The C-terminal half-fragment of the Sendai virus C protein prevents the gamma-activated factor from binding to a gamma-activated sequence site

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Received 6 May 2003; returned to author for revision 13 June 2003; accepted 18 July 2003

Abstract

Sendai virus C protein associates with the signal transducer and activator of transcription (STAT) 1 and inhibits the interferon (IFN) response. We report a molecular basis for the anti-IFN-γ mechanism of Sendai virus. The C-terminal half-fragment of the C protein (D1) retains both the STAT1-binding and the anti-IFN-γ abilities comparable to those of the full-size C. IFN-γ stimulation generates phosphorylated-STAT1 even in the presence of the C or the D1. The phosphorylated-STAT1 generated in the D1-expressing cells forms an aberrant complex, which does not bind to a γ-activated sequence (GAS) probe. Purified D1, indeed, inhibits in vitro the binding of the phosphorylated-STAT1 dimer to the GAS probe. The D1, however, binds to the STAT1 N-terminal domain, but not the DNA binding domain. These results suggest the possibility that the C protein prevents the γ-activated factor from binding to GAS elements through its interaction with the STAT1 N-terminal domain.

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Keywords: Paramyxovirus; Sendai virus; Interferon; Signaling; C protein; Gamma-activated factor; Gamma-activated sequence; STAT1

Introduction

Paramyxoviruses evolve their own strategies for antagonizing the host interferon (IFN) system (for reviews, see Garcia-Sastre, 2001; Goodbourn et al., 2000; Gotoh et al., 2001, 2002). For members of the Rubulavirus genus, virus-encoded V proteins function as an IFN antagonist (Didcock et al., 1999b; Kawano et al., 2001; Kubota et al., 2001; Nishio et al., 2001; Parisien et al., 2001) and inhibit IFN signaling by targeting the signal transducer and activator of transcription (STAT) 1 or STAT2 for proteasome-mediated degradation (Andrejeva et al., 2002; Didcock et al., 1999a, 1999b; Fuji et al., 1999; Kubota et al., 2001; Nishio et al., 2001; Parisien et al., 2001; Young et al., 2000). Depending on their target molecules, the STAT1-targeting viruses (simian viruses 5 and 41, and mumps virus) inhibit both IFN-α/β and IFN-γ signaling, whereas the STAT2-targeting virus (human parainfluenza virus type 2) inhibits only IFN-α/β signaling. On the other hand, Sendai virus (SeV) of the Respirovirus genus, uses a C protein instead of the V protein for circumventing the IFN response (Garcin et al., 1999; Gotoh et al., 1999). SeV C protein inhibits both IFN-α/β and IFN-γ signaling, but never causes degradation of STATs like the Rubulavirus V protein in most cell types (Garcin et al., 1999; Gotoh et al., 1999, 2003; Kato et al., 2001; Komatsu et al., 2000; Takeuchi et al., 2001, 2002, 2003). The SeV C protein produces a variety of effects on STATs: suppression of IFN-stimulated phosphorylation of STATs (STAT1, STAT2, and STAT3) (Komatsu et al., 2000); impairment of STAT1 dephosphorylation (Komatsu et al., 2002); and induction of formation of a STAT1 high molecular mass complex (HMMC) (Takeuchi et al., 2001). Of these effects, it has been recently found that the inhibition of
STAT2 tyrosine phosphorylation is crucial for the blockade of IFN-α signaling (Gotoh et al., 2003). In contrast to the anti-IFN-α mechanism, a molecular basis for the SeV anti-IFN-γ mechanism has not been fully understood yet. This study is designed to shed light on how the SeV C protein inhibits IFN-γ signaling.

IFN-γ signaling is initiated with the binding of dimeric IFN-γ to the IFN-γ receptor. The binding causes transphosphorylation and activation of the receptor associated kinases, JAK1 and JAK2 (for review, see Stark et al., 1998). The activated JAKs phosphorylate an IFN-γ receptor subunit, IFNGR1, on Tyr140, which provides a docking site for the SH2 domain of STAT1 (Greenlund et al., 1994, 1995; Igarashi et al., 1994). Upon binding to the docking site, STAT1 is phosphorylated on Tyr701 (Schindler et al., 1992; Shuai et al., 1992, 1993). The tyrosine-phosphorylated STAT1 (pY-STAT1) forms a homodimer termed γ-activated factor (GAF) through a reciprocal interaction between the SH2 domain and the phosphorylated Tyr701, then translocates into the nucleus, and binds to γ-activated sequence (GAS) elements to activate IFN-stimulated genes (ISGs). Two species of STAT1, STAT1α and STAT1β, are present in cells as a result of differential splicing but only STAT1α functions as a component of active GAF. Because STAT1β lacks the STAT1α C-terminal transactivation domain (TAD) (38 amino acids) containing Ser727. Phosphorylation of STAT1α on Ser727 is indeed essential for the maximal activation of transcription by GAF (Wen et al., 1995). Transcriptional activation function of GAF further requires the interaction between STAT1 and cyclic-AMP response element binding protein (CREB)-binding protein (CBP)/p300 (Zhang et al., 1996).

The SeV C protein is expressed as a nested set of four proteins, C′, C, Y1, and Y2, from overlapping open reading frames in the P/V/C gene transcripts (Curran and Kolakofsky, 1989; Giorgi et al., 1983). Translation of the C′, C, Y1, and Y2 is initiated at 81AGC, 114AUG, 183AUG, and 201AUG, respectively, and terminated at the same position, UAA228. Even the shorter forms Y1 and Y2 are capable of inhibiting the IFN response (Garcin et al., 2000; Kato et al., 2001). SeV not only inhibits IFN-α stimulated phosphorylation of STATs (Komatsu et al., 2000) but also affects IFN-γ stimulated phosphorylation of STAT1 (Komatsu et al., 2002; Takeuchi et al., 2001). The effects, however, are rather complicated. Tyrosine phosphorylation in response to short-term IFN-γ stimulation is suppressed at the early phase of infection, but the suppression is subsequently abrogated (Takeuchi et al., 2001). Consistent with the abrogation, the inhibition of tyrosine phosphorylation is not observed when a sufficient amount of the C protein is constitutively expressed in HeLa cells in the absence of the other viral proteins (Komatsu et al., 2002; Saito et al., 2002). Interestingly, once the pY-STAT1 level is elevated in either infected cells or the C-expressing cells, the high level of pY-STAT1 is maintained for a long time. This is explainable in part due to the suppressive effect of the C protein on dephosphorylation of pY-STAT1 (Komatsu et al., 2002). SeV infection also affects serine phosphorylation kinetics of STAT1 but allows STAT1 to be doubly phosphorylated on Tyr701 and Ser727 (Komatsu et al., 2002). This assures us that the C protein targets processes after the STAT1 phosphorylation for the inhibition of the IFN-γ response. The C protein physically associates with STAT1 in infected cells (Garcin et al., 2002; Takeuchi et al., 2001), resulting in formation of a STAT1 HMHC (over 2 MDa) (Takeuchi et al., 2001). This HMHC cannot be detected as a single band by native polyacrylamide gel electrophoresis (PAGE) analysis because it is too large to enter a native gel (Takeuchi et al., 2001). Therefore, detection of no GAF band in the extract containing the C protein by electrophoretic mobility shift assays (EMSAs) does not imply the loss of the GAS binding ability of the C-associated pY-STAT1, but rather indicates retention of pY-STAT1 molecules in a gel well (Takeuchi et al., 2001).

Under these circumstances, we attempted to determine the binding capacity of pY-STAT1 associated with the C or truncated C proteins, for GAS elements. The results demonstrate that the C-terminal half-fragment (aa 85–204) of the C protein, with both the STAT1 binding and the anti-IFN-γ abilities comparable to the full-size C, inhibits binding of GAF to a GAS element. We thus propose a hypothesis that the C proteins (C, Y1, and Y2) prevent GAF from binding to GAS elements through their interaction with STAT1.

Results

Feature of the C-STAT1 complex

The C protein inhibits the IFN-γ response by targeting processes downstream of the STAT1 phosphorylation (Komatsu et al., 2002; Takeuchi et al., 2001). This finding prompted us to examine the binding capacity of the C-associated pY-STAT1 for GAS elements or CBP/p300. However we were confronted with a difficulty in this analysis because the native PAGE analysis could not detect the C-associated STAT1 as a band (Takeuchi et al., 2001). The C-associated STAT1 complex (over 2 MDa) is too large to enter a native gel and consequently remains in a gel well. This implies that the binding capacity of the C-associated pY-STAT1 for GAS probe or CBP/p300 cannot be evaluated by EMSAs, whose electrophoresis and gel conditions are the same as those of the native PAGE.

To overcome this problem, we attempted to select a shorter C fragment, which could bind to STAT1 but allow STAT1 to enter a native gel, from the truncated C proteins previously generated (Gotoh et al., 2003). The C (aa 1–204), Y1 (aa 24–204), and Y2 (aa 30–204) are original products translated from the P/C/V gene transcripts in infected cells (Fig. 1). The D1 (aa 85–204) and D2 (aa 127–204) are N-terminally truncated proteins of the C, while the D3 (aa
30–126) is a C-terminally truncated protein of the Y1 (Fig. 1). These proteins are constitutively expressed as N-terminal RGSH6 (RGS-His) epitope-tagged proteins in HeLa cells by establishment of permanent cell lines (Gotoh et al., 2003). The C, Y1, Y2, and D1 that contain a C-terminal half of the C protein not only bind to STAT1 (Fig. 1) but also inhibit IFN-γ signaling (Gotoh et al., 2003). Native PAGE analysis of these cell lines revealed no conspicuous STAT1 band in the C-, Y1-, or Y2-expressing cells (Fig. 2A, lanes 2–4). In contrast, sodium dodecyl sulfate (SDS)–PAGE analysis showed expression of nearly equal amounts of STAT1 among the cell lines (Fig. 2B). These results suggested that the Y1 and Y2 had the STAT1-HMMC-inducing capacity similar to that of the C. On the other hand, a supershifted STAT1 band was seen in the D1-expressing cells (Fig. 2A, lane 5). Unfortunately, we failed to detect each of the truncated C proteins, including the D1 on this membrane, due to a high background in the immunodetection with the anti-RGS-His antibody. An anti-C antibody also did not work well to detect truncated-C specific bands, because the D1, D2, and D3 were hardly recognized by the anti-C antibody (data not shown), and the C, Y1, and Y2 associating with STAT1 probably remain at gel wells due to their STAT1-HMMC formation capacity. To obtain the data supporting that the supershift of the STAT1 band resulted from association with the D1, the following alternative experiments were conducted. First, a pull-down assay of the extracts was performed with Ni-nitrilotriacetic acid (NTA) beads that specifically bind to His epitope-tagged proteins. STAT1 was pulled down specifically from the C-, Y1-, Y2-, and D1-expressing cell extracts (Fig. 2C, lanes 2–5), indicating association of STAT1 with the C, Y1, Y2, or D1 in the extracts. Second, HeLa cell extracts were mixed in vitro with RGS-His epitope-tagged D1 (His-D1) or dihydrofolate reductase (DHFR) (His-DHFR) purified from Escherichia coli (E. coli) and then subjected to native PAGE analysis (Fig. 2D). This analysis showed that purified D1 shifted the STAT1 band to the position similar to that seen in the D1-expressing cell extract (Fig. 2D, lane 2; Fig. 2A, lane 5). These data indirectly support the idea that the supershifted band represents a STAT1–D1 complex. This property of the STAT1-D1 complex led us to a bright prospect for examination of the binding capacity of the D1-associated STAT1 to a GAS probe by EMSAs. It is unclear at present why the STAT1-D1 complex migrated significantly faster than the C-, Y1-, or Y2-STAT1 complex. One possible explanation is that the C, Y1, and Y2 form homocomplexes but the D1 is monomeric. Unfortunately, a pull-down assay with glutathione-sepharose beads and mixtures consisting of various combinations of purified His-C fragments (His-C, His-Y1, His-Y2, and His-D1) and glutathione-S-transferase (GST) fusion C fragments (GST-C, GST-Y1, GST-Y2, and GST-D1) could not provide positive evidence for this hypothesis (data not shown). This issue was not further investigated in this study.

The D1 inhibits the IFN-γ response as effectively as the full-size C

We next checked the effect of the truncated C proteins including the D1 on the IFN-γ response. The cells were
transfected with the IFN-γ-responsive luciferase reporter plasmid, and the induction of luciferase was assayed after treatment with IFN-γ (Fig. 3A). Fig. 3A clearly demonstrated that the C, Y1, Y2, and D1 inhibited the IFN-γ response, while the D2 and D3 did not. Similar results were obtained from the transient expression experiments, in which each of the truncated C proteins without RGS-His epitope tag was transiently expressed by transfection with RGS-His epitope tag minus truncated-C plasmids previously created (Gotoh et al., 2003 and data not shown). The expression levels of the truncated-C proteins in the established cell lines were distinct from one another, as shown in Fig. 3B. The level of the Y1 was the highest, whereas the levels of the C, Y2, D1, and D3 were 0.37-, 0.76-, 0.58-, and 0.60-fold as high as that of the Y1. Since the expression level of the D3 is comparable to that of the D1, the inability of the D3 to inhibit the IFN-γ response was attributable to its intrinsic property. However, the possibility could not be excluded that the lack of the inhibitory effect of the D2 is due to its low expression level, since the D2 was too small in amount and could be detected only after long exposure (Gotoh et al., 2003 and data not shown). To confirm the effect of the truncated-C proteins on IFN-γ-stimulated transcription, levels of IRF-1 as an ISG product before and after IFN-γ treatment were also examined by Western blot analysis. As shown in Fig. 3C, induction of IRF-1 was suppressed in the C-, Y1-, Y2-, and D1-expressing cells (Fig. 3C, lanes 3–10). The suppression of IRF-1 induction by the C, Y1, Y2, and D1 was incomplete, but the incompleteness was also observed in SeV-infected cells (Fig. 3D). It is unclear at present whether this incompleteness represents inability of the C protein to inhibit an alternative STAT1-independent IFN-γ pathway (Ramana et al., 2002) or a simple signaling leak on the STAT1-dependent pathway. Nevertheless, these results demonstrated that the C-terminal half of the C protein, the D1, was sufficient for exerting the anti-IFN-γ effect of the full-size C or SeV infection.

**Effect of the truncated-C proteins on IFN-γ stimulated phosphorylation of STAT1**

The C and the truncated-C proteins with the STAT1-binding ability suppress IFN-α stimulated phosphorylation of STAT1 to various degrees (Gotoh et al., 2003). This naturally demanded examination of their effects on IFN-γ-stimulated phosphorylation of STAT1. Treatment of HeLa cells with IFN-γ resulted in rapid tyrosine phosphorylation of STAT1 (Fig. 4A, mock, lanes 2–4). The elevated pY-STAT1 level decreased from 4 h posttreatment (pt) and returned nearly to the basal level by 24 h pt. The decrease in the pY-STAT1 level is explained by dephosphorylation of pY-STAT1 by a nuclear phosphatase (Haspel and Darrell, 1999; Haspel et al., 1996). In SeV-infected cells, tyrosine phosphorylation pattern was strikingly changed (Fig. 4A, SeV). When infected cells were treated with IFN-γ at 2 h postinfection (pi), the immediate elevation of the pY-STAT1 level was dramatically inhibited (Fig. 4A, lane 10). The suppression of the pY-STAT1 level by the C, Y1, Y2, and D1 was complete after 4 h pt. However, the suppression of the pY-STAT1 level by the D2 and D3 was incomplete after 4 h pt. It is unclear whether this incompleteness represents inability of the D2 and D3 to inhibit an alternative STAT1-independent IFN-γ pathway (Ramana et al., 2002) or a simple signaling leak on the STAT1-dependent pathway. Nevertheless, these results demonstrated that the C-terminal half of the C protein, the D1, was sufficient for exerting the anti-IFN-γ effect of the full-size C or SeV infection.
STAT1 level (Fig. 4A, mock, lanes 2–4) was remarkably suppressed (Fig. 4A, SeV, lanes 2–4). Instead the pY-STAT1 level inversely increased from 4 hpi (Fig. 4A, SeV, lanes 5–7). The suppressive effect on the immediate phosphorylation was not seen, when infected cells were treated with IFN-γ/H9253 at the middle phase of infection (Takeuchi et al., 2001). Consistent with this observation, IFN-γ/H9253-stimulated tyrosine phosphorylation was not inhibited in a HeLa cell line constitutively expressing a sufficient amount of the C protein (Komatsu et al., 2002). The abrogation of the inhibitory effect at the middle phase of infection partly explained the paradoxical increase in the pY-STAT1 level from 4 hpi (Fig. 4A, SeV, lanes 5–7). One should be reminded that the dephosphorylation process of pY-STAT1 is also impaired in infected cells, as well as the C-expressing cells (Komatsu et al., 2002). Since the pY-STAT1 level is determined by the balance of tyrosine phosphorylation and dephosphorylation turnovers, this impairment necessarily contributes to the prolongation of the high pY-STAT1 level in infected cells (Fig. 4A, SeV, lanes 5–7) or the C-expressing cells (Komatsu et al., 2002). These complicated effects of SeV infection and the C protein urged us to examine the effect on STAT1 phosphorylation under the two conditions; short-term and long-term IFN-γ stimulation.

First, levels of pY-STAT1, STAT1 phosphorylated on Ser 727 (pS-STAT1), and STAT1 in the cell lines after short-term (1 h) IFN-γ treatment were examined by Western blot analysis (Takeuchi et al., 2001). Consistent with the previous results (Komatsu et al., 2002), the C did not inhibit tyrosine phosphorylation (Fig. 4Ba, lanes 1–4). In contrast, the Y1, Y2, and D1 suppressed tyrosine phosphorylation (Fig. 4Ba, lanes 5–10). On the other hand, IFN-γ-stimulated serine phosphorylation was inhibited in all the truncated-C proteins with the STAT1 binding ability (the C, Y1, Y2, and D1) (Fig. 4Bb, lanes 3–10). The D2 and D3 with no STAT1-binding ability did not inhibit phosphorylation on both serine and tyrosine.
Phosphorylated on Tyr^701 and Ser^727 by short-term or long-term IFN-γ/STAT1 were generated by short-term IFN-γ-infected cells (Fig. 4A, SeV). Since small amounts of pY-D1-expressing cells (Fig. 4Bd, lanes 3), the elevation of the pY-STAT1 levels in the C-, Y1-, Y2-, and D1-expressing cells (Fig. 4Bd, lanes 3–10), as seen in SeV-infected cells (Fig. 4A, SeV). Since small amounts of pY-STAT1 were generated by short-term IFN-γ stimulation even in the Y1-, Y2-, and D1-expressing cells (Fig. 4Ba, lanes 2–5), the density of both the pY-STAT1 dimer and the GAF bands is expected to decrease in these cell lines. Indeed both bands became faint in the C-, Y2-, and D1-expressing cells (Fig. 5AB, lanes 2, 4, and 5) or disappeared in the Y1-expressing cells (Figs. 5A and B, lane 3). A pull-down assay of the extracts with Ni-NTA beads demonstrated association of the C, Y1, Y2, and D1 with the pY-STAT1 dephosphorylation process. The pY-STAT1 generated in these cell lines was also phosphorylated on Ser^727 (Fig. 4Be, lanes 4, 6, 8, and 10). In conclusion, the C and all the truncated-C proteins with the anti-IFN-γ ability allowed STAT1 to be doubly phosphorylated on Tyr^701 and Ser^727 by short-term or long-term IFN-γ stimulation. These results supported the previous conclusion that the target of the C protein for the inhibition of the IFN-γ response is present in processes after STAT1 phosphorylation.

The aberrant pY-STAT1 complex generated in the presence of the D1 does not appear to bind to the GAS DNA probe

The extracts used in Fig. 4Babc were subjected to native PAGE for Western blot analyses with anti-pY-STAT1 antibody (Fig. 5A) and to EMSAs with ^32P-labeled GAS probe (Fig. 5B). Since the C, Y1, Y2, and D1 cause STAT1 retention at gel wells or a supershift of STAT1 band (Fig. 2A, lanes 2–5), the density of both the pY-STAT1 dimer and the GAF bands is expected to decrease in these cell lines. Indeed both bands became faint in the C-, Y2-, and D1-expressing cells (Fig. 5AB, lanes 2, 4, and 5) or disappeared in the Y1-expressing cells (Figs. 5A and B, lane 3). A pull-down assay of the extracts with Ni-NTA beads demonstrated association of the C, Y1, Y2, and D1 with the pY-STAT1 (Fig. 5D, lanes 2–5). The levels of the associated pY-STAT1 (Fig. 5D, lanes 2–5) corresponded to those of pY-STAT1 present in the extracts (Fig. 5C, lanes 2–5). Importantly, the densities of the faint STAT1 dimer and GAS bands observed in the C-, Y2-, and D1-expressing cells (Figs. 5A and B, lanes 2, 4, and 5) were in inverse correlation with the expression levels of the C, Y2, and D1 proteins (Fig. 3B). This suggested that these faint bands represented populations of pY-STAT1 molecules, which did not associate with each of the truncated C proteins. Remarkably, native PAGE analysis revealed another faint band, which showed an aberrant pY-STAT1 complex, close to the top in the D1-expressing cells (Fig. 5A, lane 5, an arrow). However, EMSAs did not show any apparent band at the corresponding position (Fig. 5B, lane 5). This suggested that the aberrant pY-STAT1 complex did not bind to the GAS DNA probe.

The purified D1 inhibits binding of the pY-STAT1 homodimer to the GAS probe in vitro

To test the hypothesis that the D1 could deprive pY-STAT1 homodimers of the GAS-binding ability by inducing the aberrant pY-STAT1 complex formation, in vitro assays were performed using the purified D1. The D1 was purified from E. coli as GST fusion protein (GST-D1) or RGS-His epitope-tagged D1 (His-D1). Extracts from the IFN-γ-treated HeLa cells (containing the pY-STAT1 dimer) were mixed with GST-D1, GST, His-D1, or His-DHFR and incubated for 30 min. The mixtures were then subjected to native-PAGE (Figs. 6A and C) or EMSAs (Figs. 6B and D). One microgram of GST-D1 shifted the pY-STAT1 band (Fig. 6A, lane 2), whereas the same amount of GST did not (Fig. 6A, lane 7). A decrease in the amount of GST-D1 resulted in a supershift of the shifted pY-STAT1 band (Fig. 6A, lanes 3–6). On the contrary, an EMSA revealed no binding of these supershifted bands to ^32P-labeled GAS probe (Fig. 6B, lanes 2–6). His-D1 also caused a supershift of the pY-STAT1 band (Fig. 6C, lanes 2–7), whereas His-DHFR did not (Fig. 6C, lane 8). The positions of the pY-STAT1 bands shifted by addition of 2−4 or 2−5 μg of His-D1 appeared to be same as that of the aberrant pY-
STAT1 complex generated in the D1-expressing cells (Fig. 6C, lanes 6 and 7; Fig. 5A, lane 5, an arrow). An EMSA revealed no band (Fig. 6D, lanes 4–7) or only very faint bands (Fig. 6D, lanes 2 and 3) at these positions. These results demonstrated the ability of the purified D1 to inhibit the binding of the pY-STAT1 dimers to the GAS probe.

The D1 binds to the N-terminal domain of STAT1

To understand how the D1 inhibits the GAF-GAS binding we attempted to determine the STAT1 domain responsible for interacting with the D1. Functional domains of STAT1α and STAT1β are shown in Fig. 7A. STAT1, N-terminally truncated STAT1, and C-terminally truncated STAT1 fragments (Fig. 7A) were synthesized in the presence of [35S]methionine by the in vitro transcription and translation system (Fig. 7B). The [35S]methionine-labeled STAT1 fragments were mixed with GST-D1 or GST and subjected to pull-down assays with glutathione-sepharose beads. All the STAT1 fragments that lost the N-terminal domain (NTD) did not bind to GST-D1 (Fig. 7C, lanes 3–10), indicating that the D1 interacted with none of the DNA binding domain, the SH2 domain, and the domain containing Tyr701. On the other hand, all the C-terminally truncated STAT1 fragments, including the NTD fragment, could bind to GST-D1 (Fig. 7C, lanes 11–16). The binding was specific, because GST did not bind to [35S]methionine-labeled STAT1 fragments (Fig. 7C, lanes 2, 12, 14, and 16). These results demonstrated that the D1 associated with STAT1 through its interaction with the NTD.

Discussion

The present study proposes a hypothesis that the C protein prevents GAF from binding to GAS elements by binding to STAT1. The in vitro binding assays demonstrate that the STAT1 domain responsible for interacting with the D1 is the NTD but not the SH2 domain nor the region containing Tyr701 (Fig. 7). Not only the extract containing the C, Y1, and Y2 but also the purified D1 can convert the pY-STAT1 homodimer, which has been already formed in the extract, to the high molecular pY-STAT1 complex in vitro (Figs. 6A and C) (Takeuchi et al., 2001). Furthermore, the pY-STAT1 generated by IFN-γ stimulation efficiently translocates into the nucleus in SeV-infected cells (Komatsu et al., 2002). Since nuclear transport of pY-STAT1 requires dimerization of pY-STAT1 rather than tyrosine phosphorylation itself (Mowen and David, 1998), all these findings support the idea that the C protein does not interfere with dimerization of pY-STAT1 through the reciprocal interaction between the SH2 domain and the phosphorylated Tyr701 (Shuai et al., 1994). From this, it follows that the crucial target of the C protein for the inhibition of the IFN-γ response is likely present in processes after pY-STAT1 homodimerization. We here examined the effect of the D1 instead of the full-size C on the binding capacity of the pY-STAT1 for the GAS site and demonstrated the inhibitory activity of the D1 for the GAF-GAS binding. The D1 retains both the STAT1-binding and the anti-IFN-γ abilities, comparable to those of the full-size C (Fig. 3) (Gotoh et al., 2003). This full anti-IFN-γ capacity of the D1 is also supported by the previous finding that even a shorter N-terminally truncated fragment (aa 99–204) exhibits the anti-IFN activity (Kato et al., 2002). As a natural consequence of these findings, it is likely that the full-size C has the ability to prevent GAF from binding to GAS elements. It is unclear at present how the D1 inhibits the binding of the pY-STAT1 complex to the GAS probe. It is unlikely that the D1 and GAS probe competitively bind to STAT1, because the D1 interacts with the NTD far from the DNA binding domain of STAT1 (Fig. 7). Interestingly, the purified His-D1 prevented GAF from binding to the GAS probe, even if added to the extract after addition of the GAS probe (Gotoh et al., 2003).
unpublished result). Accordingly, the D1 may induce conformational change of the GAF DNA binding domain through its interaction with the NTD.

The STAT1 NTD is an important region for the dephosphorylation process of pY-STAT1 (Shuai et al., 1996), as well as the STAT1-CBP/p300 interaction (Zhang et al., 1996), and the stabilization of a pair of STAT1 homodimers to cooperate in binding to tandem GAS sites (Vinkemeier et al., 1996; Xu et al., 1996). Accordingly, the interaction of the C protein with the STAT1 NTD may be related to the impairment of STAT1 dephosphorylation observed in infected cells or the C-expressing cells (Komatsu et al., 2002). The CBP/p300 interacts with two regions, the NTD and the transactivation domain (TAD), of STAT1 (Fig. 7A) and plays a crucial role in the transcriptional function of GAF (Zhang et al., 1996). One can naturally hypothesize that the C protein may inhibit the IFN-γ response by preventing CBP/p300 from participating in the transcriptional process of GAF. We tried to examine effect of the C or the D1 on the STAT1-CBP/p300 interaction, but failed in detecting the STAT1-CBP/p300 interaction itself in HeLa cell extracts. Even in vitro binding assays with a GST fusion CBP fragment and a GST fusion p300 fragment (GSTCBP571-687 and GSTp300/566-664) (a gift from Dr. R. Goodman), which were reported to bind to STAT1 (Zhang et al., 1996), could not provide positive evidence for the STAT1-CBP/p300 interaction.

The C, Y1, Y2, and D1 with the STAT1-binding ability transiently inhibited serine phosphorylation of STAT1 (Fig. 4Bb). Inhibition of the tyrosine phosphorylation was also seen with the exception of the C (Fig. 4Ba). On the contrary, the D2 and D3 with no STAT1-binding capacity never exhibited the inhibitory effects on both tyrosine and serine phosphorylation of STAT1. These results suggest that the C protein affects the STAT1 phosphorylation event through its interaction with STAT1. In infected cells, IFN-γ-stimulated tyrosine phosphorylation of STAT1 was suppressed at the early phase, but this suppression was subsequently abrogated (Takeuchi et al., 2001). Since the constitutively expressed C does not suppress IFN-γ-stimulated tyrosine phosphorylation

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Fig. 7. The C-terminal half domain of the C protein interacts with the N-terminal domain of STAT1. (A) A schematic diagram of STAT1α, STAT1β, and constructs of truncated STAT1 fragments. NTD, N-terminal domain; CCD, coiled coil domain; DBD, DNA binding domain; LD, linker domain; SH2, Src homology domain 2; TAD, transactivation domain. The numbered tyrosine (Y) and serine (S) residues show the positions of phosphorylation. (B and C) The STAT1 fragments were synthesized in the presence of [35S]methionine by the in vitro transcription and translation system and then separated by SDS–11% PAGE (B). The [35S]methionine-labeled translation products were mixed with GST-D1 (D1) or GST (G) and then incubated for 1 h. After extensive wash with the binding buffer, bound proteins were eluted in SDS sample buffer and then separated by SDS–11% PAGE. The gels were treated with Amplify, dried, and exposed to X-ray films for about 1 day (B) or 1 month (C).
phosphorylation of STAT1 (Fig. 4Ba, lane 4), we speculated that the major population of the four C proteins (C', C, Y1, and Y2) expressed in infected cells might shift from the Y1 and Y2 to the C during the progress of infection. We, however, found that the C was expressed constantly as the major species during the whole course of infection (Gotoh et al., unpublished result). It is therefore still unclear how SeV inhibits IFN-γ-stimulated tyrosine phosphorylation in the early phase of infection.

Garcin et al. have recently reported that two types of interaction between the C protein and STAT1 (Garcin et al., 2003). They showed that unphosphorylated STAT1 interacted with both the C and the C', whereas pY-STAT1 specifically interacted with the C but not CΔ10–15. The latter-type interaction, however, was not observed in our SeV-HeLa system. All the Y1, Y2, and D1, which lost the region containing amino acids 10 to 15, interacted with pY-STAT1 (Fig. 5, lanes 2–5). Pull-down assays with GST-C, GST-Y1, GST-Y2, and GST-D1 further showed equal binding abilities of the four GST proteins for both STAT1 and pY-STAT1 (Gotoh et al., unpublished result). The interaction of pY-STAT1 with the C, Y1, Y2, and D1 was observed even if pY-STAT1 is generated by IFN-α stimulation, as previously described (Gotoh et al., 2003). It is unclear how the discrepancy took place. Since the other domains, except for the NTD STAT1, is not required for interacting with the D1, it is of interest to understand how that CΔ10–15-containing the Y1, Y2, D1 region loses the binding ability for pY-STAT1.

There appears to be a small population of pY-STAT1 molecules, which does not associate with the C and which can bind to the GAS probe, in the C-expressing cells (Figs. 5A and B, lane 2). Nevertheless, the inhibition of the IFN-γ response in the C-expressing cells is comparable to that in the Y1 and Y2-expressing cells (Fig. 3). The possibility therefore cannot be ruled out completely that there is another mechanism besides the inhibition of the GAF-GAS binding. This possible mechanism is now under investigation.

Materials and methods

Cells and a virus

HeLa cells were maintained in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal calf serum. HeLa cell lines expressing the N-terminally RGS-His epitope-tagged C (aa 1–204), Y1 (aa 24–204), Y2 (aa 30–204), D1 (aa 85–204), D2 (aa 127–204), or D3 (aa 30–126) were maintained in DMEM supplemented with 10% fetal calf serum and 0.8 mg/ml geneticin (G418) (Gotoh et al., 2003). SeV (strain SeVpB) is propagated in the allantoic cavity of 10-day-old embryonated eggs as previously described (Yokoo et al., 1999).

Plasmid constructs

To create a pQE32-D1 plasmid expressing His-D1 in E. coli, the D1 DNA fragment purified from pTRE2-D1 (Gotoh et al., 2003) doubly digested with BamHI and HindIII was cloned into the corresponding site of pQE-32 (Qiagen). To generate plasmids expressing full-size STAT1 or truncated forms of STAT1 under the control of the T7 promoter, full-length and truncated STAT1 DNA fragments were amplified by PCR, using pEFBOS-HA-STAT1 (Nakajima et al., 1996) (a gift from Dr. T. Hirano) as a template. Forward primers, F1 (5′-AAGCTT BamH1 ggatccAG1A-TGTCTCAGTGTACGAATA-3′), F137 (5′-AAGC- TT BamH1 ggatccAGGACAAACACAAAGACTT-3′), F18 (5′-AAGCTT BamH1 ggatccAGGCGATTTGGTGTTAGAAAGACAG-CCC-3′), F188 (5′-AAGCTT BamH1 ggatccAG1465AC-TCCACCATGTGACAGAGA-3′), and F177 (5′-AAGC- TT BamH1 ggatccAG1729TCGATCAGGGCTCTACAT-3′), and a reverse primer R250 (5′-AAGCTT NotI gcggccgctTA2253CTATGACTGTTTCTAT-3′) were used for generating full-length STAT1 and N-terminally truncated forms of STAT1. Italics indicate nucleotide sequences that are not involved in a corresponding position of pcDNA3.1 vector (Invitrogen). Finally RGS-His epitope tag was appended to the N-terminus of the corresponding position of pcDNA3.1 vector (Invitrogen). Gotoh et al. have recently reported that two types of STAT1. The PCR-amplified DNA fragment was doubly digested with BamHI and NotI and then inserted into the corresponding position of pcDNA3.1 vector (Invitrogen). Finally RGS-His epitope tag was appended to the N-terminus of STAT1 fragments by insertion of annealed oligonucleotides (5′-TATGAGAGATGCACATCTACATCAGG-3′ and 5′-GATCCCCGATGAGTTGTTGACAGTGGCATCCTCCTCATAGTAC-3′) into the KpnI and BamHI sites in the pcDNA3.1-STAT1 fragment plasmids created above.

Purification of GST-D1 and His-D1 proteins from E. coli

GST, GST-D1, His-D1, and His-DHFR proteins were expressed in E. coli (BL21) transformed with pGEX-5X-1 (Amersham Pharmacia Biotech), pGEX-D1 (Gotoh et al., 2003), pQE32-D1, and pQE-40 (Qiagen), respectively, by adding isopropyl-β-thiogalactopyranoside (final concentration of 1 mM). GST and GST-D1 were purified with glutathione-sepharose beads as described previously (Gotoh et al., 2003), while His-D1 and His-DHFR were purified with Ni-NTA agarose beads (Qiagen) according to manufacturer protocol. The final eluate was dialyzed overnight in 50 mM Tris–HCl pH 7.5, 5 mM MgCl2, 100 mM NaCl, 10% glyce-
erol, 5 mM 2-mercaptoethanol and stored at −80°C until use.

**Preparation of a mammalian cell extract**

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 Na3VO4, 1 mM DTT) containing a protease inhibitor cocktail (Sigma) as described previously (Takeuchi et al., 2001). After centrifugation, the clarified supernatant was stored at −80°C.

**Native PAGE**

Extracts (5–10 μg) were diluted three to five times with the EMSA buffer (10 mM Tris–HCl pH 7.6, 1 mM EDTA, 1 mM DTT, and 5% glycerol) containing 1 ng/μl of cold GAS oligonucleotides and then separated by 5% PAGE in 0.5 × TBE buffer as described previously (Takeuchi et al., 2001).

**Western blot analysis**

A Western blot analysis was performed, as described previously (Takeuchi et al., 2001), with anti-pY-STAT1 (no. 9171; Cell Signaling Technology, Beverly, MA), anti-pS-STAT1 (no. 06-802; Upstate Biotechnology, New York), anti-STAT1 (sc-346), anti-IRF-1 (sc497) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-RGS-His (no. 34610; Qiagen) antibody.

**Pull-down assay with Ni-NTA beads**

Cells were lysed in a lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 0.25% NP-40, 5 mM 2-mercaptoethanol) containing the protease inhibitor cocktail. After centrifugation, the clarified lysates (∼300 μg) were incubated with Ni-NTA beads at 4°C for 2 h. The beads were then washed four times with the lysis buffer containing 50 mM imidazole. Bound proteins were eluted by adding the lysis buffer containing 250 mM imidazole or a SDS–gel loading buffer and separated by SDS–7.5% PAGE for Western blot analyses.

**Reporter gene assay**

Cells in a 24-well plate were transfected with pGAS-TA-Luc (0.5 μg) (Clontech) together with pRL-TK-luc (0.03 μg) (Clontech) by using Polyfect transfection reagent (Qiagen). At 20 h posttransfection, the medium was replaced with a fresh medium or a medium containing human recombinant IFN-γ (500 IU/ml) (R&D Systems Inc., Minneapolis, MN) and incubated further for 6 h. Luciferase activities in the cell lysates were measured by the Dual-luciferase reporter assay system (Promega) according to manufacturer protocol. Relative activity was expressed by a ratio of firefly luciferase activity to renilla luciferase activity.

**EMSAs**

EMSAs were performed with a 32P-labeled GAS probe under the same conditions as previously described (Takeuchi et al., 2001). To generate a 32P-labeled GAS probe, the 5' terminus of the annealed synthetic DNAs (5'-GATCGGGAAAGGGAAAATTGTAAAGCC-3' and 5'-GATCGGGTTCTAGTTGTTTCCCTTCCCTC-3') derived from a GAS site of the human IRF-1 gene (Leonard and Sen, 1996) was labeled with [γ-32P]ATP (222 TBq/mmol; Amersham Pharmacia Biotech) by using T4 bacteriophage polynucleotide kinase.

**In vitro translation of STAT1 fragments**

To prepare [35S]methionine-labeled STAT1 fragments, in vitro transcription and translation was performed by the TNT quick coupled transcription/translation systems (Promega) with Redivue [-[35S]methionine (37 TBq/ mmol) (Amersham Pharmacia Biotech) and the pcDNA3.1-STAT1 fragment plasmids.

**Determination of STAT1 domain interacting with the D1**

Purified GST-D1 or GST (2–4 μg) was mixed with 10 μl of 50% slurry of glutathione-sepharose beads and incubated for 1 h with gentle rotation. After centrifugation, the beads were further incubated with each of in vitro translated [35S]methionine-labeled STAT1 fragments at 4°C for 1 h. The beads were then washed four times with the extraction buffer. Bound proteins were eluted by adding a SDS–gel loading buffer and separated by SDS–11% PAGE. After treatment with Amplify (Amersham Pharmacia Biotech), the gels were dried and exposed to X-ray films.

**Acknowledgments**

We thank S. Ishida and S. Kitagawa (Kubo) for excellent technical assistance and Drs. Y. Ohnishi and Y. Nagai for constant encouragement of our research. We are grateful to Dr. T. Hirano for permission of the use of pEF-BOS-HA-STAT1, Dr. M. Hibi for sending pEF-BOS-HA-STAT1, Dr. R. Goodman for permission of the use of GSTCBP571-687 and GSTp300/566-664, Dr. J. Zhang for sending GSTCBP571-687 and GSTp300/566-664, and A. Fon in Goodman’s lab for sending GSTCBP451-682, GSTCBP551-682, and GSTCBP1680-1891. We also thank Dr. Y. Kimura for the helpful discussions. This work was supported in part by the Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science and by the Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.
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