Importance of the Anti-Interferon Capacity of Sendai Virus C Protein for Pathogenicity in Mice

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Received 22 November 2006/Accepted 2 January 2007

Sendai virus (SeV) belongs to the genus Respirovirus of the subfamily Paramyxovirinae; it exclusively infects respiratory epithelial cells of rodents and causes fatal bronchopneumonia. Experimental infection of mice with SeV is frequently used as a model of viral pathogenesis of respiratory disease (25, 31, 37). SeV is an enveloped virus with a linear, nonsegmented, negative-sense RNA genome of 15,384 nucleotides and contains 10 genes in the order 3-(leader)-N-(trailer)-5. SeV is a member of the family Respiroviridae, subfamily Paramyxovirinae, and belongs to the genus Respirovirus. The unique C-terminal region of V proteins contains seven cysteine residues that are highly conserved among paramyxoviruses, forms zinc finger-like motifs, and binds Zn2+ (22, 39, 49, 56) and also binds one of the intracellular RNA sensor molecules, MDA5 (1, 59). The W protein is potentially synthesized by viruses which produce an anti-IFN capacity. Therefore, the SeV P, V, and W proteins are initiated at the AUG codon 316, whereas four initiation sites at positions 81, 16, 50). Translation of the C protein gives rise to multiple protein species by a process known as RNA editing and by the use of an overlapping open reading frame. The P protein is translated from the unedited mRNA, which is the exact copy of the P gene. The V protein is translated from the edited mRNA, in which one nontemplated G residue is cotranscriptionally inserted to the editing position. Among transcripts from the P gene, one G is inserted into approximately 20% of transcripts. Less frequently (≤5%), two G residues are inserted and the W protein is synthesized (25). Therefore, the N-terminal 316 amino acids of the P, V, and W proteins produced from the coding region before the editing site are in common, and their C termini are unique (38). The V protein is expressed by members of viruses in the subfamily Paramyxovirinae, except for some viruses belonging to the genus Respirovirus. The unique C-terminal region of V proteins contains seven cysteine residues that are highly conserved among paramyxoviruses, forms zinc finger-like motifs, and binds Zn2+ (22, 39, 49, 56) and also binds one of the intracellular RNA sensor molecules, MDA5 (1, 59). The W protein is potentially synthesized by viruses which produce an anti-IFN capacity. Therefore, the SeV P, V, and W proteins are initiated at the AUG codon 316, whereas four initiation sites at positions 81, 16, 50). Translation of the C protein gives rise to multiple protein species by a process known as RNA editing and by the use of an overlapping open reading frame. The P protein is translated from the unedited mRNA, which is the exact copy of the P gene. The V protein is translated from the edited mRNA, in which one nontemplated G residue is cotranscriptionally inserted to the editing position. Among transcripts from the P gene, one G is inserted into approximately 20% of transcripts. Less frequently (≤5%), two G residues are inserted and the W protein is synthesized (25). Therefore, the N-terminal 316 amino acids of the P, V, and W proteins produced from the coding region before the editing site are in common, and their C termini are unique (38). The V protein is expressed by members of viruses in the subfamily Paramyxovirinae, except for some viruses belonging to the genus Respirovirus. The unique C-terminal region of V proteins contains seven cysteine residues that are highly conserved among paramyxoviruses, forms zinc finger-like motifs, and binds Zn2+ (22, 39, 49, 56) and also binds one of the intracellular RNA sensor molecules, MDA5 (1, 59). The W protein is potentially synthesized by viruses which produce an anti-IFN capacity. Therefore, the SeV P, V, and W proteins are initiated at the AUG codon 316, whereas four initiation sites at positions 81, 16, 50). Translation of the C protein gives rise to multiple protein species by a process known as RNA editing and by the use of an overlapping open reading frame. The P protein is translated from the unedited mRNA, which is the exact copy of the P gene. The V protein is translated from the edited mRNA, in which one nontemplated G residue is cotranscriptionally inserted to the editing position. Among transcripts from the P gene, one G is inserted into approximately 20% of transcripts. Less frequently (≤5%), two G residues are inserted and the W protein is synthesized (25). Therefore, the N-terminal 316 amino acids of the P, V, and W proteins produced from the coding region before the editing site are in common, and their C termini are unique (38). The V protein is expressed by members of viruses in the subfamily Paramyxovirinae, except for some viruses belonging to the genus Respirovirus. The unique C-terminal region of V proteins contains seven cysteine residues that are...
P proteins have been shown to counteract IFNs (48, 53). Even though the V proteins have a conserved C terminus, the target molecule and the manner of IFN antagonism are not the same. For example, the V proteins of mumps virus, SV5, and NDV degrade signal transducer and activator of transcription 1 (STAT1) (4, 21, 52), but that of hPIV2 degrades STAT2 (45). STAT1 and STAT2 play a key role in the IFN signal transduction (JAK-STAT) pathway. The antiviral state of IFN is not induced without STAT1 or STAT2. The V proteins of measles virus and Nipah virus degrade neither STAT1 nor STAT2 but block the IFN signaling pathway by inhibiting the tyrosine phosphorylation or translocation of STAT1 and STAT2 (53, 57). Moreover, the V protein of SeV is not involved in inhibiting the JAK-STAT pathway (29). These differing actions of V protein suggest that the conserved sequence in the C terminus of V protein has another original role besides IFN antagonism and that the anti-IFN role was likely acquired after the paramyxoviruses differentiate into the five genera Respirovirus, Morbillivirus, Henipavirus, Avulavirus, and Rubulavirus (13, 44).

SeV C-, Y1-, or Y2-expressing cell lines are all capable of circumventing the activation of IFN-stimulated genes and induction of an antiviral state by IFN-α/β and IFN-γ (27). These observations suggest that IFN signaling is blocked by SeV C', C, Y1, or Y2 protein without the aid of other viral proteins and that the SeV C', C, Y1, and Y2 proteins are indistinguishable in regard to anti-IFN capacity. Analysis of stable transformants expressing various truncations from the N and C termini and of those expressing mutated C proteins in which some charged amino acids were replaced by alanine indicate that the C-terminal half of C protein was sufficient for IFN antagonism, and substitution of amino acids at positions 151, 153, and 154 of the C protein critically eliminates anti-IFN capacity (28, 29).

Along with other groups, we found that SeV C proteins participate in the blocking of IFN signaling through the inhibition of tyrosine phosphorylation of STAT2 and tyrosine dephosphorylation of phosphorylated STAT1 (14, 33, 54). These inhibitions were found to come not from the formation of the aggregate composed of the C protein, STAT1, and STAT2, as proposed before (9, 58), but from another, unidentified mechanism (29).

In a previous study, we demonstrated IFN antagonism of the SeV C protein by using the cloned gene (28, 29). However, the use of the cloned single gene did not show how much IFN antagonism is required for viruses to propagate in the cells in vitro and in vivo. A spontaneous SeV mutant, Ohita-MVC11, isolated from the virulent Ohita-M1 strain was no longer virulent for mice, which was shown to be caused mainly by a change from phenylalanine to serine at 170 (F170S) of the C protein (23). Based on the notion that the cloned SeV Ohita-MVC11 C protein lost IFN antagonism, a recombinant SeV constructed with the Z strain backbone and C reading frame of the Ohita-MVC11 strain was created and confirmed to be an avirulent virus (8). This was the first known finding to indicate the importance of IFN antagonism in the viral life cycle. However, the recombinant SeV cannot be compared with parental SeV, because this manipulation not only replaces the C reading frame but also changes the overlapped P, V, and W sequences. In addition, SeV C protein is a multifunctional protein; therefore, it is possible that one amino acid change can have multiple effects (18). In fact, the F170S substitution in SeV C is known to modify more than one function associated with the C protein (9, 10). The pinpoint disruption of anti-IFN capacity is thus required to evaluate the role of IFN antagonism in the viral life cycle.

In this study, we created a mutant SeV lacking the anti-IFN capacity of the C protein by introducing three amino acid substitutions which were not critical for other functions associated with the SeV C protein (29), and we attempted to determine the physiological role of IFN antagonism in the context of the viral life cycle. The intranasally inoculated mutant SeV Cm* could propagate in the lungs of STAT1−/− mice but was cleared from those of STAT1−/− mice. We found that the anti-IFN capacity of SeV C protein was crucial for multiplication in wild-type mice in vivo. The results conversely indicated how powerfully the innate immunity contributes to eliminating SeV in the early stages of infection.

MATERIALS AND METHODS

Plasmids and stable transformants. A plasmid, pKS-C, coding for the SeV C protein (27) was used as the PCR template to obtain two DNA fragments encoding the mutated C proteins. Briefly, the 5′ and 3′ halves of the C gene were individually amplified. The CF (5′-GAATTCCTCGAGATAGTTCTCA TTCTTAAGA-3′) and CMRI (5′-GTCGACGATGCGCATCGTCTTTCC-3′) primers were used for amplifying the 5′-half fragment, while the CmF1 (5′-GAGGACGGATCCATGCGTCTTTCC-3′) and CR (5′-GAATTCCTCGAGATAGTTCTCA TTCTTAAGA-3′) primers were used for the 3′-half fragment. The position of the primer on the P gene was numbered from the start site of the P mRNA. Each outer primer for the 5′- and 3′-half fragments contained the initiation and termination codons (underlined) for C protein. Three nucleotides (italicized) upstream of the initiation codon were modified to optimize for the translation according to Kozak’s rule as described previously (27). Mutations designed to change amino acids in the C reading frame but not in the P, V, and W reading frames were introduced in the inner primers (lowercase letters). Two amplified fragments, CF-CmRI and CMRI-CR, were purified, mixed, and further connected at the overlapping site by PCR using both termini of outer primers CF and CR. A fragment encoding the mutated C protein (Cm*) was thus created. Likewise, two inner primers, CmR2 (5′-GTCGACGATGCGCATCGTCTTTCC-3′) and CmF2 (5′-GAGGACGGATCCATGCGTCTTTCC-3′), were used instead of the CmR1 and CMRI-F1 primers. A fragment encoding the second mutated C protein (Cm**) was created. These two fragments were cut with HindIII and BamHI (as indicated by superscripts in the outer primer sequences) and were cloned into the same sites of plasmid pKS336 (GenBank accession number AF407373) to obtain plasmids pKS-Cm* and pKS-Cm**, respectively. The Cm* protein is designed to have three amino acid substitutions, K151A, E153K, and R157L, and the Cm** protein has K151A and E153K. After verification of sequences, these plasmids were used to establish stable transformants as described previously (27).

The plasmid pSeV(+) producing an SeV antigenome (24), was used to generate the wild-type SeV. The C protein mutation was introduced into pSeV(+) by replacing the 672-bp fragment of SaII–EcoRI–EcoRI within the wild-type P gene by the mutated gene encoding the Cm* protein. Thus, the plasmid pSeV Cm*(+)(2) was constructed.

Cells and viruses. 2TGH cells and STAT1-deficient deliberate U3A cells were kindly provided by G. Stark (Cleveland Center for Structural Biology, OH). Human-derived cell lines (HeLa, 2fTGH, and U3A) and monkey-derived cell lines (LLCMK2 and CV1) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Recombinant wild-type SeV strain Z (SeV Wt) and the mutant SeV Cm* were recovered from cDNAs according to the general protocol (24) and were used in the present study. Viruses were propagated in 10-day-old embryonated chicken eggs. Virus titer was measured by an immunofluorescent cell-counting assay using LLCMK2 cells and expressed as cell-infecting units (CIU) per ml (31) or was measured by plaque assays using CV1 cells. For the plaque assay, cells were overlaid with 0.5% agarose in DMEM containing 7.5 μg/ml trypsin and were then fixed at 3 days postinfection with 20% ethanol and 10% acetic acid. Plaques were visualized with 0.5% amido black 10B. VSV New Jersey strain grown in chicken embryo fibroblast was used as a challenge virus to measure antiviral activity of IFN.
Infection of cells and animals. 2FTGH and STAT1-deficient U3A cells plated in six-well plates at 2 × 10⁵ cells per well were infected with either SeV Wt or SeV Cm* at a multiplicity of infection (MOI) of 5. After 1 h of virus adsorption, plates were washed once with phosphate-buffered saline and filled with 1 ml per well of serum-free DMEM. Cells were harvested every 6 h from 0 to 30 h postinfection.

Five-week-old male STAT1+/− 129S6 mice (002045-M; Taconic Farms Inc., Hudson, NY) and their parental STAT1+/+ 129S6 mice (129SVE; Taconic Farms Inc.) were used for infection. All mice were specific pathogen free and were kept under bioclean and regulated conditions in the biosafety level 3 facility at the Institute of Laboratory Animal Science, Division for Research Support, Life Science Center, Hiroshima University, Japan. Each mouse was infected intranasally with a 25-μl inoculum containing 10⁷ CIU of virus under anesthesia after inhalation of ether and/or intraperitoneal injection of Nembutal. Infected mice were checked daily for body weight and clinical signs, and some were sacrificed at intervals for investigation of lung consolidation, which was graded from 1 to 4 according to the extent of macroscopic lung lesion; one point was added when the mouse died (25). Virus replication in the mouse lung was determined according to a method described previously (31). Briefly, homogenates of the mouse lung were made in 1 ml of DMEM per lung with the aid of a glass homogenizer on ice. Homogenates were then centrifuged at 1,500 g for 20 min, and the supernatants were used for assay of infectivity.

The lack of STAT1 in U3A cells and STAT1+/− mice was confirmed by Western blotting using anti-STAT1 antibody. Quantities of IFN-β secreted into the cell culture medium were measured by enzyme-linked immunosorbent assay (ELISA), using a human IFN-β ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

Western blotting analysis. Cytoplasmic cell extracts in radioimmuno precipitation assay buffer were run through 4 to 12% acrylamide/bis-Tris gels (NuPAGE; Invitrogen Corp., Carlsbad, CA) and electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Membranes were blocked for 1 h in 3% skim milk in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 and probed with anti-STAT1 antibody (sc-839; Santa Cruz Biotechnology). Viral proteins synthesized in the cells were probed with anti-SeV serum, which was kindly supplied by B. Gotoh (Shiga School of Medicine, Shiga, Japan). The reacted bands on the membranes were detected by ECL nitrocellulose membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

RESULTS

Anti-interferon activity of SeV C protein. SeV C protein-expressing cells cannot establish an antiviral state due to the lack of gene expression induced by type I IFN (27). The anti-IFN capacity of C protein can be eliminated by introducing mutations in the C gene. A spontaneously occurring F170S substitution in the C protein is known to abolish anti-IFN capacity (8). In a previous study, we introduced substitutions from charged amino acids to alanine at several points in the C protein and found that the C protein with K151A, E153A, and R154A substitutions (named Cm5) no longer antagonized IFN (29). It is important to evaluate how much of an advantage the anti-IFN capacity of virus has for the viral life cycle. Therefore, we attempted to generate SeV without anti-IFN capacity and compare it with the parental SeV Wt.

Introduction of K151A, E153A, and R154A substitutions in the C protein also alters the amino acid sequences of the P, V, and W proteins, because they are produced from a different reading frame of the P gene. To evaluate the anti-IFN capacity of the C protein, all SeV proteins except the C protein must be the same. Therefore, two similar mutants were designed without changing the amino acid sequences of the P, V, and W proteins, one producing a C protein with K151A, E153K, and R154L substitutions and another producing a C protein with K151A and E153K substitutions (named Cm* and Cm**, respectively).

Prior to making the recombinant SeVs, the anti-IFN capacities of the Cm* and Cm** proteins were measured in the cells. HeLa cells stably expressing Cm* or Cm** protein were treated with IFN-β at 0, 10, 10², and 10³ IU/ml and were then challenged with VSV (Fig. 1). HeLa cells transformed by the empty plasmid, pKS-336, tolerated the VSV challenge partially at 10² and completely at 10³ IU/ml of IFN pretreatment but...
not at all at lower doses. However, HeLa cells expressing the wild-type C protein detached from the culture wells at any dose of IFN tested. Yet, HeLa cells expressing the mutant C protein with K151A, E153A, and R154A (named Cm5) tolerated the VSV challenge at 10^2 and 10^3 IU/ml of IFN. The extent of cell detachment at 10^2 IU/ml of IFN differed as a result of experimental fluctuations, but IFN antagonism was significant for Cm5-expressing cells compared to C-expressing cells. These results show that the anti-IFN capacity of C protein was eliminated by substitution of three charged amino acids with alanine. Under these conditions, HeLa cells expressing Cm** protein with K151A and E153K detached from the culture wells irrespective of IFN concentration upon VSV challenge, as observed in cells expressing the wild-type C protein. Substitutions in Cm** were thus found to be insufficient to eliminate anti-IFN activity. HeLa cells expressing Cm* protein with K151A, E153K, and R157L, however, could tolerate the VSV challenge at 10^3 IU/ml of IFN, as observed for the HeLa cells expressing none of the C protein. This indicates that substitutions in Cm* were sufficient to eliminate anti-IFN activity (Fig. 1).

**Generation of recombinant SeV Cm*.** Since the Cm* protein was found to lose anti-IFN capacity, the same mutation was introduced into the plasmid generating the antigenome of SeV. A recombinant virus named SeV Cm* was generated from the mutated plasmid according to an established method (24). The mutation of SeV Cm* was confirmed by sequencing the reverse transcription-PCR product of the P gene (data not shown). Immunoblot analysis using antiserum against the P and V common region showed that the P and V proteins, both encoded by the P gene, were expressed in the parental SeV Wt- and mutant SeV Cm*-infected LLCMK2 cells. No electrophoretic difference in the mobility of proteins was observed between the two viruses. However, the C protein of SeV Cm* detected with specific antiserum was found to move faster than that of SeV Wt in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, likely due to the introduced mutation (Fig. 2A). A quantitative difference in the synthesized P protein in the cells was observed between SeV Wt- and Cm*-infected cells. Next, the plaque morphologies of the parent SeV Wt and mutant SeV Cm* were compared in CV1 cells. The sizes of plaques were not significantly different for the two viruses. The plaques of SeV Cm*, however, were unclear compared to those of SeV Wt, indicating a weaker cytopathic effect of SeV Cm* than SeV Wt (Fig. 2B).

**Confirmation of impaired anti-IFN activity of SeV Cm*.** Treatment of cells with IFN-β before VSV infection prevented the expression of viral proteins in a dose-dependent manner, due to the induction of the antiviral state of IFN (Fig. 3). This antiviral activity of IFN was no longer observed if SeV Wt was inoculated into cells prior to IFN treatment. SeV Wt infection inhibited induction of the antiviral state and allowed VSV to grow in cells. This result shows the anti-IFN activity of SeV. Mutant SeV Cm* infection, however, did not allow VSV to grow in cells (Fig. 3). The wild-type and mutant SeVs were inoculated at an MOI of 1 per cell and grew to almost the same levels at the time point just before the VSV challenge (data not shown). This result suggested that SeV Cm* has no capacity to counteract the antiviral effect of IFN. Interestingly, VSV proteins were not expressed in cells infected with SeV Cm* at any

![FIG. 2. Mutant SeV Cm* created from the plasmid.](http://jvi.asm.org/)

![FIG. 3. IFN antagonism of mutant SeV Cm*.](http://jvi.asm.org/)
dose of IFN-β or in the absence of IFN. This could be explained by the fact that SeV Cm* itself induced IFN and required no extra IFN to repress the expression of postinoculated VSV. Impaired anti-IFN capacity of SeV Cm* was thus confirmed.

Role of anti-IFN activity in viral multiplication in cultured cells. As shown by the results described above, SeV without anti-IFN capacity could replicate in cultured cells, indicating that the anti-IFN capacity was not essential for SeV to replicate in cells. It is, however, important to evaluate the extent to which the anti-IFN capacity of SeV contributes to the viral life cycle. First, we inoculated SeV Wt and SeV Cm* into human 2fTGH cells at an MOI of 5 and compared the viral growth. The amount of viral proteins expressed in the cells was lower in SeV Cm*-infected cells at any time postinoculation (p.i.) (Fig. 4). In agreement with this result, virus titers in the culture supernatants were lower in SeV Cm*-infected cells than in SeV Wt-infected cells. The peak titer of SeV Cm* was found to be approximately 1/20 of that of SeV Wt. IFN-β was detected in culture supernatants of both SeV Wt- and SeV Cm*-infected cells. Larger amounts of IFN-β were secreted in SeV Cm*-infected cells at 18 and 24 h p.i., but at 30 h p.i. almost the same amount was secreted. In relation to the amount of IFN production, IFN-inducible STAT1 and STAT2 proteins in SeV Cm*-infected cells were found to increase from 6 h and remained at high levels from 18 to 30 h p.i., whereas in SeV Wt-infected cells STAT1 and STAT2 proteins stayed at the initial level from 0 to 24 h p.i. Yet, in the late stage of infection (30 h p.i.), STAT1 and STAT2 proteins were produced even in the SeV Wt-infected cells. At the same time, SeV C protein was no longer abundant in the cells, indicating that anti-IFN is related to the intracellular amount of C protein.

However, further investigation was required to confirm that, for SeV Cm*, the impaired growth is due to the lack of anti-IFN capacity. Therefore, 2fTGH-derived STAT1-deficient U3A cells were inoculated with SeV Wt or SeV Cm*. STAT1 protein was not detected in U3A cells by the immunoblot assay (Fig. 4). The amount of viral proteins produced in the U3A cells was almost equal for SeV Wt- and SeV Cm*-infected cells. In agreement with this result, the growth kinetics and peak titers were almost identical for the two viruses. Although IFN-β was found to be secreted into the culture supernatant comparably from SeV Wt- and SeV Cm*-infected cells, the amount of IFN-inducible STAT2 protein was kept constant throughout the infection, suggesting that no IFN signal was transduced into the U3A cells, as expected. The impaired growth of SeV Cm* in IFN-responsive 2fTGH cells was found to be due to the lack of anti-IFN capacity of the C protein. Anti-IFN capacity of the SeV C protein was proven to be required for increasing viral replication in cultured cells.

Role of anti-IFN capacity in viral multiplication in mice. To evaluate the involvement of the anti-IFN capacity of SeV C protein for in vivo infection, we intranasally inoculated SeV Wt or SeV Cm* in 129S6 mice. SeV Wt infection prevented body weight gain, caused body weight loss, and created lung lesion from 3 to 9 days p.i. (Fig. 5). The titer of SeV Wt in the lungs reached approximately 10^7 CIU/ml at 1 day p.i., and a high viral load was maintained throughout the observation period.
In contrast, mice inoculated with SeV Cm* gained weight, as observed in mock-inoculated mice. The titer of SeV Cm* in the lungs dropped from the initial day without increasing and reached an undetectable level at 5 days p.i. No lung lesion was observed during the experimental period. These results indicate that SeV Cm* is apathogenic for mice.

To verify that the clearance of SeV Cm* from the lungs was caused by the lack of anti-IFN capacity of the C protein, STAT1 knockout 129S6 (129S6 STAT1−/−) mice were inoculated with SeV Cm* (Fig. 5). The lack of STAT1 proteins in the 129S6 STAT1−/− mouse was confirmed by immunoblot assay (Fig. 6A). 129S6 STAT1−/− mice inoculated with SeV Wt showed body weight loss and lung lesions. The STAT1−/− genotype of the host mouse did not appear to change the pathogenicity of SeV Wt. In contrast, 129S6 STAT1−/− mice infected with SeV Cm* showed body weight loss and lung lesions as observed in SeV Wt-infected mice (Fig. 5 and 6B). SeV Cm* was thus found to be pathogenic for the 129S6 STAT1−/− mice but apathogenic for the parental 129S6 mice (Fig. 6B), indicating that the apathogenic feature of SeV Cm* for in vivo infection was due to the lack of anti-IFN capacity of the SeV C protein. The anti-IFN capacity of SeV has so far proven to be essential for multiplication in vivo.

DISCUSSION

Several viral proteins have been shown to counteract the antiviral action of IFN. In viruses of the subfamily Paramyxovirinae, the V, W, C, and P proteins produced from the P gene are involved in IFN antagonism (6, 13). Findings obtained from the cloned gene product are valuable but do not simply apply to viruses, because live viruses cannot be explained by the sum of viral gene products (42, 43, 44). Therefore, it is important to evaluate the role associated with viral proteins in the context of the viral life cycle. In this study we evaluated the anti-IFN capacity of the SeV C protein. Several recombinant SeVs with deletions or mutations in the C protein have been generated previously by the our group (12, 18, 37) and by others (8, 10, 11). SeVs with four C knockouts (C, C, Y1, and Y2) and two C knockouts (C and C) show poor growth in cultured cells and no growth in mouse lungs, indicating an indispensable role of SeV C proteins, especially for in vivo
pathogenesis (18, 37). However, the SeV C protein is a multifunctional protein, playing roles in viral RNA synthesis inhibition (3, 15, 20), apoptosis inhibition (34), and virus assembly (18, 55) as well as IFN antagonism. Whether the C, C Y1, and Y2 proteins have their own allotted functions or share functions without distinction remains to be determined (7, 27). Therefore, it was challenging to evaluate anti-IFN capacity alone by using the mutant viruses obtained so far.

Our studies reveal that viral RNA synthesis inhibition is governed by amino acids scattered across the C-terminal half of the SeV C protein, while IFN antagonism occurs in specific amino acids which are common to SeV C proteins (Fig. 1). Based on these notions, SeV Cm* lacking anti-IFN activity was created. In addition, two hosts (IFN-responsive STAT1+/+ parental hosts and nonresponsive STAT1−/− hosts) were used pairwise. This excluded additional effects accompanying the SeV C protein or unexpected error during gene manipulation. Thus, it was shown that the anti-IFN capacity of SeV C protein was required for maintaining high viral yield in vitro and was dispensable for propagation in mice in vivo (Fig. 4 and 5).

A short peptide in the N terminus of the SeV C protein was shown to have STAT1-degrading activity, thereby playing a role in counteracting IFN (11). This is important because C and C proteins with a short peptide in the N terminus can be distinguished functionally from Y1 and Y2 proteins without a short peptide. This notion was expected to explain why four different C proteins are conserved among the SeV genomes and expressed in infected cells. In the present study, although the Cm* protein with an intact short peptide in the N terminus was produced in infected cells, SeV Cm* was excluded from the mouse lungs at early stages of infection. This finding suggests that the presence of a short peptide at the N terminus of the C protein was not enough to antagonize antiviral activity of IFNs. An alternate role for the different C proteins should therefore be investigated.

The V proteins of other paramyxoviruses were shown to counteract the antiviral activity of IFNs (6, 13). In the case of SeV, the V protein, despite the fact that it has a conserved cysteine-rich motif, did not inhibit the signal transduction of IFN. For example, HeLa cells stably expressing V protein did not block the antiviral activity of IFNs (29). In addition, mutant SeVs lacking whole V protein or producing the V protein without the cysteine-rich motif in the C terminus can grow as well as the parental wild-type SeV in cultured cells (27, 28). Moreover, both V mutants of SeV can grow like the wild-type SeV in the lungs of mice until 1 day postinoculation, although the amounts of mutant viruses start to decline thereafter (25, 26). Recently, our group found that clearance of V mutants from the lungs of mice after 1 day p.i. occurred through interferon regulatory factor 3-induced innate antiviral immunity but not via IFN (32). Collectively these results show that in the immediate-early stage of SeV infection, it is the C protein of SeV (not the V protein) that plays a critical role in blocking the antiviral activity of IFNs.

STAT1 is a key protein of IFN signaling in the JAK-STAT pathway. STAT1 knockout mice have been shown to be more susceptible to viruses than are the parental mice, suggesting the importance of IFNs in the self-defense mechanism (5). Recently, two intracellular RNA sensor molecules, RIG-I and MDA5, that recognize RNA viruses and an intermediary molecule (IPS-1, MAVS, or VISA, which receives a signal from sensor molecules and transfers it toward interferon regulatory factor 3) were discovered (1, 59). Mice with knockouts of these proteins were then shown to be highly sensitive to RNA viruses (30, 36). On the other hand, the host defense mechanism placed selective pressure on the viruses to acquire antagonism. Anti-IFN capacity has been described for many viruses, as described above (6). Multiple proteins of a virus are indicated to counteract the antiviral action of IFN. It is therefore necessary to evaluate the anti-IFN capacities of viral proteins in the context of the viral life cycle.

ACKNOWLEDGMENTS

This work was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

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