained from Clontech (Mountain View, CA, USA). To construct the IFN-γ-inducible reporter plasmid pGAS-Luc, three copies of the GAS from human IRF-1 gene, 5′-AGCCTGATTTCCCCGAAATGA-3′, were inserted in the MluI and BglII sites upstream of luciferase gene of the pGL3-Promoter reporter plasmid (Promega, Madison, WI, USA). The plasmid phRL-TK(Int−) (Promega) encodes the *Renilla* luciferase gene constitutively expressed from a thymidine kinase promoter, and was used as an internal control.

Antibodies

Mouse monoclonal antibody 8G (Masuda et al., 2006) recognizing MV-N was used for the immunofluorescence assay. Rabbit polyclonal antibody against MV-N (Hagiwara et al., 2008) was used to detect MV-N, CDV-N and RPV-N for Western blot analysis. Rabbit polyclonal antibody was used for detecting MV-V (unpublished data). Rabbit polyclonal antibody against STAT1 p84/p91 (E-23), STAT2 (C-20) and rabbit anti-phospho-STAT2 (Tyr690) were purchased from Santa Cruz (Santa Cruz, CA, USA). Rabbit anti-phospho-STAT1 (Tyr701) was obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-phospho-Jak1 (Tyr1022/1023) was obtained from Invitrogen. Rabbit anti-phospho-Jak2 (Tyr1007/1008) and mouse monoclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Millipore (Billerica, MA, USA). The horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (Dako, Glostrup, Denmark) was used as secondary antibodies for Western blot analysis. Alexa Fluor 568-conjugated goat anti-mouse IgG (H + L) antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) antibody were obtained from Invitrogen for the immunofluorescence assay.

Luciferase reporter gene assay

HEK-293T cells (1×10^5 per well) were plated in 24-well plates. One day later, they were transfected with pISRE-Luc (0.2 μg/well) or pGAS-Luc (0.004 μg/well), phRL-TK(Int−) reference plasmid (0.001 μg/well) and empty pCAGGS vector, or pCAGGS encoding the appropriate protein (0.6 μg/well or the indicated amounts in the legend to Fig. 2.). Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. The medium was changed 24 h after transfection, and universal type I IFN (PBL InterferonSource, Piscataway, NJ, USA) or recombinant human IFN-γ (Sigma) was added to the medium at 1000 U/ml. After a further 24 h of incubation, luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) following the manufacturer's protocol. Each transfection experiment was conducted in triplicate. Reporter activity was calculated as the ratio of firefly luciferase activity to the reference *Renilla* luciferase activity. The results are expressed as fold induction, which is the value relative to the value of control experiments without IFN treatment. A two-tailed Student's *t*-test was used for two-group comparisons. To confirm the gene expressions, Western blot was performed using the cell lysates.

Western blot analysis

HEK-293 or HEK-293T cells (7×10^5 per well) were plated in 6-well plates. One day later, they were transfected with empty pCAGGS vector, pCAG-MV-N plasmid or pCAG-NiV-P plasmid (1 μg/well) using FuGENE 6 (Roche Diagnostics, Mannheim, Germany). Cells were treated with 1000 U/ml universal type I IFN or IFN-γ 24 h post transfection, for 10, 20, or 30 min. The cells were lysed with cell lysis buffer (20 mM HEPES-NaOH (pH 7.5), 1% Nonidet P-40, 120 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 1 mM NaF) and the lysates were centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatants were recovered, and protein concentration was determined by BCA protein assay (Pierce, Rockford, IL, USA). The proteins were separated by 5%, 7% or 10% SDS-PAGE, and transferred to a PVDF membrane (Immobilon-P; Millopore). The membrane was blocked in 4% BlockAce (Dainihon Pharmaceutics, Osaka, Japan) in phosphate-buffered saline (PBS) or 5% bovine serum albumin (BSA) (Sigma) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature. After overnight incubation at 4 °C with a primary antibody, the membranes were washed in TBST for 15 min and incubated at room temperature with a secondary antibody for 1 h. The membranes were washed in TBST for 15 min and detected using ECL or ECL Plus Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). The chemiluminescent signals were detected and visualized using a LAS-1000 mini (Fujifilm, Tokyo, Japan).

Nondenaturing polyacrylamide gel analysis

HEK-293 or HEK-293T cells grown in 60-mm dishes to approximately 80% confluence were transfected with empty pCAGGS vector or with pCAG-MV-N plasmid using FuGENE 6. At 24 h post transfection, cells were treated with 1000 U/ml universal type I IFN or IFN-γ for 6 h. The cells were lysed as mentioned above. The extracts were separated on 8% polyacrylamide gel in Tris–Glycine Native running buffer, and then transferred to a PVDF membrane with transfer buffer for native PAGE (48 mM Tris, 39 mM glycine, 20% methanol, 0.1% SDS). The membrane was blocked and immunoblotted as described above.

Immunofluorescence

COS-7 cells (4×10^4 per well) were seeded on coverslips on 24-well plates and transfected with empty vector or pCAGGS encoding the appropriate protein (0.2 μg/well) using FuGENE 6. At 24 h post transfection, cells were treated with 2000 U/ml universal type I IFN or 1000 U/ml IFN-γ for 30 min at 37 °C. Transfected cells were washed three times with ice-cold PBS, fixed in 4% paraformaldehyde for 30 min, permeabilized for 4 min with PBS containing 0.1% Triton X-100, and blocked for 30 min in PBS containing 2% BSA and 0.1% Triton X-100. The cells were then incubated overnight with a primary antibody against STAT1 or STAT2 and a monoclonal antibody against MV-N in PBS containing 2% BSA and 0.1% Triton X-100 at 4 °C, before being washed in PBS. They were subsequently incubated with an appropriate secondary antibody for 1 h at room temperature. After three washes in TBST, the fluorescent signals were visualized by confocal laser microscopy (Olympus, Tokyo, Japan). Hoechst 33342 (Lonza, Basel, Switzerland) was used as a nuclear stain. The subcellular localization of STAT1 and STAT2 was divided into two groups, displaying mainly nuclear or homogenous (nuclear + cytosolic). The number of cells was determined by counting about 50 cells each in random microscopic fields. Each experiment was conducted in triplicate.

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