Respirovirus C protein inhibits activation of type I interferon receptor-associated kinases to block JAK-STAT signaling

Yoshinori Kitagawa[1], Mayu Yamaguchi[1], Miki Kohno[1,2], Madoka Sakai[1,2], Masae Itoh[2], and Bin Gotoh[1]*

Division of Microbiology and Infectious Diseases, Department of Pathology, Shiga University of Medical Science, Seta, Otsu, Shiga 520-2192, Japan[1]; Nagahama institute of Bio-Science and Technology, Nagahama, Shiga 526-0829, Japan[2]

***Corresponding author: Bin Gotoh**

Mailing address and present address: Division of Microbiology and Infectious Diseases, Department of Pathology, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga 520-2192, Japan.

Phone: 81-77-548-2176 Fax: 81-77-548-2176 E-mail: bing@belle.shiga-med.ac.jp

Abstract

Respirovirus C protein blocks the type I interferon-stimulated activation of the JAK-STAT pathway. It has been reported that C protein inhibits interferon-α-stimulated tyrosine phosphorylation of STATs, but the underlying mechanism is poorly understood. Here we show that C protein of Sendai virus, a member of the *Respirovirus* genus, binds to the IFN-α/β receptor subunit (IFNAR2) and inhibits interferon-α-stimulated tyrosine phosphorylation of the upstream receptor-associated kinases, JAK1 and TYK2. Analysis of various Sendai virus C mutant (Cm) proteins demonstrates the importance of the inhibitory effect on receptor-associated kinase phosphorylation for blockade of JAK-STAT signaling. Furthermore, this inhibitory effect and the IFNAR2 binding capacity were observed for all the respirovirus C proteins examined. Our results suggest that respirovirus C protein inhibits activation of the receptor-associated kinases JAK1 and TYK2 possibly through interaction with IFNAR2.

Keywords; respirovirus, Sendai virus, C protein, interferon, JAK-STAT pathway, JAK1, TYK2

Introduction

The *Respirovirus* genus in the family *Paramyxoviridae* includes human parainfluenza virus type 1 (HPIV1) and human parainfluenza virus type 3 (HPIV3), which are important in the field of pediatrics medicine [1]. HPIV1 is known as an infectious agent causing viral croup syndrome. HPIV3 can cause severe lower respiratory tract infection like human respiratory syncytial virus, particularly in infants with congenital heart diseases and low-birth-weight babies. Pathogenesis of these respiroviruses involves complicated processes affected by multiple factors. Of such factors, viral evasion strategies against the host interferon (IFN) system have been recently paid a lot attention.

Type I IFNs, IFN-α and IFN-β, are produced and secreted by virus infected cells and induce an anti-viral state in nearby cells via activation of the JAK-STAT signaling pathway by binding to the IFN receptor consisting of two subunits, IFN-α/β receptor subunit (IFNAR) 1 and IFNAR2 [2-4]. The binding brings the receptor-associated kinases, JAK1 and TYK2, into close proximity, resulting in cross-phosphorylation of JAK1 and TYK2. These activated kinases phosphorylate specific tyrosine residues of STAT2 and STAT1. Phosphorylated STAT2 and STAT1 leave the receptor as heterodimer, which associates with IRF9 to form interferon-stimulated gene factor 3 (ISGF3). ISGF3 is translocated into the nucleus and binds to the promoter containing IFN-stimulated response element (ISRE) to activate IFN-stimulated genes such as the anti-viral PKR gene.

Sendai virus (SeV), a murine respirovirus, was the first case in which respirovirus accessory protein C was found to block the type I IFN JAK-STAT signaling pathway [5,6]. Subsequent studies have demonstrated that this anti-IFN activity is a characteristic common to all the members of the *Respirovirus* genus including HPIV1 and

HPIV3 [7-10], suggesting its important role in survival of members of the *Respirovirus* genus through evolution. Silencing the C gene or eliminating its anti-IFN activity by the reverse-genetic technology resulted in attenuation of the virus virulence, demonstrating that the anti-IFN activity is deeply involved in viral pathogenesis [9,11-13]. Understanding of viral immune evasion mechanism thus will contribute to not only elucidation of viral pathogenesis but also development of effective vaccines and antiviral agents.

Twenty years have passed since the anti-IFN activity of SeV was discovered. Nevertheless, full understanding of its molecular mechanism has not yet been reached. Garcin et al. reported the significance of STAT1 degradation induced by expression of the SeV C protein in some cell types [14,15]. However STAT1 degradation has not been observed in a variety of cell types such as HeLa and HEK293T cells, and also in any type of the cells expressing the C protein of HPIV1 and HPIV3 [7,8,10,16]. Therefore, it is clear that there is a mechanism by which the C protein blocks the JAK-STAT signaling pathway without leading to STAT1 degradation. Previous studies performed in our lab have demonstrated that the SeV C protein binds to STAT1 and inhibits IFN-α-stimulated tyrosine-phosphorylation of STAT2 and STAT1 [15,17-19]. Analysis of the C mutant proteins has demonstrated the significance of the inhibition of tyrosine-phosphorylation of STAT2, and has raised the possibility that STAT1 is a target of the SeV C protein [19]. Afterwards target molecule of the SeV C protein has become uncertain because it was found that several C mutant proteins, which exhibited the decreased STAT1-binding capacity, retained the ability to block the type I IFN JAK-STAT signaling pathway [20]. It also remains unclear what is the real target of the HPIV1 and HPIV3 C proteins and how the HPIV1 and HPIV3 C proteins inhibit type I

IFN-stimulated JAK-STAT pathway, although it has been reported that the HPIV1 C protein binds to STAT1 and inhibits phosphorylation of STAT1 and STAT2 [21], and that the HPIV3 C protein inhibits phosphorylation of STAT1 [7].

Under these circumstances, we attempted to find out target molecules of the respirovirus C proteins for the inhibition of the JAK-STAT signaling to elucidate the underlying molecular mechanism. It was found that the SeV C protein interacted with IFNAR2 and JAK1 as well as STAT1, and inhibited IFN-α-stimulated phosphorylation of the upstream receptor-associated kinases, JAK1 and TYK2. Analysis of various SeV C mutant proteins and other respirovirus C proteins has ruled out the possibility of STAT1 and JAK1 as a major target, and have demonstrated the importance of the inhibition of tyrosine-phosphorylation of the receptor-associated kinases JAK1 and TYK2.

Materials and Methods

Cells and a virus

HEK293T and U3A (STAT1-null 2fTGH) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum [22]. Vesicular stomatitis virus (VSV) was propagated in Vero cells [23].

Plasmids

In order to express viral or cellular protein with or without FLAG, V5 or Glutathione-S-transferase (GST) tag, mammalian expression plasmids were created by insertion of a DNA fragment carrying the respective gene into the multicloning site downstream of the cytomegalovirus enhancer chicken β-actin hybrid promoter of pCA7.

The DNA fragment encoding viral protein or one of the human signaling components constituting the JAK-STAT pathway was created by polymerase chain reaction (PCR) or reverse transcription (RT)-PCR. SeV and HPIV1 express multiple species of the C protein, because their C open reading frames contain four translational start sites to produce a nested set of four carboxy-coterminal four proteins, C', C, Y1, and Y2 [24]. C is the most abundant protein of four proteins expressed in infected cells. Therefore C-expression plasmids for SeV and HPIV1 were created by insertion of DNA fragments encoding C but not C' with or without FLAG or V5 tag into pCA7. Mutations were introduced by PCR-based overlap mutagenesis in the same way as before [25]. Sequence fidelity of all the plasmids was confirmed by sequence analysis. pIRESpuro3 plasmid carrying the puromycin-resistant gene was purchased from Clontech Laboratories, Mountain View, CA.

Luciferase reporter gene assay

ISRE promoter-driven firefly luciferase (Fluc) reporter plasmid (pISRE-TA-Luc) (Clontech Laboratories, Mountain View, CA) (80 ng/well) and pRL-TK (Promega Corporation, Madison, WI) (10 ng/well) were transfected into HEK293T cells (~1.0 x 10^5) cultured in a 24-well plate in triplicate together with a plasmid expressing wild type or mutant C protein (50 ng/well) by using polyethyleneimine (Polysciences, Warrington, PA) [25,26]. The total mass of transfected DNA was held constant in all experiments by adding an appropriate amount of pCA7 empty plasmid. At 24 h post-transfection, transfected cells were treated with recombinant human IFN-α2b (1,000 U/ml; Schering-Plough, Kenilworth, NJ) for 6 h, and then lysed. Luciferase activities of the cell lysates were measured by the dual-luciferase reporter assay system (Promega Corporation, Madison, WI) according to the manufacturer's protocol. Relative luciferase

activity was determined as the ratio of Fluc activity to Renilla luciferase (Rluc) activity.

Immunoprecipitation and GST pull-down assay

HEK293T or U3A cells (~5.0 x 10^5/well) in a 6-well plate were transfected with various combinations of plasmids (500 ng/well each), using polyethyleneimine. At 24 h post-transfection, cells were lysed in 400 μl of a lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail). For immunoprecipitation, the cell lysates were incubated with anti-V5 mouse monoclonal antibody (mAb) (SV5-Pk1: Invitrogen, Carlsbad, CA), anti-FLAG mouse mAb (1E6: Wako Pure Chemical Industries, Osaka, Japan), or anti-myc mouse mAb (2276: Cell signaling Technology, Danvers, MA) together with SureBeads Protein G (Bio-Rad, Hercules, CA) at 4˚C for 2 hr. In some experiments, protein products synthesized *in vitro* by the TNT SP6 high-yield wheat germ protein expression system (Promega Corporation, Madison, WI) were used in place of cell lysates [27]. For GST pull-down assay, the cell lysates were incubated with Glutathione Sepharose beads (GE Healthcare Life Sciences, Buckinghamshire, England) at 4˚C for 2 hr. After washing the beads five times with the lysis buffer, proteins were eluted from the beads by boiling with Laemmli sample buffer [50 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 10% glycerol and 5% 2-mercaptoethanol], and then subjected to immunoblot analysis.

Immunoblot analysis

Samples were resolved by SDS-(10-15%)-polyacrylamide gel electrophoresis, and then electroblotted onto a membrane filter (Immobilon-P: Millipore, Burlington, MA). The membrane was blocked in PVDF Blocking Reagent (Toyobo, Osaka, Japan) before incubation at 4˚C overnight with anti-VSV mouse serum, anti-C rabbit serum,

157 anti-FLAG mouse mAb (1E6), anti-V5 mouse mAb (SV5-Pk1), anti-GST mouse mAb
158 (5A7; Wako Pure Chemical Industries, Osaka Japan), anti-STAT1 rabbit polyclonal Ab
159 (sc-346; SantaCruz Biotechnology, Dallas, TX), anti-STAT2 rabbit polyclonal Ab
160 (4594; Cell signaling Technology, Danvers, MA), anti-phospho-STAT2 (Tyr690) rabbit
161 polyclonal Ab (07-224; Millipore, Burlington, MA), or anti-phospho-STAT1 (Tyr701)
162 rabbit mAb (7649; Cell signaling Technology, Danvers, MA), anti-JAK1 rabbit mAb
163 (3344; Cell signaling Technology, Danvers, MA), anti-TYK2 rabbit mAb (14193; Cell
164 signaling Technology, Danvers, MA), anti-phospho-JAK1 goat polyclonal Ab
165 (sc-16773; SantaCruz Biotechnology, Dallas, TX), anti-phospho-TYK2 rabbit mAb
166 (68790; Cell signaling Technology, Danvers, MA), or anti-GAPDH rabbit mAb (5147:
167 Cell signaling Technology, Danvers, MA). The membrane was then incubated at room
168 temperature for 2 h with horseradish peroxidase-conjugated anti-mouse IgG Ab,
169 anti-rabbit IgG Ab (GE Healthcare Life Sciences, Buckinghamshire, England), or
170 anti-goat IgG Ab (Jackson ImmunoResearch, West Grove, PA). Immunoreactive bands
171 were visualized by using the ECL select substrate (GE Healthcare Life Sciences,
172 Buckinghamshire, England).
173
174 **Results**
175 **STAT1 is not a major target of the SeV C protein**
176 The SeV C protein binds to STAT1 and inhibits IFN-α-stimulated
177 tyrosine-phosphorylation of STAT1 and STAT2 [15,17,18,28]. These findings suggest
178 that STAT1 is one of the target molecules of the C protein. However, subsequent study
179 revealed that several SeV C mutant proteins retained the ability to block the type I IFN
180 JAK-STAT pathway, although exhibited the decreased STAT1-binding capacity [20]. To

confirm whether this result is correct, we have constructed a variety of plasmids expressing the C mutant protein (Fig.1 A). A series of Cm proteins has point mutations, which result in replacement of two or three charged amino acids with alanine [20]. C_{F170S} has a single point mutation, which causes amino acid substitution of serine for phenylalanine at the position 170. This mutation is derived from an avirulent mutant SeV generated through successive passages of a highly virulent field strain, Ohita-M1 [29]. Initially, we examined effect of these C mutant proteins on IFN-α-stimulated activation of the promoter containing ISRE. pISRE-TA-Luc and internal control pRL-TK were transfected into HEK293T cells along with one of the C mutant proteins. Transfected cells were treated with IFN-α for 6 h, and then subjected to luciferase reporter assay. As shown in Fig.1 B, Cm3, Cm4, Cm6, Cm7, and Cm9 retained the inhibitory effect on IFN-α-stimulated activation of the Fluc gene, comparable to that of wild type C. In contrast, inhibitory effect was not observed for Cm5, Cm8, and C_{F170S}, indicating that Cm5, Cm8, and C_{F170S} has lost the ability to block the type I IFN JAK-STAT pathway. This finding was also confirmed by examining effect on establishment of the IFN-α-induced antiviral state in cells (Fig.1 C). HEK293T cells were transfected with one of the C mutant proteins, and subsequently treated with IFN-α. After IFN-α treatment for 24 h, the cells were infected with VSV, one of the IFN-sensitive viruses. At 6 h post-infection, the level of VSV proteins was estimated by immunoblot analysis. As expected, the level of viral protein synthesis was comparable between Cm3, Cm4, Cm6, Cm7, Cm9, and wild type C, whereas it was significantly suppressed in Cm5, Cm8, and C_{F170S}.

We also tested the ability of the C mutant proteins to bind to STAT1 (Fig.1 D). HEK293T cells were transfected with one of the FLAG-tagged C mutants, and then

205 subjected to immunoprecipitation with anti-FLAG antibody. As shown in Fig.1 D, STAT1
206 was co-precipitated in cells transfected with Cm3 and Cm4 as efficiently as in cells
207 transfected with wild type C, whereas only a negligible amount of STAT1 was
208 co-precipitated in Cm5, Cm6, Cm7, and Cm8. It should be noted that Cm6 and Cm7,
209 which retained the inhibitory effect on JAK-STAT signaling (Fig.1 B), exhibited the
210 decreased STAT1 binding capacity. Furthermore, intermediate levels of STAT1 were
211 co-precipitated in Cm9 and C_{F170S}, although there was a marked contrast between them in
212 the ability to block JAK-STAT signaling (Fig.1 BC). From these results, we have
213 concluded that STAT1 is not a major target of the SeV C protein.
214
215 **IFNAR2 and JAK1 are potential targets of the SeV C protein**
216 To find a molecular target of the SeV C protein, we investigated the interaction between
217 the C protein and components of the type I IFN JAK-STAT signaling pathway. SeV C was
218 transfected into HEK293T cells along with one of the FLAG-tagged signaling
219 components, and then transfected cells were subjected to immunoprecipitation. As shown
220 in Fig.2 A, the C protein was co-precipitated with anti-FLAG antibody in cells transfected
221 with FLAG-tagged IFNAR2, JAK1, and STAT1. Conversely, V5-tagged IFNAR2, JAK1,
222 and STAT1 were co-precipitated with anti-FLAG antibody when FLAG-tagged C was
223 transfected into HEK293T cells along with one of the V5-tagged signaling components
224 (Fig.2 B). To rule out the possibility that the C-IFNAR2 and C-JAK1 interactions were
225 mediated by endogenous STAT1, immunoprecipitation experiments were carried out for
226 U3A (STAT1-null 2fTGH) cells transfected with FLAG-C and one of the V5-tagged
227 signaling components (Fig.2 CD). FLAG-C was co-precipitated with anti-V5 antibody
228 even in STAT1-null cells transfected with IFNAR2-V5 and FLAG-C or with JAK1-V5

and FLAG-C. Co-transfection of exogenous STAT1 into U3A cells did not affect the amount of co-precipitated FLAG-C in cells transfected with IFNAR2-V5 and FLAG-C (Fig.2 C). In contrast, the amount of co-precipitated FLAG-C was decreased by co-transfection of STAT1 in cells transfected with JAK1-V5 and FLAG-C (Fig.2 D), suggesting that JAK1 and STAT1 may compete with each other for binding to the C protein. Taken together, these results suggest that the C-IFNAR2 and C-JAK1 interactions are not mediated by STAT1. Thus IFNAR2 and JAK1 were found to be potential targets of the C protein.

The SeV C protein interacts with cytoplasmic domain of IFNAR2 and with kinase domain of JAK1

We next attempted to identify domains of IFNAR2 and JAK1 responsible for interaction with the SeV C protein. HEK293T cells were transfected with SeV C and one of FLAG-tagged IFNAR2 deletion mutants (Fig.3 A), and then subjected to immunoprecipitation. As shown in Fig.3 B, the C protein was co-precipitated with anti-FLAG antibody in cells transfected with FLAG-tagged $IFNAR2_{1\text{-}346}$, and $IFNAR2_{1\text{-}462}$, and not $IFNAR2_{1\text{-}265}$, suggesting that the C protein interacts with aa 265-346 region of the IFNAR2 cytoplasmic tail. Multiple bands were observed for FLAG-tagged IFNAR2 deletion mutants. This is probably due to protein modifications such as glycosylation, precise nature of which was not determined. Importance of the aa 265-346 region was also supported by GST pull-down assay using extracts from cells transfected with FLAG-C and one of the GST-tagged IFNAR2 deletion mutants (Fig.3 C). As shown in Fig.3 D, FLAG-C was co-purified with GST-$IFNAR2_{266\text{-}515}$ but neither with GST-$IFNAR2_{347\text{-}515}$ nor with GST-$IFNAR2_{463\text{-}515}$. Immunoprecipitation experiments

were further performed for HEK293T cells transfected with SeV C and one of V5-tagged JAK1 deletion mutants (Fig.4 A). As shown in Fig.4 B, FLAG-C was co-precipitated with anti-V5 antibody in cells transfected with V5-tagged JAK1$_{427\text{-}1154}$, JAK1$_{556\text{-}1154}$, or JAK1$_{859\text{-}1154}$, indicating that the C protein interacts with the kinase domain (aa 859-1154) of JAK1.

Molecular target of the SeV C protein

The C-JAK1 and C-IFNAR2 interactions are not mediated by STAT1 as described above (Fig.2 C), but the possibility remains that they are mediated by unknown intracellular molecules other than STAT1. To determine whether their interactions were direct, immunoprecipitation experiments were carried out for products synthesized *in vitro* by the wheat germ cell-free expression system. IFNAR2$_{266\text{-}515}$-FLAG, JAK1$_{859\text{-}1154}$-FLAG, and V5-C were synthesized by the wheat germ transcription/translation system (Input in Fig.5). They were mixed in various combinations, and subjected to immunoprecipitation (Fig.5). V5-C was co-precipitated with anti-FLAG antibody in mixtures of V5-C and IFNAR2$_{266\text{-}515}$-FLAG. Conversely IFNAR2$_{266\text{-}515}$-FLAG was co-precipitated with anti-V5 antibody. These results suggest that the C-IFNAR2 interaction is direct. In contrast, co-precipitation of V5-C and JAK1$_{859\text{-}1154}$-FLAG was not observed for mixtures of V5-C and JAK1$_{859\text{-}1154}$-FLAG, raising the possibility that the C-JAK1 interaction is mediated by unknown cellular molecules.

To determine whether the C-JAK1 and C-IFNAR2 interactions are essential for the blockade of type I IFN JAK-STAT signaling, the ability of the C mutant proteins to bind to JAK1 or IFNAR2 was tested by immunoprecipitation experiments. As shown in Fig.6 A, JAK1-V5 was co-precipitated with anti-FLAG antibody in cells co-transfected

277 with FLAG-tagged Cm3, Cm4, Cm6, or Cm9, whereas only a negligible amount of
278 JAK1-V5 was co-precipitated in Cm5, Cm7, Cm8, or C_{F170S}. It should be noted that Cm7,
279 which retained full inhibitory effect on JAK-STAT signaling as described above (Fig.1
280 BC), exhibited the decreased JAK1 binding capacity, demonstrating that JAK1 is not a
281 major target of the SeV C protein. On the other hand, IFNAR2-V5 was co-precipitated
282 with anti-FLAG antibody in cells transfected with any of the FLAG-tagged C mutant
283 proteins (Fig.6 B). To check whether binding of SeV C and C mutant proteins to IFNAR2
284 is specific, we determined whether SeV P protein binds to IFNAR2 as a control. As
285 shown in Fig.6 C, IFNAR2-V5 was not co-precipitated in cells co-transfected with
286 FLAG-tagged SeV P protein. These results neither have supported nor have ruled out the
287 hypothesis that IFNAR2 is a major target of the C protein.
288
289 **The SeV C protein prevents neither STAT2 nor JAK1 from interacting with**
290 **IFNAR2**
291 Cytoplasmic tail of IFNAR2 is the region with which JAK1 and STAT2 interact [30-32],
292 It raised the possibility that the C protein might prevent JAK1 or STAT2 from interacting
293 with IFNAR2. Immunoprecipitation experiments were performed using extracts from
294 cells transfected with STAT2-V5 and IFNAR2-FLAG or with JAK1-V5 and
295 IFNAR2-FLAG to monitor STAT2-IFNAR2 and JAK1-IFNAR2 interactions (Fig.7).
296 IFNAR2-FLAG were co-precipitated with anti-V5 antibody in either case (the second
297 lanes, Fig.7 AB), indicating that both STAT2 and JAK1 interact with IFNAR2. However,
298 co-transfection with C or one of the C mutant proteins did not affect the amount of
299 IFNAR2-FLAG co-precipitated (Fig.7 AB). These results suggest that the C protein
300 prevents neither IFNAR2-JAK1 interaction nor IFNAR2-STAT2 interaction.

301

302 **The SeV C protein inhibits type I IFN-stimulated tyrosine-phosphorylation of the**
303 **receptor-associated kinases**

304 Formation of the ISGF3 complex requires phosphorylation of tyrosine residues in the
305 C-terminal regulatory domain of STAT1 and STAT2. This phosphorylation is inhibited in
306 cells expressing the C protein [18,19]. We thus checked phosphorylation status of both
307 STATs in cells transfected with one of the C mutant proteins at 15 min after IFN-α
308 stimulation (Fig.8). Cm3, Cm4, Cm6, Cm7, and Cm9 retained inhibitory effect on
309 IFN-α-stimulated tyrosine-phosphorylation of STAT1 and STAT2, comparable to that of
310 wild type C, whereas Cm5, Cm8, and C_{F170S} exhibited the decreased inhibitory effect.
311 This is in good agreement with the result of Fig.1 BC. STAT1 and STAT2 are
312 phosphorylated by the receptor-associated kinases, JAK1 and TYK2. Since these kinases
313 are activated by cross-phosphorylation through IFN-α-mediated association of IFNAR1
314 and IFNAR2, we tested effect of the C mutant proteins on IFN-α-stimulated
315 tyrosine-phosphorylation of JAK1 and TYK2. As shown in Fig.8,
316 tyrosine-phosphorylation of both JAK1 and TYK2 was inhibited in cells transfected with
317 C, Cm3, Cm4, Cm6, Cm7, and Cm9. This result is also in good agreement with the result
318 of Fig.1 BC. Taken together, these findings have demonstrated the importance of the
319 inhibitory effect on tyrosine-phosphorylation of the receptor-associated kinases for the
320 blockade of JAK-STAT signaling.

321

322 **Common characteristics of respirovirus C proteins**

323 The C protein of HPIV1 and HPIV3 belonging to the same *Respirovirus* genus blocks
324 the type I IFN JAK-STAT pathway [7,21]. These findings were confirmed by the

reporter assay as shown in Fig.9 A. To determine whether the underlying molecular mechanism is common to members of the *Respirovirus* genus, we examined interaction of the HPIV1, HPIV3, and BPIV3 C proteins with components of the JAK-STAT pathway. Immunoprecipitation experiments showed that HPIV1, HPIV3, and BPIV3 C proteins were capable of binding to IFNAR2 (Fig.9 B). In contrast, the HPIV1, HPIV3, and BPIV3 C proteins exhibited only a little or negligible binding capacity for STAT1 and JAK1 (Fig.9 CD). We also examined effect of the HPIV1, HPIV3, and BPIV3 C proteins on IFN-α-stimulated tyrosine-phosphorylation of the signaling components. Inhibition of tyrosine-phosphorylation of STAT1, STAT2, and the receptor-associated kinases, JAK1 and TYK2, was observed for the HPIV1, HPIV3, and BPIV3 C proteins as well (Fig.9 E). These results suggest that the abilities of the C protein to bind to IFNAR2 and to inhibit the receptor-associated kinase activation are common characteristics of members of the *Respirovirus* genus.

Discussion

The present study has demonstrated that the respirovirus C protein inhibits activation process of the receptor-associated kinases, JAK1 and TYK2. This finding is consistent with our previous observation that IFN-α-stimulated tyrosine-phosphorylation of TYK2 is partly suppressed in SeV-infected cells [33], and explains how the C protein inhibits IFN-α-stimulated tyrosine-phosphorylation of STAT1 and STAT2. There is no difference between the SeV, HPIV1, HPIV3, and BPIV3 C proteins in their abilities to inhibit type I IFN-stimulated tyrosine-phosphorylation of JAK1 and TYK2. This suggests that the underlying mechanism has been conserved between respiroviruses and has played a critical role in virus survival through evolution, although amino acid sequence identity

between SeV C and HPIV3 C or between SeV C and BPIV3 C is low at present (38.4% or 35.3%, respectively) [24].

IFNAR2 is the only signaling component, to which all the respirovirus C proteins examined can bind (Fig.9 BCD), suggesting that target molecule is IFNAR2. However, convincing evidence could not be obtained, because all the SeV C mutant proteins created here have retained the IFNAR2 binding capacity. The possibility remains that unknown molecules functioning near the receptor or receptor-associated kinases is a target of the C protein. It is also unclear how the C protein inhibits cross-activation of JAK1 and TYK2. Since the SeV C protein binds to the cytoplasmic tail of IFNAR2 nearby cell membrane (Fig.3), we hypothesized that the C protein could prevent JAK1 or STAT2 from interacting with IFNAR2. However, immunoprecipitation experiments showed that neither IFNAR2-STAT2 interaction nor IFNAR2-JAK1 interaction was affected by expression of the C protein (Fig.7 AB). It is possible that the C protein might hinder type I IFN-mediated association between IFNAR1 and IFNAR2, which is required for cross-activation of the receptor-associated kinases, or might affect distribution of IFNAR2 by inhibiting transport of IFNAR2 from rough endoplasmic reticulum to the cell surface. These possibilities should be taken into consideration in the future study.

Immunoprecipitation experiments did not detect interaction of the HPIV1 C protein with STAT1 (Fig.9 C). This result, although seemingly conflicts with the previous finding by Schomacker et al. [21], is reconcilable with it. The C open reading frame of HPIV1 and SeV unlike HPIV3 and BPIV3 contains four translational start sites to produce a nested set of carboxy-coterminal four proteins termed C', C, Y1, and Y2, which are listed in descending order in size. Schomacker et al. reported that they tried to

373 identify C binding partners by several methods including yeast-two-hybrid assays and
374 immunoprecipitation, but failed at first [21]. They could succeed in
375 co-immunoprecipitate STAT1 with C' (largest form of the C protein) but not C only
376 when C' was over-expressed in 293T cells and washing conditions for the
377 immunoprecipitation were adjusted. These results may suggest the possibility that the
378 N-terminal region (aa 1-15) of C'(aa 1-219), which C (aa 16-219) does not have, is
379 responsible for the C'-STAT1 interaction. Thus their findings do not necessarily conflict
380 with our results obtained using plasmids expressing C but not C'.
381 Analysis of the SeV C mutant proteins has demonstrated that the C-STAT1 and
382 C-JAK1 interactions are not required for the blockade of the JAK-STAT pathway (Fig.1
383 and Fig.6). Do the C-STAT1 and C-JAK1interactions make no contribution to the
384 blockade of the type I IFN JAK-STAT signaling pathway? Oda et al. have determined
385 the crystal structure of the N-terminal domain of STAT1 associated with the C-terminal
386 half of the C protein [34], and have proposed the hypothesis that one molecule of the C
387 protein might associate with the dimeric structure formed between the N-terminal
388 domains of STAT1 and STAT2, thereby leading the STAT1-STAT2 heterodimer into an
389 anti-parallel form, which is easily dephosphorylated [35]. It is also possible that the C
390 protein might inhibit kinase activity through interaction with the kinase domain of
391 JAK1. However, it would be necessary to isolate C mutant proteins that retain the
392 binding capacity for only one of three binding proteins (IFNAR2, JAK1, and STAT1) to
393 assess contribution of the C-JAK1 and C-STAT1 interactions to the signaling inhibition.
394 The *Paramyxoviridae* family includes the *Respirovirus*, *Morbillivirus*,
395 *Henipavirus*, *Rubulavirus*, and *Avulavirus* genera. Members of the *Respirovirus* genus
396 uses the C protein and not the V protein as an IFN antagonist that blocks the type I IFN

JAK-STAT pathway, whereas members of the other genera use the V protein instead of the C protein. The present study has demonstrated that inhibition of activation of the receptor-associated kinases is a common characteristic of the respirovirus C proteins. The V protein of PIV5, mumps virus and HPIV2 in the *Rubulavirus* genus and Newcastle disease virus in the *Avulavirus* genus promotes degradation of either STAT1 or STAT2 [36-39], whereas the V protein of measles virus in the *Morbillivirus* genus inhibits STAT1 and STAT2 phosphorylation without STAT degradation [40,41]. The V protein of Hendra and Nipah viruses in the *Henipavirus* genus binds to both STAT1 and STAT2, inhibits their phosphorylation, and induces their cytoplasmic aggregates [42,43]. Thus, there may be common specific mechanism at least within the same genus.

Knockout of the C gene results in attenuation of virus pathogenicity. Therefore, the recombinant virus, whose C gene is silenced, is a candidate for attenuated virus vaccine. However, the C-knockout recombinant SeV and HPIV1 show too poor growth in cell culture and hence cannot be prepared as vaccines [13,44]. The C protein is a multi-functional protein that exerts anti-IFN effect [5,6,45-47], regulates viral RNA synthesis [48,49], facilitates virus budding [44,50-53], and inhibits virus-induced apoptosis [54]. Such various functions collectively contribute to virus pathogenicity, resulting in over-attenuation of the recombinant viruses. Thus, moderately attenuated recombinant viruses, which could be created by silencing only a single function with the other functions remained, might be suitable for vaccine. For this purpose, it would be necessary to determine domains or amino acid residues important for maintaining each function of the C protein.

In conclusion, the present study has uncovered that members of the *Respirovirus* genus have evolved the C proteins as an IFN antagonist, which inhibits IFN-α-stimulated

tyrosine-phosphorylation of the upstream receptor-associated kinases possibly through interaction with IFNAR2 to block the type I IFN JAK-STAT signaling pathway.

Acknowledgements

We thank Komatsu T. (Aichi) for helpful discussion. Sequence analysis was performed using the ABI PRISM 3130xl Genetic Analyzer in the Central Research Laboratory, Shiga University of Medical Science. This work was supported by JSPS KAKENHI Grant Number JP19K08928, and by grants from the Shiga University of Medical Science and from the Yakult Honsha, Japan.

Author Contributions

YK, MI, and BG designed study and analysed data. YK, MY, MK, and MS performed experiments. YK and BG wrote the manuscript.

References

[1] Adams, M.J. et al. (2017). Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2017). Arch Virol 162, 2505-2538.

[2] Platanias, L.C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 5, 375-386.

[3] Villarino, A.V., Kanno, Y. and O'Shea, J.J. (2017). Mechanisms and consequences of Jak-STAT signaling in the immune system. Nat Immunol 18, 374-384.

[4] Stark, G.R., Cheon, H. and Wang, Y. (2018). Responses to Cytokines and Interferons that Depend upon JAKs and STATs. Cold Spring Harb Perspect Biol 10, a028555.

[5] Garcin, D., Latorre, P. and Kolakofsky, D. (1999). Sendai virus C proteins counteract the interferon-mediated induction of an antiviral state. J Virol 73, 6559-6565.

[6] Gotoh, B., Takeuchi, K., Komatsu, T., Yokoo, J., Kimura, Y., Kurotani, A., Kato, A. and Nagai, Y. (1999). Knockout of the Sendai virus C gene eliminates the viral ability to prevent the interferon-alpha/beta-mediated responses. FEBS Lett 459, 205-210.

[7] Malur, A.G., Chattopadhyay, S., Maitra, R.K. and Banerjee, A.K. (2005). Inhibition of STAT 1 phosphorylation by human parainfluenza virus type 3 C protein. J Virol 79, 7877-7882.

[8] Bousse, T., Chambers, R.L., Scroggs, R.A., Portner, A. and Takimoto, T. (2006). Human parainfluenza virus type 1 but not Sendai virus replicates in human

459 respiratory cells despite IFN treatment. Virus Res 121, 23-32.
460 [9] Bartlett, E.J., Hennessey, M., Skiadopoulos, M.H., Schmidt, A.C., Collins, P.L.,
461 Murphy, B.R. and Pickles, R.J. (2008). Role of interferon in the replication of
462 human parainfluenza virus type 1 wild type and mutant viruses in human ciliated
463 airway epithelium. J Virol 82, 8059-8070.
464 [10] Chambers, R. and Takimoto, T. (2009). Host specificity of the anti-interferon
465 and anti-apoptosis activities of parainfluenza virus P/C gene products. J Gen
466 Virol 90, 1906-1915.
467 [11] Van Cleve, W. et al. (2006). Attenuating mutations in the P/C gene of human
468 parainfluenza virus type 1 (HPIV1) vaccine candidates abrogate the inhibition of
469 both induction and signaling of type I interferon (IFN) by wild-type HPIV1.
470 Virology 352, 61-73.
471 [12] Kato, A., Kiyotani, K., Kubota, T., Yoshida, T., Tashiro, M. and Nagai, Y. (2007).
472 Importance of the anti-interferon capacity of Sendai virus C protein for
473 pathogenicity in mice. J Virol 81, 3264-3271.
474 [13] Bartlett, E.J. et al. (2008). Human parainfluenza virus type 1 C proteins are
475 nonessential proteins that inhibit the host interferon and apoptotic responses and
476 are required for efficient replication in nonhuman primates. J Virol 82,
477 8965-8977.
478 [14] Garcin, D., Curran, J., Itoh, M. and Kolakofsky, D. (2001). Longer and shorter
479 forms of Sendai virus C proteins play different roles in modulating the cellular
480 antiviral response. J Virol 75, 6800-6807.
481 [15] Garcin, D., Marq, J.B., Strahle, L., le Mercier, P. and Kolakofsky, D. (2002). All
482 four Sendai Virus C proteins bind Stat1, but only the larger forms also induce its

483 mono-ubiquitination and degradation. Virology 295, 256-265.
484 [16] Mao, H., Chattopadhyay, S. and Banerjee, A.K. (2009). N-terminally truncated
485 C protein, CNDelta25, of human parainfluenza virus type 3 is a potent inhibitor
486 of viral replication. Virology 394, 143-148.
487 [17] Takeuchi, K., Komatsu, T., Yokoo, J., Kato, A., Shioda, T., Nagai, Y. and Gotoh,
488 B. (2001). Sendai virus C protein physically associates with Stat1. Genes Cells 6,
489 545-557.
490 [18] Komatsu, T., Takeuchi, K., Yokoo, J. and Gotoh, B. (2002). Sendai virus C
491 protein impairs both phosphorylation and dephosphorylation processes of Stat1.
492 FEBS Lett 511, 139-144.
493 [19] Gotoh, B., Komatsu, T., Takeuchi, K. and Yokoo, J. (2003). The C-terminal
494 half-fragment of the Sendai virus C protein prevents the gamma-activated factor
495 from binding to a gamma-activated sequence site. Virology 316, 29-40.
496 [20] Kato, A. et al. (2004). Characterization of the amino acid residues of sendai
497 virus C protein that are critically involved in its interferon antagonism and RNA
498 synthesis down-regulation. J Virol 78, 7443-7454.
499 [21] Schomacker, H., Hebner, R.M., Boonyaratanakornkit, J., Surman, S.,
500 Amaro-Carambot, E., Collins, P.L. and Schmidt, A.C. (2012). The C proteins of
501 human parainfluenza virus type 1 block IFN signaling by binding and retaining
502 Stat1 in perinuclear aggregates at the late endosome. PLoS One 7, e28382.
503 [22] Kitagawa, Y., Zhou, M., Yamaguchi, M., Komatsu, T., Takeuchi, K., Itoh, M. and
504 Gotoh, B. (2010). Human metapneumovirus M2-2 protein inhibits viral
505 transcription and replication. Microbes Infect 12, 135-145.
506 [23] Kitagawa, Y., Sakai, M., Shimojima, M., Saijo, M., Itoh, M. and Gotoh, B.

(2018). Nonstructural protein of severe fever with thrombocytopenia syndrome phlebovirus targets STAT2 and not STAT1 to inhibit type I interferon-stimulated JAK-STAT signaling. Microbes Infect 20, 360-368.

[24] Nagai, Y. (1999). Paramyxovirus replication and pathogenesis. Reverse genetics transforms understanding. Rev Med Virol 9, 83-99.

[25] Kitagawa, Y. et al. (2011). A tryptophan-rich motif in the human parainfluenza virus type 2 V protein is critical for the blockade of toll-like receptor 7 (TLR7)- and TLR9-dependent signaling. J Virol 85, 4606-4611.

[26] Kitagawa, Y., Yamaguchi, M., Zhou, M., Nishio, M., Itoh, M. and Gotoh, B. (2013). Human parainfluenza virus type 2 V protein inhibits TRAF6-mediated ubiquitination of IRF7 to prevent TLR7- and TLR9-dependent interferon induction. J Virol 87, 7966-7976.

[27] Kitagawa, Y., Sakai, M., Funayama, M., Itoh, M. and Gotoh, B. (2017). Human Metapneumovirus M2-2 Protein Acts as a Negative Regulator of Alpha Interferon Production by Plasmacytoid Dendritic Cells. J Virol 91, e00579-17.

[28] Gotoh, B., Takeuchi, K., Komatsu, T. and Yokoo, J. (2003). The STAT2 activation process is a crucial target of Sendai virus C protein for the blockade of alpha interferon signaling. J Virol 77, 3360-3370.

[29] Itoh, M., Isegawa, Y., Hotta, H. and Homma, M. (1997). Isolation of an avirulent mutant of Sendai virus with two amino acid mutations from a highly virulent field strain through adaptation to LLC-MK2 cells. J Gen Virol 78, 3207-3215.

[30] Li, X., Leung, S., Kerr, I.M. and Stark, G.R. (1997). Functional subdomains of STAT2 required for preassociation with the alpha interferon receptor and for signaling. Mol Cell Biol 17, 2048-2056.

[31] Wagner, T.C. et al. (2002). Interferon signaling is dependent on specific tyrosines located within the intracellular domain of IFNAR2c. Expression of IFNAR2c tyrosine mutants in U5A cells. J Biol Chem 277, 1493-1499.

[32] Usacheva, A., Sandoval, R., Domanski, P., Kotenko, S.V., Nelms, K., Goldsmith, M.A. and Colamonici, O.R. (2002). Contribution of the Box 1 and Box 2 motifs of cytokine receptors to Jak1 association and activation. J Biol Chem 277, 48220-48226.

[33] Komatsu, T., Takeuchi, K., Yokoo, J., Tanaka, Y. and Gotoh, B. (2000). Sendai virus blocks alpha interferon signaling to signal transducers and activators of transcription. J Virol 74, 2477-2480.

[34] Oda, K., Matoba, Y., Irie, T., Kawabata, R., Fukushi, M., Sugiyama, M. and Sakaguchi, T. (2015). Structural Basis of the Inhibition of STAT1 Activity by Sendai Virus C Protein. J Virol 89, 11487-11499.

[35] Oda, K., Oda, T., Matoba, Y., Sato, M., Irie, T. and Sakaguchi, T. (2017). Structural analysis of the STAT1:STAT2 heterodimer revealed the mechanism of Sendai virus C protein-mediated blockade of type 1 interferon signaling. J Biol Chem 292, 19752-19766.

[36] Didcock, L., Young, D.F., Goodbourn, S. and Randall, R.E. (1999). The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. J Virol 73, 9928-9933.

[37] Parisien, J.P., Lau, J.F., Rodriguez, J.J., Sullivan, B.M., Moscona, A., Parks, G.D., Lamb, R.A. and Horvath, C.M. (2001). The V protein of human parainfluenza virus 2 antagonizes type I interferon responses by destabilizing signal transducer and activator of transcription 2. Virology 283, 230-239.

555 [38] Nishio, M., Garcin, D., Simonet, V. and Kolakofsky, D. (2002). The carboxyl
556 segment of the mumps virus V protein associates with Stat proteins in vitro via a
557 tryptophan-rich motif. Virology 300, 92-99.
558 [39] Huang, Z., Krishnamurthy, S., Panda, A. and Samal, S.K. (2003). Newcastle
559 disease virus V protein is associated with viral pathogenesis and functions as an
560 alpha interferon antagonist. J Virol 77, 8676-8685.
561 [40] Takeuchi, K., Kadota, S.I., Takeda, M., Miyajima, N. and Nagata, K. (2003).
562 Measles virus V protein blocks interferon (IFN)-alpha/beta but not IFN-gamma
563 signaling by inhibiting STAT1 and STAT2 phosphorylation. FEBS Lett 545,
564 177-182.
565 [41] Palosaari, H., Parisien, J.P., Rodriguez, J.J., Ulane, C.M. and Horvath, C.M.
566 (2003). STAT protein interference and suppression of cytokine signal
567 transduction by measles virus V protein. J Virol 77, 7635-7644.
568 [42] Rodriguez, J.J., Parisien, J.P. and Horvath, C.M. (2002). Nipah virus V protein
569 evades alpha and gamma interferons by preventing STAT1 and STAT2 activation
570 and nuclear accumulation. J Virol 76, 11476-11483.
571 [43] Rodriguez, J.J., Wang, L.F. and Horvath, C.M. (2003). Hendra virus V protein
572 inhibits interferon signaling by preventing STAT1 and STAT2 nuclear
573 accumulation. J Virol 77, 11842-11845.
574 [44] Kurotani, A., Kiyotani, K., Kato, A., Shioda, T., Sakai, Y., Mizumoto, K.,
575 Yoshida, T. and Nagai, Y. (1998). Sendai virus C proteins are categorically
576 nonessential gene products but silencing their expression severely impairs viral
577 replication and pathogenesis. Genes Cells 3, 111-124.
578 [45] Komatsu, T., Takeuchi, K., Yokoo, J. and Gotoh, B. (2004). C and V proteins of

579 Sendai virus target signaling pathways leading to IRF-3 activation for the
580 negative regulation of interferon-beta production. Virology 325, 137-148.
581 [46] Takeuchi, K., Komatsu, T., Kitagawa, Y., Sada, K. and Gotoh, B. (2008). Sendai
582 virus C protein plays a role in restricting PKR activation by limiting the
583 generation of intracellular double-stranded RNA. J Virol 82, 10102-10110.
584 [47] Yamaguchi, M., Kitagawa, Y., Zhou, M., Itoh, M. and Gotoh, B. (2014). An
585 anti-interferon activity shared by paramyxovirus C proteins: inhibition of
586 Toll-like receptor 7/9-dependent alpha interferon induction. FEBS Lett 588,
587 28-34.
588 [48] Cadd, T., Garcin, D., Tapparel, C., Itoh, M., Homma, M., Roux, L., Curran, J.
589 and Kolakofsky, D. (1996). The Sendai paramyxovirus accessory C proteins
590 inhibit viral genome amplification in a promoter-specific fashion. J Virol 70,
591 5067-5074.
592 [49] Tapparel, C., Hausmann, S., Pelet, T., Curran, J., Kolakofsky, D. and Roux, L.
593 (1997). Inhibition of Sendai virus genome replication due to promoter-increased
594 selectivity: a possible role for the accessory C proteins. J Virol 71, 9588-9599.
595 [50] Hasan, M.K., Kato, A., Muranaka, M., Yamaguchi, R., Sakai, Y., Hatano, I.,
596 Tashiro, M. and Nagai, Y. (2000). Versatility of the accessory C proteins of
597 Sendai virus: contribution to virus assembly as an additional role. J Virol 74,
598 5619-5628.
599 [51] Sakaguchi, T., Kato, A., Sugahara, F., Shimazu, Y., Inoue, M., Kiyotani, K.,
600 Nagai, Y. and Yoshida, T. (2005). AIP1/Alix is a binding partner of Sendai virus
601 C protein and facilitates virus budding. J Virol 79, 8933-8941.
602 [52] Irie, T., Shimazu, Y., Yoshida, T. and Sakaguchi, T. (2007). The YLDL sequence

603 within Sendai virus M protein is critical for budding of virus-like particles and
604 interacts with Alix/AIP1 independently of C protein. J Virol 81, 2263-2273.
605 [53] Irie, T., Nagata, N., Yoshida, T. and Sakaguchi, T. (2008). Recruitment of
606 Alix/AIP1 to the plasma membrane by Sendai virus C protein facilitates budding
607 of virus-like particles. Virology 371, 108-120.
608 [54] Koyama, A.H., Irie, H., Kato, A., Nagai, Y. and Adachi, A. (2003). Virus
609 multiplication and induction of apoptosis by Sendai virus: role of the C proteins.
610 Microbes Infect 5, 373-378.
611

612 **Figure legends**

613 **Fig.1**

614 Effect of the SeV C mutant proteins on type I IFN JAK-STAT signaling, and their
615 STAT1-binding capacity. (A) Amino acid sequence of SeV C_{F170S} and C mutant (Cm)
616 proteins. Charged amino acids shown in bold letters have been replaced by A in a series of
617 the Cm proteins. F at the position 170 has been replaced by S in the C_{F170S} protein. (B) C
618 or one of the C mutants was transfected into HEK293T cells along with pISRE-TA-Luc
619 and pRL-TK. At 24 h post-transfection, cells were treated with IFN-α2b (1,000 U/ml) for
620 6 h, and then subjected to luciferase reporter assay. Mean values from three independent
621 experiments are shown with standard deviations as error bars. (C) HEK293T cells were
622 transfected with FLAG-tagged C or one of the C mutants. At 24 h post-transfection, cells
623 were treated with IFN-α2b (1,000 U/ml) for 24 h, and then infected with VSV at a
624 multiplicity of infection of 10. At 6 h post-infection, cells were lysed and subjected to
625 immunoblot analysis (IB) with anti-VSV antibody. (D) HEK293T cells were transfected
626 with FLAG-tagged C or one of the C mutants. At 24 h post-transfection, cells were lysed,
627 and then subjected to immunoprecipitation (IP) with anti-FLAG antibody followed by IB
628 with anti-FLAG or anti-STAT1 antibody. A portion of each whole cell lysate prepared for
629 IP was also subjected to IB.

630 **Fig.2**

631 Interaction of the SeV C protein with components of the type I IFN JAK-STAT pathway.
632 HEK293T (A, B) or U3A (C, D) cells were transfected with indicated plasmids. At 24 h
633 post-transfection, cells were lysed, and then subjected to IP with anti-FLAG (A, B) or
634 anti-V5 (C, D) antibody followed by IB with anti-FLAG, anti-C, anti-V5, or anti-STAT1
635 antibody. A portion of each whole cell lysate prepared for IP was also subjected to IB.

Fig.3

Interaction of the SeV C protein with IFNAR2 deletion mutants. (A and C) Schematic diagram of FLAG-tagged or GST-tagged IFNAR2 deletion mutants. SP; signal peptide, ED; extracellular domain, TM; transmembrane domain, CT; cytoplasmic tail. (B and D) HEK293T cells were transfected with indicated plasmids. At 24 h post-transfection, cells were lysed, and then subjected to IP with anti-FLAG antibody or GST pull-down assay followed by IB with anti-C or anti-FLAG antibody. A portion of each whole cell lysate prepared for IP or GST pull-down assay was also subjected to IB.

Fig.4

Interaction of the SeV C protein with JAK1 deletion mutants. (A) Schematic diagram of V5-tagged JAK1 deletion mutants. FERM; 4.1 protein, ezrin, radixin, moesin domain, SH2; src homology 2 domain. (B) HEK293T cells were transfected with indicated plasmids. At 24 h post-transfection, cells were lysed, and then subjected to IP with anti-V5 antibody followed by IB with anti-V5 or anti-FLAG antibody. A portion of each whole cell lysate prepared for IP was also subjected to IB.

Fig.5

Interaction between SeV C and signaling components synthesized *in vitro*. $JAK1_{859\text{-}1154}$-FLAG, $IFNAR2_{266\text{-}515}$-FLAG, and V5-C were synthesized by the wheat germ cell-free expression system. The *in vitro* transcription/translation products were mixed in various combinations and then subjected to IP with anti-FLAG or anti-V5 antibody followed by IB with anti-V5 or anti-FLAG antibody. A portion of *in vitro* transcription/translation products (shown as Input) was also subjected to IB.

Fig.6

Interaction of the SeV C mutant proteins with JAK1 or IFNAR2. JAK1-V5 (A) or

660 IFNAR2-V5 (B and C) was transfected into HEK293T cells along with FLAG-tagged P,
661 FLAG-tagged C, or one of the FLAG-tagged C mutants. At 24 h post-transfection, cells
662 were lysed and then subjected to IP with anti-FLAG antibody or anti-myc antibody
663 followed by IB with anti-FLAG or anti-V5 antibody. A portion of each whole cell lysate
664 prepared for IP was also subjected to IB. P; SeV phosphoprotein

665 **Fig.7**

666 Effect of the SeV C and C mutant proteins on STAT2-IFNAR2 or JAK1-IFNAR2
667 interactions. STAT2-V5 and FLAG-IFNAR2 (A) or JAK1-V5 and FLAG-IFNAR2 (B)
668 were transfected into HEK293T cells along with C or one of the C mutant proteins. At 24
669 h post-transfection, cells were lysed, and then subjected to IP with anti-V5 antibody
670 followed by IB with anti-FLAG or anti-V5 antibody. A portion of each whole cell lysate
671 prepared for IP was also subjected to IB.

672 **Fig.8**

673 Effect of the SeV C mutant proteins on IFN-α-stimulated tyrosine-phosphorylation of
674 STAT1, STAT2, JAK1, and TYK2. Indicated plasmids were transfected into HEK293T
675 cells along with pIRESpuro3 carrying the puromycin-resistant gene. At 24 h
676 post-transfection, cells were incubated in a medium containing puromycin (10 μg/ml) for
677 24 h. After removal of puromycin, surviving cells were treated with IFN-α2b (1,000
678 U/ml) for 15 min, and then subjected to IB with anti-phospho-JAK1, anti-JAK1,
679 anti-phospho-TYK2, anti-TYK2, anti-phospho-STAT1, anti-STAT1,
680 anti-phospho-STAT2, anti-STAT2, anti-FLAG, or anti-GAPDH antibody.

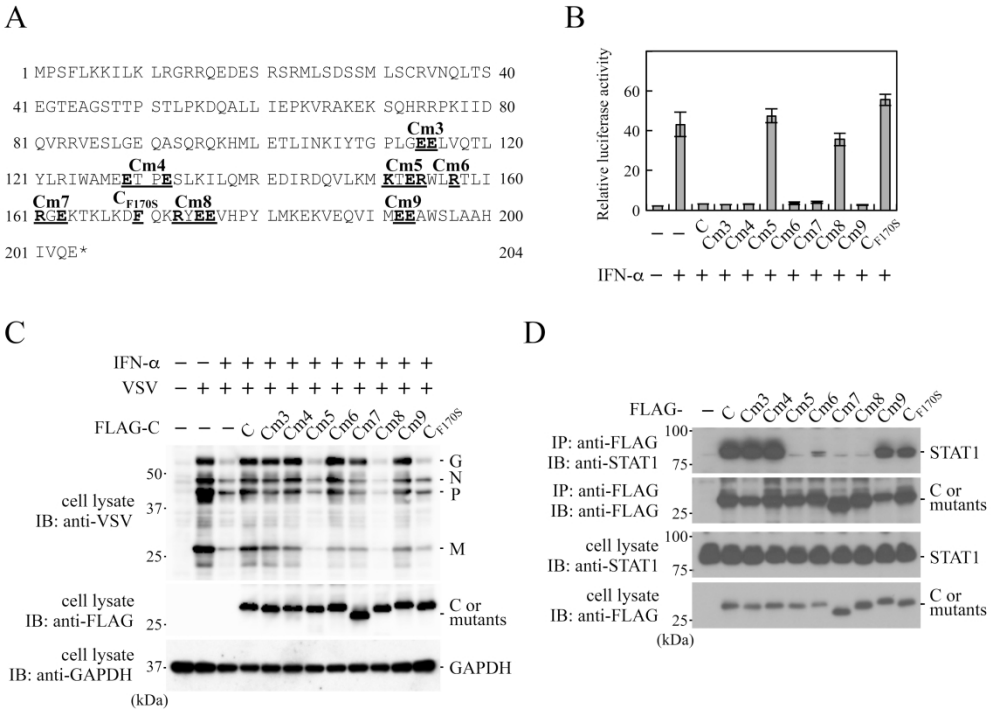
681 **Fig.9**

682 Effect of HPIV1-C, HPIV3-C, and BPIV3-C on type I IFN JAK-STAT signaling, and
683 their interaction with signaling components. (A) Indicated plasmids were transfected into

HEK293T cells along with pISRE-TA-Luc and pRL-TK. At 24 h post-transfection, cells were treated with IFN-α2b (1,000 U/ml) for 6 h, and then subjected to luciferase reporter assay. Mean values from three independent experiments are shown with standard deviations as error bars. (B-D) HEK293T cells were transfected with indicated plasmids. At 24 h post-transfection, cells were subjected to IP with anti-FLAG antibody followed by IB with anti-STAT1, anti-FLAG, or anti-V5 antibody. A portion of each whole cell lysate prepared for IP was also subjected to IB. (E) Indicated plasmids were transfected into HEK293T cells along with pIRESpuro3. At 24 h post-transfection, cells were incubated in a medium containing puromycin (10 μg/ml) for 24 h. After removal of puromycin, surviving cells were treated with IFN-α2b (1,000 U/ml) for 15 min, and then subjected to IB with indicated antibodies. SeV-V; a translation product of the V mRNA, which is transcribed from the SeV P gene through a process known as RNA editing.

Figure 1

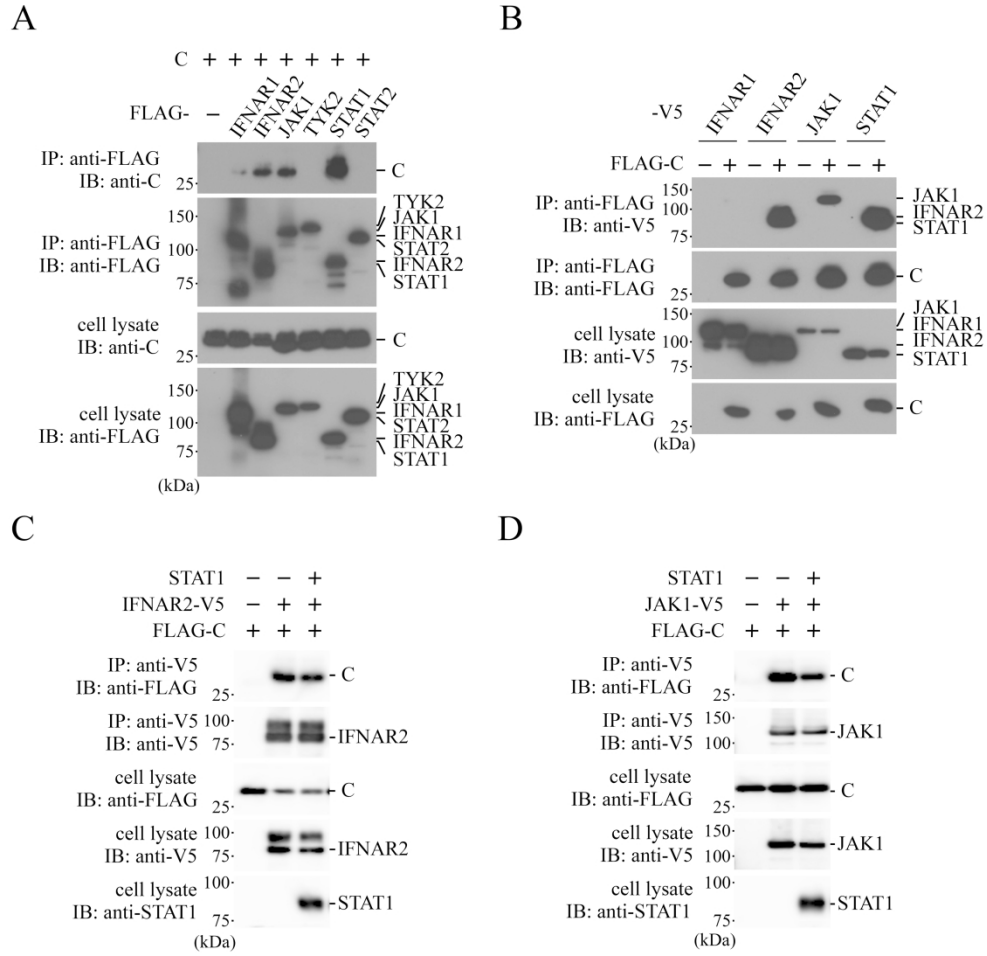
Kitagawa, Y., *et al.*,



Effect of the SeV C mutant proteins on type I IFN JAK-STAT signaling, and their STAT1-binding capacity.

175x137mm (600 x 600 DPI)

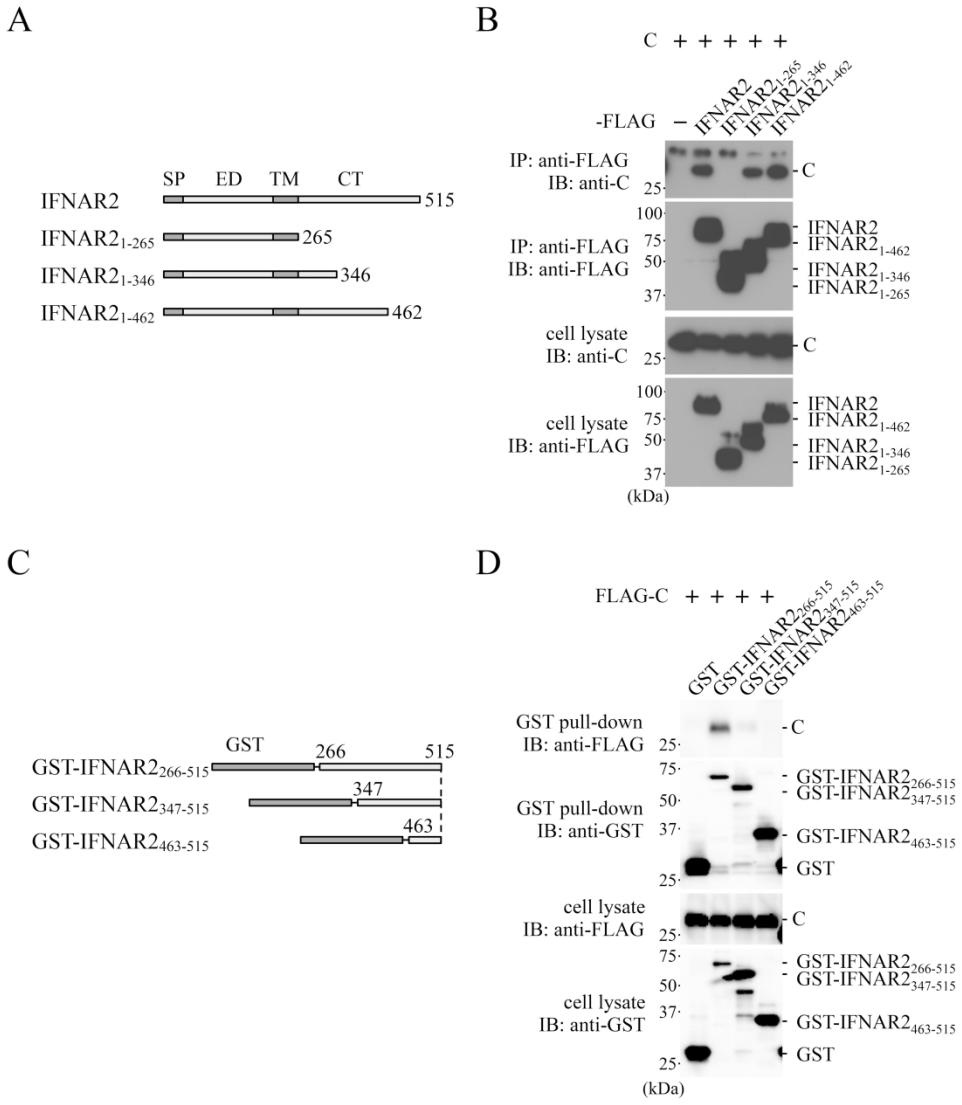
Figure 2 Kitagawa, Y., *et al*.,



Interaction of the SeV C protein with components of the type I IFN JAK-STAT pathway.

156x162mm (600 x 600 DPI)

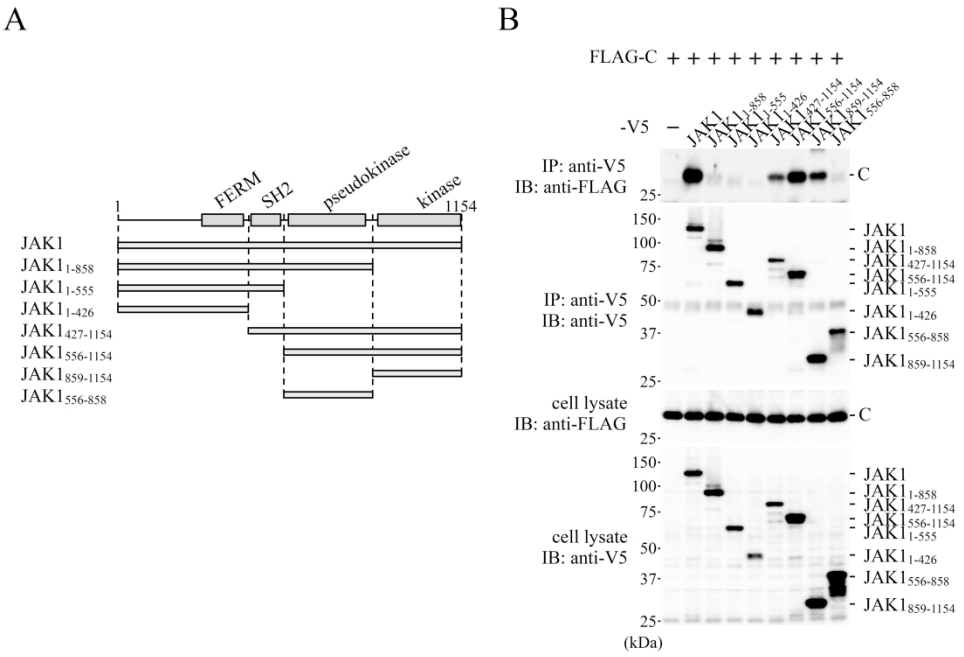
Figure 3 Kitagawa, Y., *et al.*,



Interaction of the SeV C protein with IFNAR2 deletion mutants.

156x187mm (600 x 600 DPI)

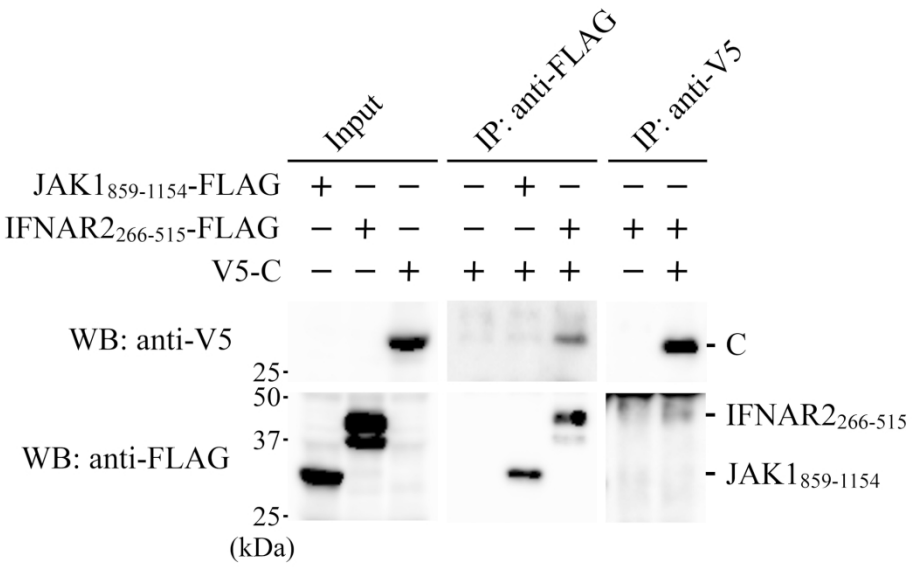
Figure 4 Kitagawa, Y., *et al.*,



Interaction of the SeV C protein with JAK1 deletion mutants.

168x125mm (600 x 600 DPI)

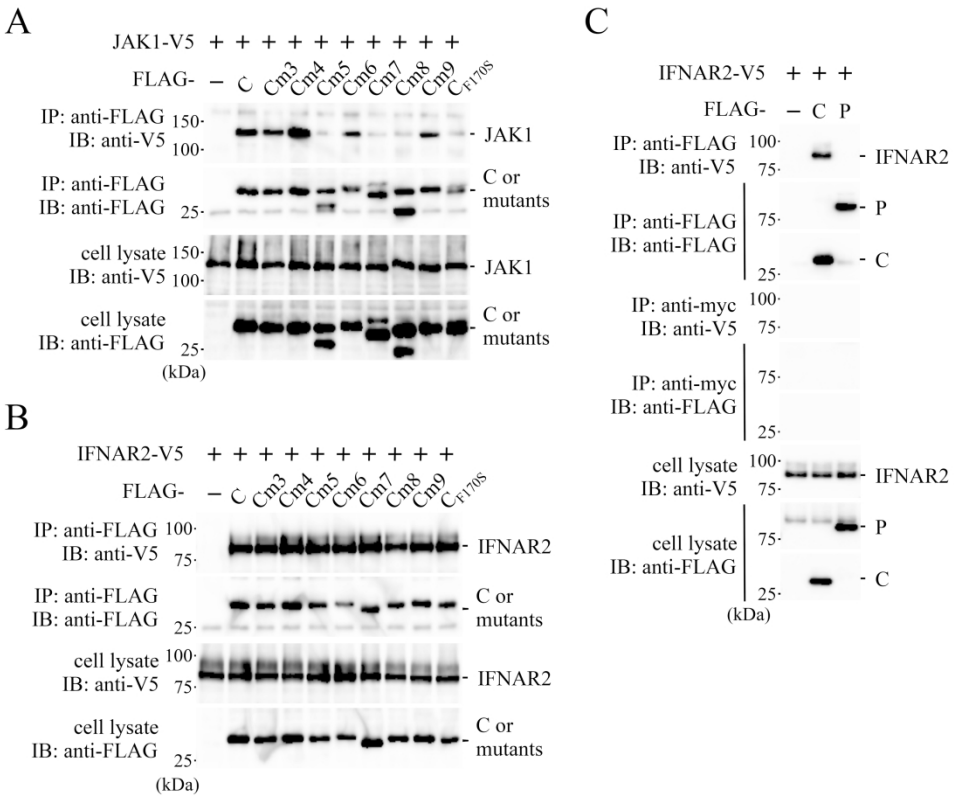
Figure 5 Kitagawa, Y., *et al*.,



Interaction between SeV C and signaling components synthesized in vitro.

100x75mm (600 x 600 DPI)

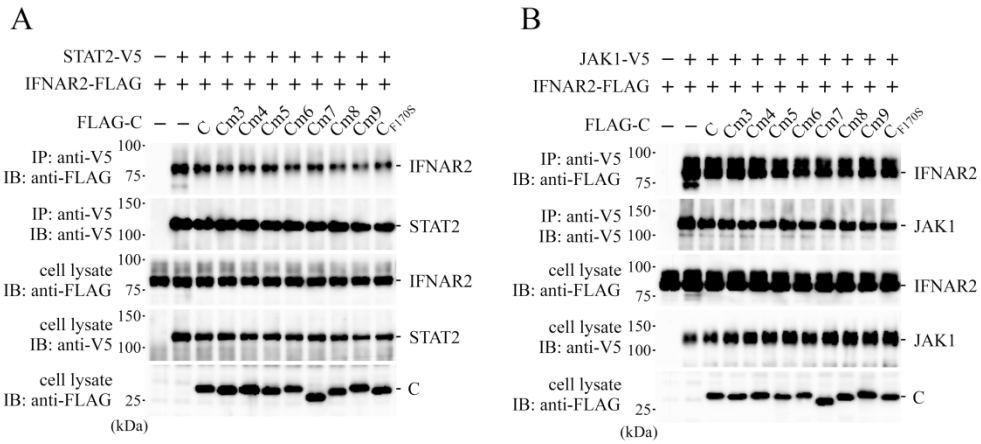
Figure 6 Kitagawa, Y., *et al*.,



Interaction of the SeV C mutant proteins with JAK1 or IFNAR2.

149x137mm (600 x 600 DPI)

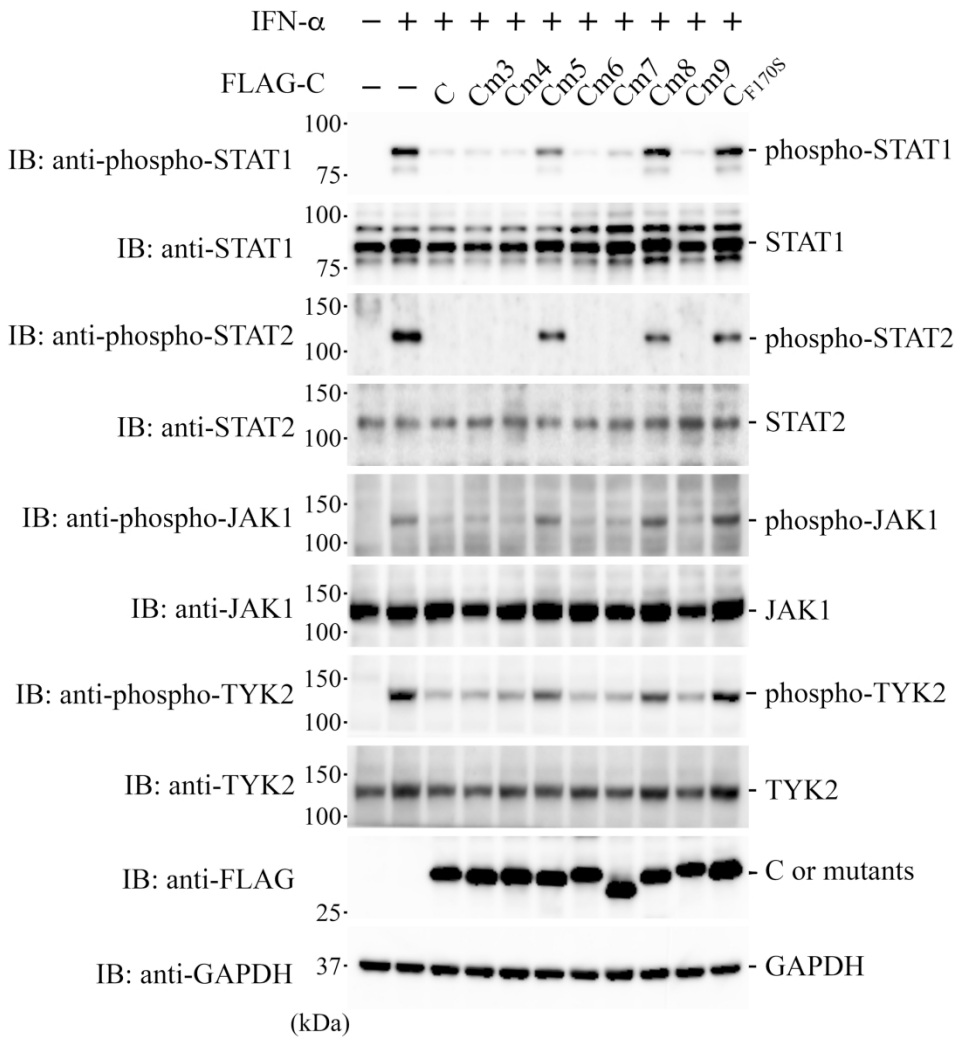
Figure 7 Kitagawa, Y., *et al*.,



Effect of the SeV C and C mutant proteins on STAT2-IFNAR2 or JAK1-IFNAR2 interactions.

175x93mm (600 x 600 DPI)

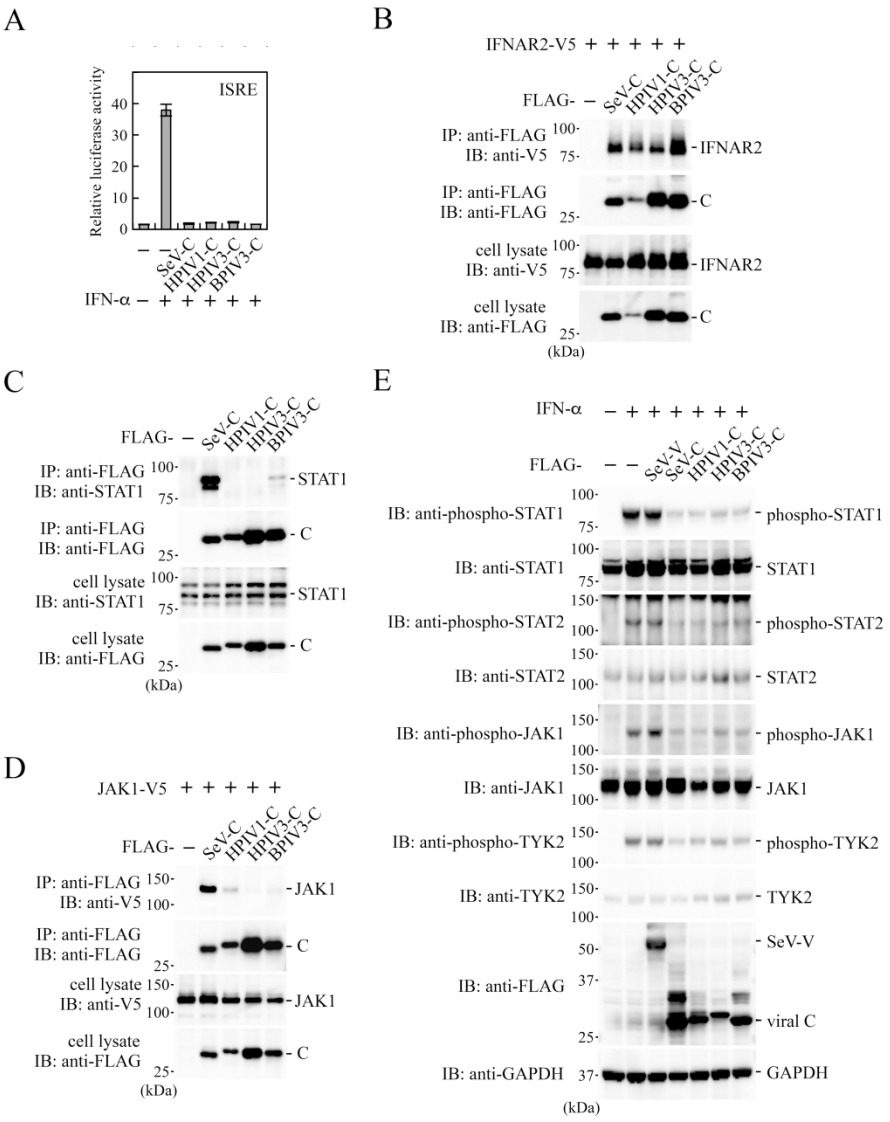
Figure 8 Kitagawa, Y., *et al.*,



Effect of the SeV C mutant proteins on IFN-α-stimulated tyrosine-phosphorylation of STAT1, STAT2, JAK1, and TYK2.

106x131mm (600 x 600 DPI)

Figure 9 Kitagawa, Y., *et al*.,



Effect of HPIV1-C, HPIV3-C, and BPIV3-C on type I IFN JAK-STAT signaling, and their interaction with signaling components.

162x212mm (600 x 600 DPI)