Sendai virus C protein impairs both phosphorylation and dephosphorylation processes of Stat1

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Abstract Sendai virus expresses C protein that blocks interferon (IFN) signaling. We previously reported suppression of IFN-stimulated tyrosine phosphorylation of signal transducers and activators of transcription (Stats) in infected cells. However this conclusion has remained controversial. To settle it, we re-examined the effect of C protein expression on phosphorylation of Stat1 in detail. IFN-stimulated tyrosine phosphorylation of Stat1 was doubtlessly suppressed early in infection, but the suppression was incomplete, suggesting the importance of the unknown blocking mechanism that inactivates the tyrosine-phosphorylated (pY)-Stat1 generated as the signaling leak. Interestingly, the dephosphorylation process of pY-Stat1 was also impaired. These effects on both phosphorylation and dephosphorylation processes were attributable to the function of the C protein. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Paramyxovirus; Sendai virus; Interferon; Stat1; Dephosphorylation; Phosphorylation

1. Introduction

Interferons (IFNs) establish an antiviral state in cells by inducing antiviral proteins such as double-stranded RNA-dependent protein kinase, $2'–5'$ oligoadenyl synthetase and Mx proteins [1–3]. Induction of the antiviral proteins results from transcriptional activation of IFN-stimulated genes (ISGs) via the IFN signaling pathway [1]. Binding of IFN-α/β to type I IFN receptor causes tyrosine phosphorylation of receptor-associated 'Janus' tyrosine kinases (JAK family), Jak1 and Tyk2. The activated JAKs tyrosine-phosphorylate the signal transducer and activator of transcription (Stat) 2 and Stat1. The tyrosine-phosphorylated (pY)-Stat2 and pY-Stat1 then form a heterodimer, translocate into the nucleus and combine IFN responsive factor 9 (p48) to form IFN-stimulated gene factor 3 (ISGF3) and activate transcription of ISGs. On the other hand, IFN-γ uses a similar but distinct pathway. Binding of IFN-γ to the type II IFN receptor facilitates transphosphorylation and activation of Jak1 and Jak2, which in turn tyrosine-phosphorylate Stat1. The pY-Stat1 forms a homodimer, termed IFN-γ-activated factor (GAF), translocates into the nucleus and activates transcription of ISGs. Transcriptional activation by GAF and ISGF3 requires co-factors such as cyclic AMP response element binding protein binding protein (CBP)/p300 to activate transcription of ISGs [4,5]. The pY-Stat1 is ultimately inactivated through dephosphorylation by a nuclear phosphatase [6,7]. Two forms of Stat1, Stat1α and Stat1β, are present. Stat1β is translated from a spliced form of Stat1α mRNA and lacks the COOH-terminal 31 amino acids of Stat1α [8,9]. This C-terminal region contains the serine residue that is phosphorylated in response to IFNs. The serine phosphorylation is required for maximal transactivation function of GAF but not ISGF3 [10].

With the evolution of the host IFN system, viruses have co-evolved mechanisms which antagonize the antiviral effects of IFNs [11–18]. The family Paramyxoviridae is not exceptional as well [11]. The Paramyxoviridae contain important pathogens of children and infant, including human parainfluenza viruses (hPIVs), mumps virus and measles virus. Recent studies revealed that members of the two genera, Rubulavirus and Respirovirus, have evolved mechanisms that inhibit IFN signaling [11]. Indeed, simian virus (SV) 5, SV41, hPIV2, and mumps virus in the Rubulavirus inhibit IFN signaling by decreasing the level of either Stat1 or Stat2 [19–28]. The anti-IFN protein was found to be the V protein encoded by the P gene [20,23–26]. On the other hand, Sendai virus (SeV) in the other genus Respirovirus decreases none of the signaling components in most cell lines except for NIH3T3 mouse embryo fibroblast cells [29–31]. Thus more extensive studies are required to elucidate the underlying mechanism employed by SeV.

SeV is an enveloped virus that possesses a single-stranded negative-sense RNA genome which contains six tandemly linked genes. Of six genes, the P gene is unique in giving rise to a plethora of viral polypeptides including C and V proteins by means of overlapping frames and by pseudotemplated nucleotide addition known as the RNA editing [32]. The C open reading frame (ORF) overlaps in the +1 frame relative to the P ORF and produces a nested set of four C proteins, C', C, Y1 and Y2, referred to collectively as the C proteins [33,34]. Initiation codons of C', C, Y1 and Y2 are [81]ACG, [114]AUG, [183]AUG and [201]AUG, respectively. Translation of all the four C proteins terminates at the same posi-

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Abbreviations: CBP, cyclic AMP response element binding protein; DMEM, Dulbecco's minimal essential medium; GAF, γ-activated factor; hPIV, human parainfluenza virus; IFN, interferon; ISG, IFN-stimulated gene; ISGF3, IFN-stimulated gene factor 3; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; pi, post-infection; pS, serine-phosphorylated; pt, post-treatment; pY, tyrosine-phosphorylated; SDS, sodium dodecyl sulfate; SeV, Sendai virus; Stat, signal transducer and activator of transcription; SV, simian virus; WT, wild-type

tion UAA[728]. Recent development in reverse genetic technology succeeded in creating C knockout SeVs [35–38] and revealed that the C gene is required for preventing the IFN responses [30,39]. Although the importance of [114]AUG initiated C protein for blocking IFN signaling had been emphasized [39], studies using a series of knockout viruses including four C knockout viruses [37,40] and using cells expressing individual C protein demonstrated that not only C but also Y1 and Y2 proteins have the capacity to inhibit the IFN signaling pathway [30,31,41]. However, debate still continues as to whether Y1 and Y2 proteins are able to rescue VSV replication from the antiviral effects of IFN [29,41].

Studies on the molecular mechanism by which SeV interferes with IFN signaling have so far been analyzed on infected cells [28,42,43]. The first important finding is that tyrosine phosphorylation of Stats in immediate response to IFN-α was inhibited in the early phase of infection [42]. Young et al., however, reported a distinct conclusion about this [28]. They suggested that SeV does not inhibit tyrosine phosphorylation of Stat1 but does serine phosphorylation of Stat1 in response to autocrine IFN induced by infection [28]. To resolve this discrepancy, we decided to examine more precisely the effect of SeV infection on phosphorylation of Stat1. We show here that SeV suppresses definitely tyrosine phosphorylation of Stat1 by short-time stimulation with IFN in the early phase of infection. However, the suppression was incomplete. The C protein contributed to not only the suppression of tyrosine phosphorylation but also the impairment of dephosphorylation of pY-Stat1. The present study thus emphasizes the importance of another blocking mechanism, which inactivates transcriptional activity of the pY-Stat1 generated as the signaling leak.

2. Materials and methods

2.1. Cells and viruses

HeLa cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum. A HeLa cell clone, named C+, which constitutively expresses the C protein [41], was grown in DMEM supplemented with 10% fetal calf serum and 100 μg/ml blasticidin. The human cell lines, U118 [44] and HEC-1B [45,46], were cultured in MEM supplemented with 10% fetal calf serum, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. SeVpB, a temperature sensitive mutant [47], was propagated in the allantoic cavity of 10-day-old embryonated eggs and was used for most of experiments unless otherwise mentioned. Titers of SeVpB were determined as described previously [48]. Recombinant SeVs, wild-type (WT) (cDNA-derived Z strain) and 4C(−) [37,49], propagated in Vero cells were titrated by the hemadsorption test as described previously [30]. rSeV was used at the appropriate dilution so that all cells were infected.

2.2. Chemicals and antibodies

Recombinant human IFN-α-2a and IFN-γ were purchased from Takeda Chemical Industries, Osaka, Japan, and and from R&D Systems Inc., Minneapolis, MN, USA, respectively. Staurosporine was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Anti-phospho-(Tyr[701]) Stat1 (anti-pY-Stat1) rabbit polyclonal antibody (no. 9171) was from New England Biolabs, Inc., Beverly, MA, USA. Anti-phospho-(Ser[727]) Stat1 (anti-serine-phosphorylated (pS)-Stat1) rabbit polyclonal antibody (no. 06-802) was from Upstate Biotechnology, New York, USA. Anti-Stat1 mouse monoclonal antibody SC-464 was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. FITC-labeled anti-rabbit IgG goat serum was from Tago, Burlingame, USA.

2.3. Western blot analysis

Cells were lysed with extraction buffer (50 mM HEPES, pH 7.6, 300 mM NaCl, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 2 μg/m pepstatin) [50]. After centrifugation, the clarified supernatant was stored as the total extract at −80°C. Total cell extracts (20–50 μg protein) were electrophoresed in 6% sodium dodecyl sulfate (SDS)–polyacrylamide gels [51]. Proteins in the gels were transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit (Amersham Pharmacia Biotech) was used as a secondary antibody. The proteins were detected using ECL detection reagent (Amersham Pharmacia Biotech).

3. Results

3.1. IFN-independent tyrosine phosphorylation of Stat1 in SeV-infected cells

Young et al. observed elevation of the pY-Stat1 level in SeV-infected human diploid fibroblast 2fTGH cells at 16 h post-infection (pi) in the absence of exogenously added IFN [28]. They interpreted this tyrosine phosphorylation as a result of stimulation with autocrine IFN-α/β produced in response to infection and concluded that SeV did not inhibit IFN-stimulated tyrosine phosphorylation of Stat1. To confirm the result, HeLa cells were infected with SeV and then harvested at 2 and 20 h pi. The levels of pY-Stat1 in the extracts were estimated by Western blot analysis. Consistent with their result, the pY-Stat1 level in HeLa cells obviously elevated at 20 h pi in the absence of exogenously added IFN (Fig. 1). To determine whether this elevation is due to the response to autocrine IFN-α/β, experiments were made in an IFN non-responder cell line, HEC-1B [45,46], as well as in an IFN non-producer cell line, U118 [44]. Interestingly, similar elevation was observed in both cell lines (Fig. 1). These results revealed that SeV infection per se could cause IFN-independent tyrosine phosphorylation of Stat1. It is therefore difficult to determine whether the elevation of the pY-Stat1 levels in infected HeLa or 2fTGH cells in the absence of exogenously added IFN was due to stimulation with autocrine IFN or due to the IFN-independent tyrosine phosphorylation.

3.2. Leaky blockage in IFN-mediated tyrosine phosphorylation of Stat1

Treatment of cells with IFN causes immediate tyrosine phosphorylation of Stat1. We previously presented the data
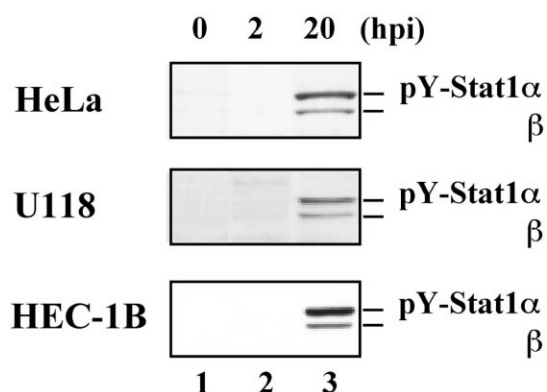

Fig. 1. IFN-independent tyrosine phosphorylation of Stat1 in SeV-infected cells. Confluent monolayers of HeLa, U118 and HEC-1B cells were infected with SeV at a multiplicity of infection of 10, and harvested at indicated time points. Then the total cell extracts were subjected to 6% SDS–PAGE followed by Western blot analysis with anti-pY-Stat1 antibody. Results are representative of three independent experiments.
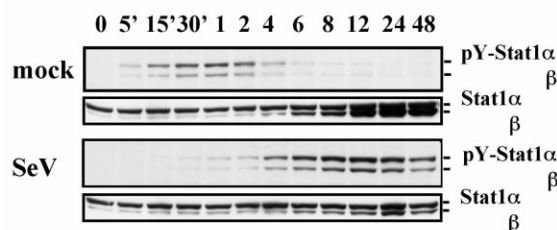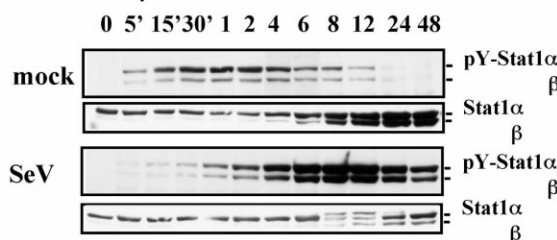
A. IFN-α

B. IFN-γ

C. mock

Fig. 2. Effect of SeV infection on tyrosine phosphorylation of Stat1 in response to continuous stimulation with IFN. HeLa cells were mock-infected or infected with SeV 2 h before addition of IFN-α (1000 U/ml) (A) or IFN-γ (500 U/ml) (B), or mock treatment (C). Cells were harvested at indicated time points after addition of IFN. The total extracts were subjected to 6% SDS–PAGE followed by Western blot analysis with anti-pY-Stat1 or anti-Stat1 antibody. Results for A–C are representative of three independent experiments.

indicating that this process in response to either IFN-α or IFN-γ was impaired at 2 h pi [42,43]. Suppression of IFN-α-stimulated tyrosine phosphorylation of Stat1 was also observed at 20 h pi, but the inhibitory effect for IFN-γ stimulation was abrogated by 20 h pi [43]. This conclusion was drawn from estimation of the pY-Stat1 levels induced by short-time (30 min) incubation with IFN at 2 h and 20 h pi.

To see more precisely the effect of SeV infection on IFN-stimulated phosphorylation of Stat1, we examined the level of pY-Stat1 after continuous stimulation with IFN. Either IFN-α or IFN-γ was added to the medium of infected or mock-infected cells at 2 h pi and then incubated for the indicated times (Fig. 2). Treatment of mock-infected cells with IFN-α resulted in immediate increase in the intracellular level of pY-Stat1, which reached a peak within 1 h and then decreased from 2 h post-treatment (pt) (Fig. 2A, mock). The decrease in the pY-Stat1 level is due to rapid dephosphorylation of pY-Stat1 by a nuclear phosphatase [6,7]. When SeV-infected cells were treated with IFN-α, the phosphorylation pattern strikingly changed. The pY-Stat1 level retained nearly below the detection level for 2 h after addition of IFN-α (Fig. 2B), confirming the previous finding [42]. Surprisingly, the level of pY-Stat1 gradually increased from 4 h pt and reached a peak at 12 h pt. Levels of pY-Stat1 are determined by the balance of tyrosine phosphorylation and dephosphorylation turnovers. On the other hand, SeV suppresses tyrosine phos-

phorylation caused by short-time stimulation with IFN-α in not only early but also middle phases of infection [43]. Therefore it is likely that the increase in the pY-Stat1 level from 4 h pt resulted from accumulation of pY-Stat1 due to impairment of the dephosphorylation process of the pY-Stat1 generated as the signaling leak (see Section 3.4). A similar pattern was observed for stimulation with IFN-γ (Fig. 2B), although the phosphorylation levels were higher than those for stimulation with IFN-α. In the absence of exogenously added IFNs, the pattern was also similar although the phosphorylation level was lower (Fig. 2C). Levels of Stat1 were not markedly affected throughout infection (Fig. 2), demonstrating that the early block was not due to degradation of Stat1. Stat1α was detected as double bands at 8 and 12 h pt with IFN-γ in infected cells (Fig. 2B). pY-Stat1 migrates slower than unphosphorylated Stat1 on SDS–polyacrylamide gel electrophoresis (PAGE) [52]. Indeed the upper band migrated exactly at the same position as the pY-Stat1α did, indicating that a large proportion of Stat1 was tyrosine-phosphorylated at 8 h or 12 h pt with IFN-γ in infected cells. From these results we concluded that SeV suppresses tyrosine phosphorylation in immediate response to either IFN-α or IFN-γ in the early phase of infection but the suppression was incomplete. It is unclear why the levels of pY-Stat1 decreased in infected cells at 24 and 48 h pt, compared with those at 8 and 12 h pt. It is, however, evident that the decrease did not correlate with changes of the Stat1 levels (Fig. 2).

3.3. Effect of SeV infection on serine phosphorylation of Stat1

Young et al. reported that the intracellular level of pS-Stat1 was below the detection level at 16 h pi in infected cells in the absence of exogenously added IFNs [28], suggesting that SeV inhibits autocrine IFN-α/β-stimulated serine phosphorylation of Stat1. To confirm their result, we also examined the effect of SeV infection on serine phosphorylation of Stat1 using the
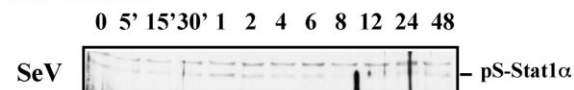
A. IFN-α

B. IFN-γ

C. mock

Fig. 3. Effect of SeV infection on serine phosphorylation of Stat1 in response to continuous stimulation with IFN. Experimental conditions were the same as those described in Fig. 2. The total extracts were subjected to 6% SDS–PAGE followed by Western blot analysis with anti-pS-Stat1 antibody. Results for A–C are representative of three independent experiments.

same samples that were analyzed in Fig. 2 (Fig. 3). Treatment of mock-infected cells with IFN-α resulted in a more gradual increase in the level of pS-Stat1 (Fig. 3A), compared with that in the pY-Stat1 level (Fig. 2A). In contrast, no serine phosphorylation was observed at any incubation periods in infected cells irrespective of the presence of exogenously added IFN-α (Fig. 3A,C), demonstrating that SeV suppresses serine phosphorylation in response to either IFN-α or autocrine IFN. Since serine phosphorylation of Stat1 is not required for the transcriptional activity of ISGF3, this suppression never contributes to the inhibition of IFN signaling. Treatment of mock-infected cells with IFN-γ exhibited a similar pattern to the case of stimulation with IFN-α (Fig. 3B). In SeV-infected cells, the level of pS-Stat1 increased from 6 h pt, reached a peak at 8 h pt and returned to the basal level by 48 h pt. As a whole, a suppressive effect of SeV infection on IFN-γ-mediated serine phosphorylation was observed, but the levels of pS-Stat1 at 6 and 8 h pt were comparable to those in mock-infected cells (Fig. 3B). Since the pS-Stat1 band observed at 6 and 8 h pt migrated at the same position as the pY-Stat1 band did (data not shown), they are doubly phosphorylated at the tyrosine and serine residues. Thus these effects of SeV infection on serine phosphorylation of Stat1 never account for the inhibition of IFN signaling.
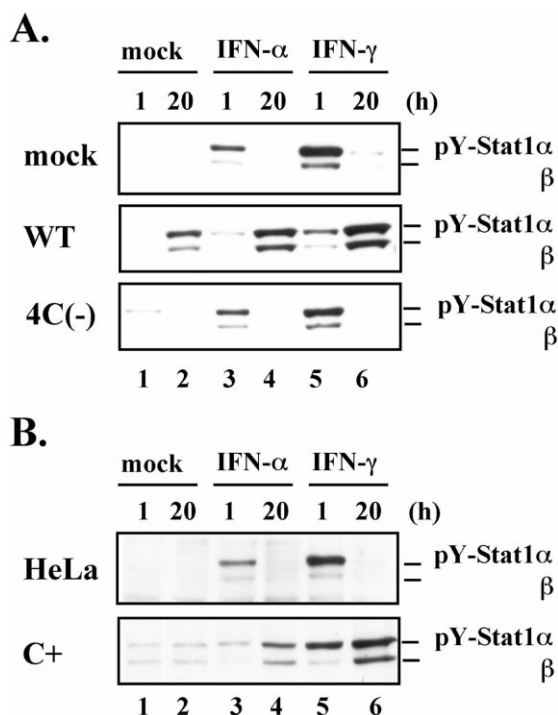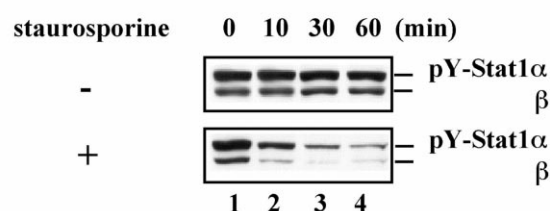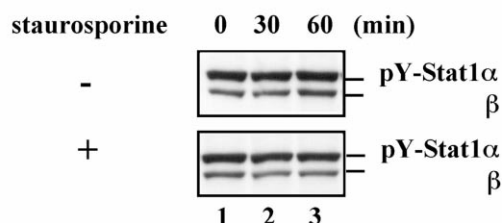

Fig. 4. The C protein is responsible for the inhibitory effects on tyrosine phosphorylation of Stat1 as well as the accumulation of pY-Stat1. In A, HeLa cells were mock-infected or infected with either WT or 4C(−) virus. Then either IFN-α (1000 U/ml) or IFN-γ (500 U/ml) was added to the media at 2 h pi. The cells were harvested at 1 h and 20 h after addition of IFN. In B, HeLa or C+ cells were treated with IFN-α (1000 U/ml) or IFN-γ (500 U/ml) and harvested at indicated times. The total cell extracts were subjected to 6% SDS–PAGE followed by Western blot analysis with anti-pY-Stat1 antibody. Results for A and B are representative of three independent experiments.
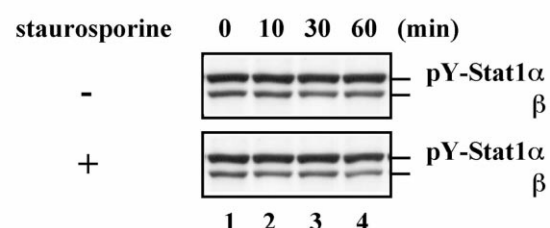

Fig. 5. Impairment of the dephosphorylation process of pY-Stat1 by the C protein. HeLa cells were mock-infected (A) or infected with SeV (B), and then treated with IFN-γ at 2 h pi for 1 h or 20 h, respectively. C+ cells (C) were treated with IFN-γ for 20 h. Then the cells (A–C) were mock-treated (−) or treated with staurosporine (500 nM) (+) for indicated times. The total cell extracts were analyzed by 6% SDS–PAGE followed by Western blot analysis with anti-pY-Stat1 antibody. Results for A–C are representative of three independent experiments.

3.4. *The C protein is responsible for the suppression of tyrosine phosphorylation of Stat1 as well as the accumulation of pY-Stat1*

To determine whether the effect on tyrosine phosphorylation of Stat1 is attributable to the function of the C protein, we examined the effect of infection with the C knockout SeV, 4C(−) virus, which does not express any of four C proteins, on IFN-stimulated tyrosine phosphorylation of Stat1. Cells were infected with WT or 4C(−) virus and then incubated with IFN-α or IFN-γ for 1 or 20 h. As shown in Fig. 4A, Stat1 was tyrosine-phosphorylated in response to either IFN-α or IFN-γ in 4C(−)-infected cells (Fig. 4A, lanes 3 and 5). Notable elevation of pY-Stat1 levels was observed for 20 h incubation in WT-infected cells, whereas no pY-Stat1 accumulated in 4C(−) virus-infected cells (Fig. 4A, lanes 2, 4 and 6). These results demonstrated that the C protein was required for the suppression of tyrosine phosphorylation of Stat1 in response to short-time stimulation with IFN as well as the accumulation of pY-Stat1 caused by continuous IFN stimulation. The C+ cells constitutively expressing the SeV C protein display unresponsiveness to IFNs comparable to SeV-infected cells [41]. C+ cells were treated with IFN-α or IFN-γ

for 1 or 20 h. Immediate response to IFN-α (Fig. 4B, lane 3) but not IFN-γ (Fig. 4B, lane 5) was suppressed in C+ cells. This result was in good agreement with the inhibition pattern previously observed in the middle phase of infection [43]. Continuous stimulation of C+ cells with either IFN-α or IFN-γ resulted in obvious elevation of pY-Stat1 levels (Fig. 4B, lanes 4 and 6). Neither virus infection nor expression of the C protein decreased the levels of Stat1 (data not shown). These results demonstrated that the effects of SeV infection on phosphorylation of Stat1 were attributable to the function of the C protein. Interestingly, the very low level of pY-Stat1 was detected in C+ cells without any treatment (Fig. 4B, lanes 1 and 2). Thus the C protein may be responsible in part for the elevation of pY-Stat1 in SeV-infected U118 or HEC-1B cells.

3.5. Impairment of the dephosphorylation process of pY-Stat1

Staurosporine, known as a powerful kinase inhibitor, was used to determine whether the dephosphorylation process was impaired in infected cells or C+ cells. Since staurosporine inhibits phosphorylation of Stat1, the dephosphorylation process of pY-Stat1 would consequently become visible [6]. When the pY-Stat1 level in mock-infected cells reached a peak by treatment with IFN-γ for 1 h (Fig. 5A, lane 1), staurosporine was added to the media. As shown in Fig. 5A, the pY-Stat1 level decreased immediately after addition of staurosporine and almost disappeared within 60 min (Fig. 5A, lanes 2–4), indicating prompt inactivation of pY-Stat1 through dephosphorylation by the nuclear phosphatase. Treatment of cells with staurosporine did not affect the level of Stat1 (data not shown). In contrast, the level of pY-Stat1 generated in infected HeLa cells or C+ cells by continuous stimulation with IFN-γ (Fig. 5B,C) or IFN-α (data not shown) was maintained even in the presence of staurosporine. These results

a. HeLa   **b. HeLa / IFN-γ**
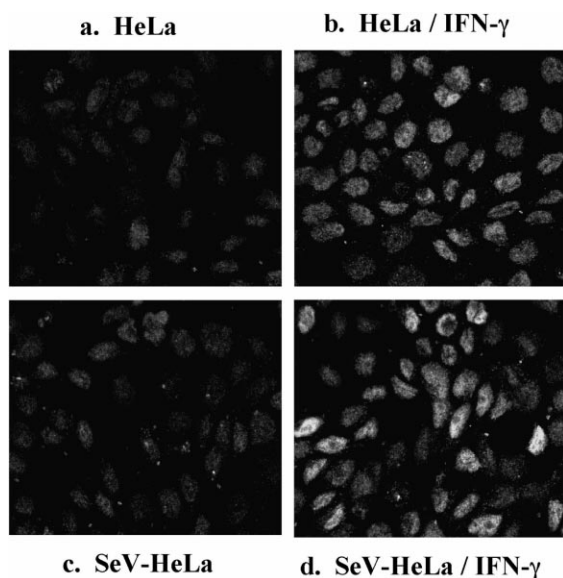
c. SeV-HeLa   **d. SeV-HeLa / IFN-γ**

Fig. 6. Translocation of pY-Stat1 into the nucleus in infected cells. Confluent monolayers of HeLa cells were mock-infected (a, b) or infected with SeV (c, d). At 20 h pi, the cells were then mock-treated (a, c) or treated with IFN-γ for 30 min (b, d). The cells were fixed, permeabilized and then subjected to immunofluorescent staining with anti-pY-Stat1 antibody according to the manufacturer's instructions. Results for a–d are representative of two independent experiments.

suggested that the C protein impaired the dephosphorylation process of pY-Stat1.

3.6. The C protein never impaired translocation of pY-Stat1 into the nucleus

The dephosphorylation process of pY-Stat1 takes place in the nucleus [6]. To exclude the possibility that the impairment of the dephosphorylation process is due to a block in the translocation of Stat1 into the nucleus, the immunofluorescent staining experiment was performed using anti-pY-Stat1 antibody. There was little fluorescence without IFN treatment (Fig. 6a,c), while obvious fluorescence in the nucleus was detected in both mock-infected (Fig. 6b) and infected cells (Fig. 6d) with IFN-γ treatment, indicating that upon IFN-γ stimulation pY-Stat1 translocated into the nucleus even in the presence of the C protein.

4. Discussion

The present study showed that continuous stimulation of infected cells with IFN-α or IFN-γ resulted in generation of a considerable amount of pY-Stat1. Under this condition, pY-Stat1 generated by IFN-γ stimulation was doubly phosphorylated on both serine and tyrosine. Since infected cells are unresponsive even to continuous IFN stimulation, these results strongly suggest the presence of the second blocking mechanism that inactivates the leak pY-Stat1. These effects on phosphorylation were attributable to the functions of the C protein. The second mechanism is thought to be indispensable for the block in IFN signaling. If so, does the suppression of tyrosine phosphorylation of Stat1 observed in the early phase of infection contribute to the inhibition of IFN signaling? Since the amount of the C protein is very low early in infection (2 h pi), the second blocking mechanism may not have been established in this early phase. It is therefore possible that in the context of infection the suppression of tyrosine phosphorylation plays a critical role until the second blocking mechanism has been established.

The C protein impaired the dephosphorylation process of the leak pY-Stat1. The role of this impairment in the signaling block remains to be elucidated. The C protein physically associates with both unphosphorylated Stat1 and pY-Stat1 [43]. Therefore the C-Stat1 association may contribute to the impairment of the dephosphorylation process, although the possibility cannot be excluded that the C protein directly inhibits the function of the nuclear phosphatase. N-terminal mutants of Stat1 impair the dephosphorylation process, suggesting that the N-terminal region affects phosphatase access [53]. On the other hand, Stat1 interacts with CBP/p300 through two separate regions, N-terminal and C-terminal regions, of Stat1. Thus the C protein may prevent factors such as the nuclear phosphatase and CBP/p300 from being accessible to Stat1 through interaction with domains nearby the N-terminal region of Stat1. The analysis on the interacting domains of the C protein and Stat1 is now in progress.

How does the C protein suppress IFN-stimulated tyrosine phosphorylation of Stats? The C protein may be also responsible for the effects of SeV infection on IFN-mediated serine phosphorylation of Stat1, because the phosphorylation patterns observed in 4C(−) virus-infected cells and in C+ cells were similar to those in mock-infected cells and in SeV-infected cells, respectively (data not shown). There may be a

common underlying mechanism between them. Tyrosine phosphorylation of Stat2 and Stat3 in addition to Stat1 is suppressed in response to IFN-α in the early phase of infection as described previously [42]. Activation of Tyk2 was partially impaired in this phase [42]. Thus it is possible that the C protein acts at the JAK or receptor level. Interestingly, in the middle phase of infection, tyrosine phosphorylation of JAKs in response to either IFN-α or IFN-γ was inhibited (unpublished results), although Stat1 was efficiently tyrosine-phosphorylated in response to IFN-γ stimulation, suggesting the presence of JAK-independent tyrosine phosphorylation in response to IFN-γ in infected cells. The molecular basis for this mechanism may be linked to that for the blocking mechanism that suppresses IFN-stimulated tyrosine phosphorylation of Stat1.

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