Inhibition of the gamma interferon response by a Sendai virus C protein mutant with no STAT1-binding ability

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Abstract **Sendai virus C protein interacts with the signal transducer and activator of transcription (STAT) 1. This interaction is believed to be essential for the Sendai virus inhibition of the interferon (IFN) response. We here analyzed C^{F170S} (a C protein mutant with the F170S mutation) with no STAT1-binding ability. C^{F170S} lacked the ability to inhibit the IFN-α response, but retained the ability to inhibit the IFN-γ response. IFN-γ stimulation caused STAT1 phosphorylation, formation of the gamma-activated factor capable of binding to a gamma-activated sequence DNA probe, and STAT1 nuclear translocation, even in the presence of C^{F170S}. These results suggest that C protein has the STAT1-binding-independent anti-IFN-γ mechanism, which targets processes after the STAT1 nuclear translocation event.**

Keywords: Sendai virus; C protein; STAT1; Gamma-interferon; Immune system; Evasion

1. Introduction

Interferons (IFNs) are cytokines, which play a pivotal role in the host defense against virus infection. To antagonize the host IFN system, viruses in the *Paramyxovirinae* have acquired the ability to block IFN signaling during evolution [1–4]. Viral proteins responsible for this anti-IFN function are accessory proteins, V and/or C proteins encoded by the P gene. Most, if not all, of the paramyxovirus IFN antagonists (V and/or C proteins) interact with the signal transducer and activator of transcription (STAT) 1, a key component common to both IFN-α/β and IFN-γ signaling pathways, and the STAT1-interaction appears to be essential for their anti-IFN function. For example, V protein of simian virus 5, mumps virus or human parainfluenza virus type 2 interacts with host cellular molecules including STAT1, thereby inhibiting IFN signaling through proteasome-mediated degradation of STAT1 or STAT2 [5–14]. On the contrary, interaction of Nipah virus or Hendra virus V protein with STAT1 does not lead to STAT degradation but results in the inhibition of the STAT nuclear translocation, thereby blocking IFN signaling [15,16]. Similarly, Sendai virus (SeV) C protein also interacts with STAT1 [17,18] and blocks IFN signaling [19,20], but the C-STAT1 interaction never results in STAT1 degradation in many cell types including HeLa, HEC-1B, and U118 cell lines [6,17,19–23] except for some cell types [18,24–26]. The aim of this study is to better understand how SeV C protein inhibits the IFN response without degrading STAT1.

IFN-α/β binds to type I IFN receptor, causing phosphorylation of STAT1 on Tyr^{701} and of STAT2 on Tyr^{690} following activation of receptor-associated JAK kinases, JAK1 and TYK2 [27]. The tyrosine phosphorylated (pY) STAT1 and pY-STAT2 form a heterodimer, translocate into the nucleus, and combine with IFN regulatory factor (IRF)-9 to form a heterotrimer complex termed IFN-stimulated gene factor 3 (ISGF3). On the other hand, binding of IFN-γ to Type II IFN receptor causes phosphorylation of STAT1 on Tyr^{701} following activation of receptor-associated kinases, JAK1 and JAK2. The pY-STAT1 forms a homodimer termed gamma-activated factor (GAF) and translocates into the nucleus. The ISGF3 or GAF then binds to IFN-stimulated response elements (ISREs) or gamma-activated sequence (GAS) sites, respectively, in the promoters of the IFN-stimulated genes (ISGs). Maximal activation of the GAF-mediated transcription requires phosphorylation of STAT1 on Ser^{727} besides Tyr^{701} [28]. The phosphorylated STAT1 is eventually inactivated through dephosphorylation by nuclear phosphatases, such as TC45 (the nuclear isoform of the ubiquitously expressed T-cell protein tyrosine phosphatase) [29] or SHP-2 (SH2 domain-containing protein tyrosine phosphatase 2) [30], rapidly relocated back to the cytoplasm and takes part in subsequent activation–inactivation cycle [31–33].

SeV C protein is encoded by the C open reading frame (ORF) that overlaps the P ORF in +1 frame on the P gene transcripts [34]. The SeV C ORF produces a nested set of C′, C, Y1 and Y2, which are collectively referred to as C protein [35]. Translation of C′, C, Y1 and Y2 initiates at different positions, ^{81}ACG, ^{114}AUG, ^{183}AUG, and ^{201}AUG, respectively, but terminates at the identical position, ^{726}UAA. To elucidate significance of the C-STAT1 interaction for the inhibition of the IFN response, we

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Abbreviations: CBP, CREB-binding protein; DMEM, Dulbecco's minimum essential medium; EMSA, electrophoretic mobility shift assay; GAF, gamma-activated factor; GAS, gamma-activated sequence; GST, glutathione *S*-transferase; IFN, interferon; ISG, IFN-stimulated gene; IRF, IFN regulatory factor; ISGF3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response element; NTA, Ni–nitrilotriacetic acid; ORF, open reading frame; SOV, sodium orthovanadate; PAGE, polyacrylamide gel electrophoresis; pS, serine phosphorylated; pY, tyrosine phosphorylated; SDS, sodium dodecyl sulfate; SeV, Sendai virus; STAT, signal transducer and activator of transcription

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created truncated C fragments, as well as a C protein mutant, C^{F170S} [19,36–38], which has a single amino acid substitution of F to S at position 170 (F170S mutation) on the STAT1-binding domain [39]. Analysis of these mutants showed a good correlation between the STAT1-binding ability and the ability to inhibit the IFN-α response and further suggested that the STAT1-interaction contributed to the inhibitory effect on IFN-α-stimulated phosphorylation of STAT1 and STAT2. This analysis also demonstrated that the near-complete inhibition of tyrosine phosphorylation of STAT2 was crucial for the blockade of IFN-α signaling [39]. For the inhibition of the IFN-γ response, it was found that the C-terminal half fragment of C protein prevented the GAF from binding to a GAS DNA probe in vitro [40]. This result suggests that C protein prevents formation of the GAF capable of binding to GAS sites though interacting with STAT1. All these findings suggest importance of the STAT1-C interaction for the SeV inhibition of the IFN response. However, during analysis of the C mutant proteins, we unexpectedly found that C^{F170S}, which has no STAT1-binding ability, retained the ability to inhibit the IFN-γ response.

In this article, we study on a molecular basis for the anti-IFN-γ mechanism of C^{F170S}. The results suggest that SeV C protein can inhibit the IFN-γ response even without interacting with STAT1 and this novel STAT1-interaction-independent mechanism targets processes after the STAT1 nuclear translocation event.

2. Materials and methods

2.1. Cells

HeLa cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum. HeLa-C (H-C) and HeLa-C^{F170S} (H-C^{F170S}) cells were maintained in DMEM supplemented with 10% fetal calf serum and 0.8 mg/ml geneticin (G418) [39].

2.2. Reporter gene assay

Cells in a 96 well plate were transfected with 30 ng of either pGAS-TA-Luc or pISRE-TA-Luc (Clontech), and 5 ng of pRL-TK-luc (Clontech) together with 30 ng of pEFneo empty vector [41] (a gift from H. Asao), pEFneo-C or pEFneo-C^{F170S} [39] by using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals). At 20 h post-transfection, the cells were mock-treated or treated with either human recombinant IFN-γ (500 IU/ml) (R&D Systems Inc., Minneapolis, MN) or IFN-α-2a (1000 IU/ml; Takeda Chemical Industries, Osaka, Japan) for 6 h and measured for luciferase activities by using a Dual-Glo Luciferase assay system (Promega) according to the manufacturer's protocol. Firefly luciferase activity, expressed in relative light units, was normalized to *Renilla* luciferase activity.

2.3. Western blot analysis

Cells were lysed in an extraction buffer (10 mM HEPES, pH 7.9, 300 mM NaCl, 0.25% NP-40, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate (SOV; Na_3VO_4), and 1 mM DTT) containing a protease inhibitor cocktail (Sigma) [17] unless otherwise mentioned. Western blot analysis was performed, as described previously [17], with an anti-pY-STAT1 (No. 9171), (Cell Signaling Technology, Beverly, MA), anti-serine phosphorylated (pS)-STAT1 (No. 06-802) (Upstate Biotechnology, NY), anti-STAT1 (sc-346 or sc-464), anti-IRF-1 (sc-497) (Santa Cruz Biotechnology, CA) or anti-RGS-His (No. 34610) (Qiagen) antibody.

2.4. Pull down assay with glutathione S-transferase fusion proteins

Glutathione *S*-transferase (GST), GST fusion C (GST-C) and GST fusion C^{F170S} (GST-C^{F170S}) were purified from *Escherichia coli* as described previously [39]. Extracts (~150 μg) from HeLa cells treated with IFN-γ (500 IU/ml) for 1 h were mixed with 10 μl of 50% slurry of GST, GST-C or GST-C^{F170S} (2–4 μg) conjugated glutathione–Sepharose beads (Amersham Pharmacia Biotech) and incubated for 1 h at 4 °C with gentle rotation. The beads were then washed four times with the extraction buffer. Proteins bound to the beads were eluted by adding sodium dodecyl sulfate (SDS)-gel loading buffer and separated by SDS–7.5% polyacrylamide gel electrophoresis (PAGE).

2.5. Pull down assay with Ni–nitrilotriacetic acid beads

Cells were lysed in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.25% NP-40, and 5 mM 2-mercaptoethanol) containing the protease inhibitor cocktail. The lysates clarified by centrifugation (250 μg) were incubated with Ni–nitrilotriacetic acid (NTA) beads (Qiagen) at 4 °C for 1–2 h. The beads were then washed four times with the lysis buffer containing 20 mM imidazole. Proteins bound to the beads were eluted by adding SDS-gel loading buffer and separated by SDS–7.5%PAGE.

2.6. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with a ^{32}P-labeled synthetic DNA probe derived from GAS of the human IRF-1 gene [42] as previously described [17,40].

3. Results

3.1. The F170S mutation does not abolish the anti-IFN-γ ability of C protein

We previously showed that SeV C protein interacted with STAT1 through its C-terminal half domain (aa 85–204) [40]. The F170S mutation on this C-terminal region abolished not only the STAT1-binding ability but also the ability to inhibit the IFN-α response [18,24,39]. This finding suggests importance of the C-STAT1 interaction for the inhibition of the IFN-α response. To clarify its significance for the inhibition of the IFN-γ response, we compared the effect of C^{F170S} and original C on activation of the IFN-γ-responsive reporter gene. HeLa cells were transfected with either empty vector (pEFneo), C-expression vector (pEFneo-C), or C^{F170S}-expression vector (pEFneo-C^{F170S}) together with an IFN-γ-responsive reporter plasmid (pGAS-TA-Luc) and the induction of luciferase activity was assayed after IFN-γ treatment. Contrary to our expectation, activation of the IFN-γ-responsive promoter was significantly suppressed by C^{F170S}, as well as C, although the suppression by C^{F170S} was slightly weaker (Fig. 1A). This result conflicted with the previous data presented by Garcin et al. [24]. To confirm the anti-IFN-γ ability of C^{F170S}, we next examined the effect of C^{F170S} on the IFN-γ-mediated induction of ISG products such as IRF-1 and STAT1 by using previously established H-C and H-C^{F170S}, which are HeLa cell lines that stably express $RGSH_6$ epitope tagged C and C^{F170S}, respectively [39]. The expression levels of C and C^{F170S} were almost the same (Fig. 1B). Levels of IRF-1 and STAT1 in each cell line before and after IFN-γ treatment were estimated by Western blot analysis (Fig. 1C). Consistent with the result of the reporter gene experiment, induction of STAT1 and IRF-1 was significantly suppressed in both H-C and H-C^{F170S}, although C^{F170S} again appeared to be slightly inferior in suppression. Taken together, these results demonstrate that C^{F170S} retains the anti-IFN-γ ability almost comparable to that of C. This unexpected finding urged us to reexamine the effect of C^{F170S} on the IFN-α response. As shown in Fig. 1D, response of H-C^{F170S} to IFN-α was closer to that of the control HeLa rather than that of H-C. In addition, notable elevation of the STAT1 level was observed in H-C^{F170S} after IFN-α treatment (Fig. 1E). These results confirm the previous conclusion that C^{F170S} lacks most of the anti-IFN-α ability [39].

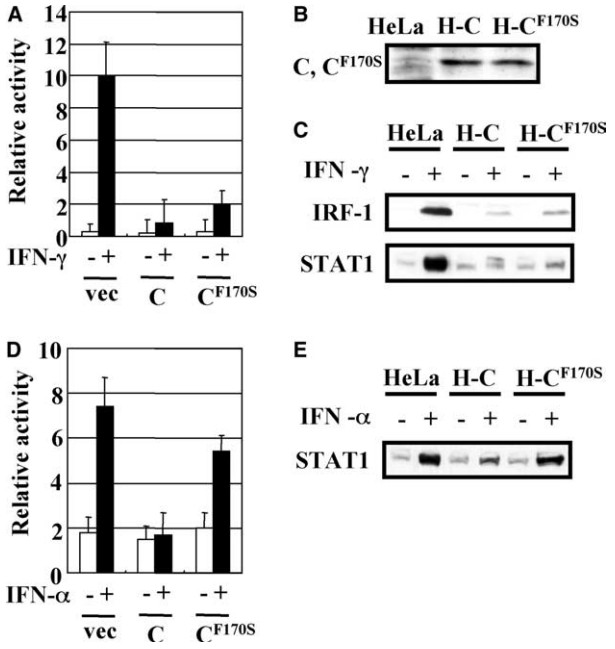

Fig. 1. Effect of C and C^{F170S} on the IFN response. Panels A and D: HeLa cells were transfected with empty pEFneo vector (vec), pEFneo-C or pEFneo-C^{F170S} together with pRL-TK-luc and either pGAS-TA-Luc (Panel A) or pISRE-TA-Luc (Panel D). Cells were then mock-treated or treated with IFN-γ (500 IU/ml) (Panel A) or IFN-α (1000 IU/ml) (Panel D) for 6 h. Luciferase activity was measured as described in Section 2. Data represent the mean values of the normalized luciferase activities from quadruplicate samples. Panel B: The extracts from HeLa, H-C or H-C^{F170S} cells were subjected to SDS–12.5%PAGE for Western blot analysis with an anti-RGS His antibody. Panels C and E: HeLa, H-C, and H-C^{F170S} cells were mock-treated (–) or treated with IFN-γ (500 IU/ml) (Panel C) or IFN-α (1000 IU/ml) (Panel E) for 16 h. Proteins in the cell extracts were separated by XV Pantera system (SDS-7.5%-Perfect NT Gel) (DRC, Tokyo, Japan) for Western blot analysis with an anti-IRF-1 or anti-STAT1 (sc-464) antibody. Results are representatives of three independent experiments.

3.2. Effect of C^{F170S} on IFN-γ-stimulated phosphorylation of STAT1

Discovery of the anti-IFN-γ activity of C^{F170S} raised an essential question of how C^{F170S} inhibited the IFN-γ response without interacting with STAT1. We first examined the effect on IFN-γ-stimulated phosphorylation of STAT1. HeLa, H-C, and H-C^{F170S} cells were treated with IFN-γ for various periods and the levels of pY-STAT1, pS-STAT1 and STAT1 were estimated by Western blot analysis (Fig. 2). The level of pY-STAT1 in the control HeLa cells reached a maximum at 2–4 h of IFN-γ stimulation and then gradually declined thereafter. In contrast, in H-C and H-C^{F170S}, pY-STAT1 continued to accumulate and persisted at higher levels for at least 24 h of IFN-γ stimulation (Fig. 2). The reason why pY-STAT1 accumulated in the presence of C or C^{F170S} will be discussed later. In all cell lines, STAT1 was serine-phosphorylated in response to IFN-γ stimulation, although the levels of pS-STAT1 in H-C on the whole appeared to be slightly lower (Fig. 2). These results indicate that neither C nor C^{F170S} inhibits IFN-γ stimulated phosphorylation of STAT1 on both Tyr^{701} and Ser^{727}.

3.3. Does C^{F170S} specifically bind to pY-STAT1?

Garcin et al. [26] have recently shown that C^{F170S} does not bind to unphosphorylated STAT1 but binds to pY-STAT1, and emphasized importance of the N-terminal region present only in the larger forms, C′ and C, for the C-pY-STAT1 interaction. If this is the case, it is possible that this specific interaction between C^{F170S} and pY-STAT1 may be involved in the inhibition of the IFN-γ response. However, we had previously obtained conflicting data [39]. The pull down assay of IFN-α-treated cell extracts with GST-C^{F170S} had showed that C^{F170S} did not bind to either unphosphorylated STAT1 or pY-STAT1. Nevertheless, this experiment does not completely exclude the possibility that C^{F170S} may interact with pY-STAT1 generated by IFN-γ stimulation, because pY-STAT1 in the IFN-γ-treated cell extract is present predominantly as a homodimer, whereas it is present as the heterotrimer ISGF3 or STAT1–STAT2 heterodimer in the IFN-α-treated cell extract. Thus, we performed a pull down assay using IFN-γ-treated HeLa cell extracts. As shown in Fig. 3A, both STAT1 and pY-STAT1 bound to GST-C, whereas neither of them bound to GST-C^{F170S}. Similar results were obtained under the conditions where sodium salt concentration in the binding and washing buffers was reduced from 300 to 100 mM (data not shown).

GST fusion proteins purified from *E. coli* may be different, in conformation, from native C synthesized in mammalian cells. Thus, we also conducted a pull down assay using Ni–NTA beads and extracts from H-C and H-C^{F170S} that express native $RGSH_6$ epitope tagged C and C^{F170S}, respectively. Both STAT1 and pY-STAT1 were pulled down again from H-C cell extracts, whereas neither of them was pulled down from H-C^{F170S} cell extracts (Fig. 3B and C). These results ensure no specific interaction of C^{F170S} with STAT1.

3.4. C^{F170S} does not inhibit either the GAF formation or STAT1 nuclear translocation

We next examined the effect of C^{F170S} on the downstream IFN-γ signaling of the STAT1 phosphorylation event. After the phosphorylation event, pY-STAT1 forms a homodimer, termed GAF, capable of binding to a GAS site. EMSA with a

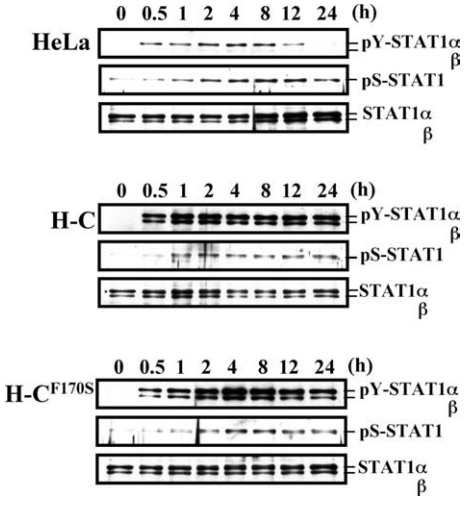

Fig. 2. Effect of C and C^{F170S} on IFN-γ-stimulated phosphorylation of STAT1. HeLa, H-C, or H-C^{F170S} cells were treated with IFN-γ (500 IU/ml) and then harvested at the indicated time points. Proteins in the cell extracts were separated by SDS–7%PAGE for Western blot analysis with an anti-pY-STAT1 antibody, anti-pS-STAT1 antibody and anti-STAT1 (sc-346) antibody.

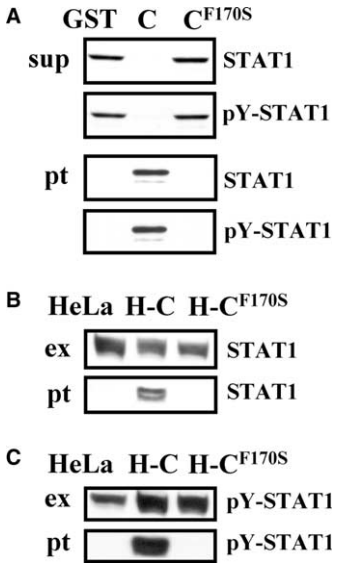

Fig. 3. C^{F170S} does not bind to either unphosphorylated STAT1 or pY-STAT1. Panel A: IFN-γ-treated HeLa cell extracts were incubated with GST, GST-C, or GST-C^{F170S} immobilized on glutathione–Sepharose beads for 1 h. The mixtures were then divided by centrifugation into supernatant (sup) and pellet (pt) fractions. Proteins in each fraction were separated by SDS–7.5%PAGE for Western blot analysis with an anti-STAT1 (sc-464) or anti-pY-STAT1 antibody. Panel B: Extracts (ex) from HeLa, H-C or H-C^{F170S} cells were subjected to pull down assay with Ni–NTA beads. Ni–NTA beads were collected by centrifugation as a pellet (pt) fraction. Panel C: The extracts from HeLa, H-C, or H-C^{F170S} cells treated with IFN-γ (500 IU/ml) for 1 h were subjected to pull down assay as in Panel B. Proteins were separated by the XV Pantera system for Western blot analysis with an anti-STAT1 (sc-464) (Panel B) or anti-pY-STAT1 antibody (Panel C).

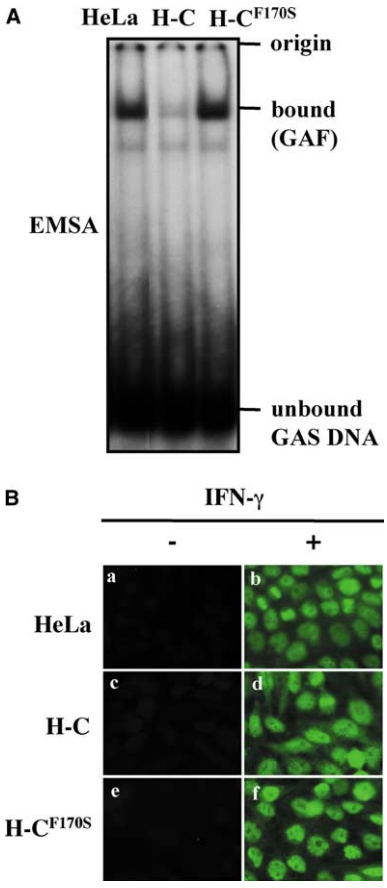

Fig. 4. C^{F170S} does not inhibit either formation of the GAF or the STAT1 nuclear translocation. Panel A: HeLa, H-C or H-C^{F170S} cells were treated with IFN-γ (500 IU/ml) for 1 h. The cell extracts were subjected to EMSA with a ^{32}P-labeled GAS probe. Panel B: HeLa, H-C, or H-C^{F170S} cells were mock-treated (–) or treated with IFN-γ (1000 IU/ml) for 3 h and then fixed. Cells were stained by the immunofluorescent staining method with an anti-pY-STAT1 antibody according to the manufacturer's protocol (Cell Signaling Technology, Beverly, MA).

^{32}P-labeled GAS DNA probe confirmed a previous result; disappearance of the GAF band in the presence of C (Fig. 4A) [17,40]. In contrast, GAF complex was formed in the presence of C^{F170S}, which could bind to the GAS DNA probe (Fig. 4A). Upon IFN-γ stimulation, the GAF translocates into the nucleus. Immunofluorescent staining with an anti-pY-STAT1 antibody showed nuclear translocation of pY-STAT1 generated in response to IFN-γ stimulation even in the presence of C or C^{F170S} (Fig. 4B). Taken together, these results indicate that C^{F170S} targets processes after the STAT1 nuclear translocation event for the inhibition of the IFN-γ response.

3.5. *C^{F170S} retains the ability to inhibit the STAT1 dephosphorylation event*

To identify a target molecule of C^{F170S} for the inhibition of the IFN-γ response, proteins binding to $RGSH_6$ epitope tagged C^{F170S} or C were pulled down with Ni–NTA beads from large amounts of H-C^{F170S} or H-C cell extracts, and separated by SDS-gradient PAGE followed by silver-staining. Unfortunately, we failed to detect any specific band common to both H-C and H-C^{F170S} extracts.

Instead, we took notice of the aberrant accumulation of pY-STAT1 in response to IFN-γ stimulation as a common feature of C and C^{F170S} (Fig. 2). Since the overall level of pY-STAT1 is determined by balance of phosphorylation and dephosphorylation events, prolonged phosphorylation of STAT1 may result from either an increase in JAK kinase activity or a decrease in phosphatase activity. Indeed, the accumulation of pY-STAT1 in the presence of C had been previously found to be due to the inhibition of the pY-STAT1 dephosphorylation event [23]. We thus determined whether C^{F170S} retained this ability. To monitor the rate of STAT1 dephosphorylation, we used staurosporine, a kinase inhibitor that blocks the continuous phosphorylation of STAT1 by JAKs. After treatment with IFN-γ for 1 h, media were replaced with fresh media containing staurosporine (Fig. 5A). In HeLa cells, the pY-STAT1 level rapidly decreased to near a basal level within 1 h after addition of staurosporine. In contrast, the pY-STAT1 level in H-C or H-C^{F170S} maintained even 1 h after treatment with staurosporine. It should be noted that the levels of pY-STAT1 in H-C or H-C^{F170S} in the absence of staurosporine were higher than those in control HeLa. When HeLa cells were pre-incubated with a phosphatase inhibitor, SOV, and then treated with IFN-γ for 1 h in the presence of SOV, the pY-STAT1 level in control HeLa was enhanced, whereas no enhancement was observed in H-C or H-C^{F170S} (Fig. 5B). Taken together, these results indicate that both C and C^{F170S} have the common ability to inhibit STAT1 dephosphorylation.

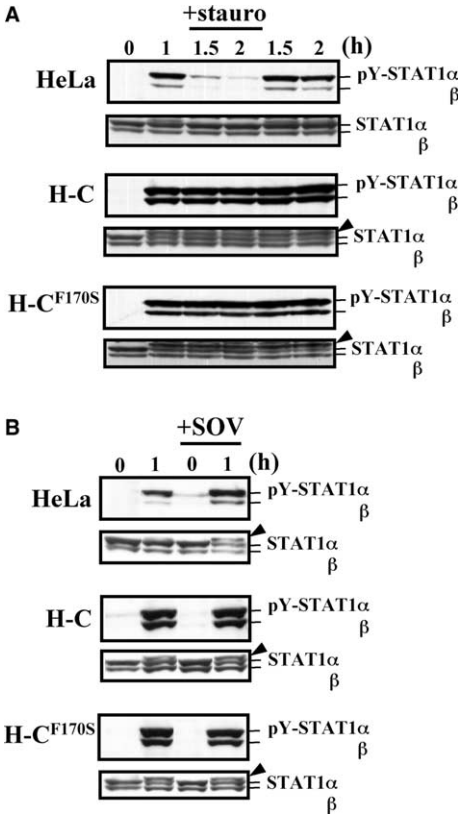

Fig. 5. C^{F170S} retains the ability to inhibit STAT1 dephosphorylation. Panel A: HeLa, H-C or H-C^{F170S} cells were stimulated with IFN-γ (500 IU/ml) for indicated time periods with or without addition of 100 nM staurosporine (stauro) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) after 1 h of IFN-γ treatment. Levels of pY-STAT1 and STAT1 were determined by Western blot analysis with an anti-pY-STAT1 or anti-STAT1 (sc-346) antibody. Panel B: HeLa, H-C or H-C^{F170S} cells were treated with IFN-γ (500 IU/ml) for indicated time periods with or without addition of 1 mM SOV before 1.5 h of the IFN-γ treatment. Extracts were analyzed as in Panel A. Arrowheads indicate positions of pY-STAT1 detected by anti-STAT1 antibody.

4. Discussion

In this article, we show that C protein can inhibit the IFN-γ response without interacting with STAT1. The F170S mutation on the STAT1-binding domain abolishes the STAT1-binding ability of C protein [18,39]. The abrogation of the STAT1-binding ability, however, does not abolish the ability to inhibit the IFN-γ response (Fig. 1). Regarding the STAT1-binding ability of C^{F170S}, Garcin et al. [26] presented conflicting data indicating the binding ability of C^{F170S} for pY-STAT1 but not unphosphorylated STAT1. They further claimed that the C-pY-STAT1 interaction required an N-terminal region spanning amino acids 10–15 within the longer form of C protein (C/C′). To determine whether C^{F170S} could bind to pY-STAT1, we conducted several experiments. The pull down assay with GST-C^{F170S} synthesized in *E. coli* or with $RGSH_6$ epitope tagged C^{F170S} synthesized in HeLa cells showed that STAT1 did not bind to C^{F170S} irrespective of its phosphorylation status (Fig. 3). The EMSA showed no shift of the GAF band in the presence of C^{F170S} (Fig. 4A), supporting the notion that there are no molecules interacting with the GAF. Furthermore, our previous study revealed that the N-terminally truncated C fragments – Y1 (aa 24–204), Y2 (aa 30–204) and D1 (aa 85–204) – retained the ability to bind to both unphosphorylated and phosphorylated STAT1 molecules [39,40]. This indicates that the N-terminal region (aa 10–15) of C protein is not essential for the C-STAT1 interaction. On the contrary, the STAT1 domain responsible for interacting with the D1 domain was found to be located at its N-terminal region but not its C-terminal region containing the phosphorylation site of Tyr^{701} [40]. All these results are consistent with the inability of C^{F170S} to bind to STAT1. At present, we cannot provide any reasonable explanations for the discrepancies between their and our results. The possibility cannot be ruled out that the difference in the cell type and/or SeV strain analyzed might affect the results.

The analysis of IFN-γ signaling in the presence of C^{F170S} (Figs. 2 and 4) demonstrates that C^{F170S} does not inhibit either STAT1 phosphorylation, formation of the GAF, or STAT1 nuclear translocation. Thus, the novel anti-IFN-γ mechanism, independent of the STAT1-binding, targets a process after the STAT1 nuclear translocation event; probably the GAF-mediated transcriptional process itself. It is likely that C^{F170S} interacts with an unknown cellular molecule that participates in and plays a crucial role in the GAF-mediated transcriptional process. Through interacting with that target molecule, C^{F170S} may prevent the GAF from binding to GAS sites in the chromosomal DNA or may inhibit the subsequent process. One of such candidates is CREB-binding protein (CBP)/p300, a cofactor that regulates the transactivation function of the GAF [43]. Co-precipitation experiments, however, failed to detect interaction between CBP/p300 and C (unpublished data). Human parainfluenza virus type 3 closely related to SeV also inhibits the IFN-γ response, but does not inhibit formation of the GAF complex [6]. No shift of the GAF band was observed [6]. These results might be explainable, given that the target molecule of human parainfluenza virus type 3 would be identical to that of SeV C^{F170S}.

To find a clue about how C^{F170S} would inhibit the GAF-mediated transcription, we took notice of a common feature of C and C^{F170S}; the accumulation of pY-STAT1 in response to IFN-γ stimulation (Fig. 2). The accumulation was found to be at least in part due to the inhibition of the STAT1 dephosphorylation event (Fig. 5). Table 1 summarizes features of mutant C proteins, including truncated C proteins previously characterized, Y1, Y2, D1, D2 (aa 127–204), and D3 (aa 30–126) [39]. There is a good correlation between the ability to inhibit the IFN-γ response and the ability to cause the pY-STAT1 accumulation. Accordingly, the inhibition of the

Table 1
Features of C mutant proteins

	STAT1-binding ability	Accumulation of pY-STAT1	Inhibition of the IFN-γ response
C	+	+	+
C^{F170S}	–	+	+
Y1[a]	+	+	+
Y2[a]	+	+	+
D1[a]	+	+	+
D2[a]	–	–	–
D3[a]	–	–	–

C (aa 1–204), Y1 (aa 24–204), Y2 (aa 30–204), D1 (aa 85–204), D2 (aa 127–204), D3 (aa 30–126).
[a] Results from [39,40].

STAT1 dephosphorylation event may be related to the novel STAT1-binding-independent anti-IFN-γ mechanism. It is unclear, at present, whether the inhibition of the STAT1 dephosphorylation is essential for the inhibition of the STAT1-binding-independent mechanism, or only an epiphenomenon.

Previous studies showed that the C-terminal half fragment of C protein had the ability to prevent the GAF from binding to a GAS element in vitro. This finding suggests that C protein blocks IFN-γ signaling by inhibiting formation of the GAF capable of binding to the GAS probe through the STAT1 interaction. C protein thus appears to inhibit the IFN-γ response at two different phases; the formation of the GAS-binding-active GAF (STAT1-binding-dependent phase) and the subsequent process after the STAT1 nuclear translocation (STAT1-binding-independent phase). The inferiority of C^{F170S} to C in the IFN-γ ability (Fig. 1A and C) suggests significance of the STAT1-binding dependent phase for the anti-IFN-γ ability. However, to precisely assess its significance, isolation of C protein mutants will be needed, which retain the STAT1-binding ability but lose the latter STAT1-binding-independent mechanism.

As shown in Fig. 1D and E, C^{F170S} has lost most of the anti-IFN-α ability [39], but appears to still retain a slight inhibitory effect on the IFN-α response. This slight inhibition was reproducibly observed. It is possible that this inhibition may also result from the novel STAT1-binding-independent mechanism. Finally, the possibility should be kept in mind, although unlikely, that C^{F170S} might have acquired the STAT1-binding-independent mechanism, which original C might not have.

Which molecule is a target of the STAT1-binding-independent mechanism? Identification of the target would be important for full understanding of not only the SeV strategy for evading the IFN response but also mechanism of the GAF-mediated transcription.

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