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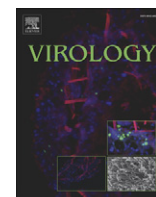

Measles virus C protein suppresses gamma-activated factor formation and virus-induced cell growth arrest

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Measles virus (MeV) produces two accessory proteins, V and C, from the P gene. These accessory proteins have been reported to contribute to efficient virus proliferation through the modulation of host cell events. Our previous paper described that Vero cell-adapted strains of MeV led host cells to growth arrest through the upregulation of interferon regulatory factor 1 (IRF-1), and wild strains did not. In the present study, we found that C protein expression levels varied among MeV strains in infected SiHa cells. C protein levels were inversely correlated with IRF-1 expression levels and with cell growth arrest. Forced expression of C protein released cells from growth arrest. C-deficient recombinant virus efficiently upregulated IRF-1 and caused growth arrest more efficiently than the wild-type virus. C protein preferentially bound to phosphorylated STAT1 and suppressed STAT1 dimer formation. We conclude that MeV C protein suppresses IFN-γ signaling pathway via inhibition of phosphorylated STAT1 dimerization.

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Introduction

Measles is a highly contagious acute febrile disease characterized by Koplik's spots and generalized maculopapular rash. Despite the availability of live attenuated vaccines, measles is still a severe problem and the cause of childhood mortality in developing countries. Alveobronchiolitis and encephalitides are the major causes of death from measles infection. Measles virus (MeV) is a member of the genus Morbillivirus of the family Paramyxoviridae. The MeV genome consists of six genes encoding the proteins N (nucleoprotein), P (phosphoprotein), M (matrix protein), F (fusion protein), H (hemagglutinin), and L (large protein) (Griffin, 2007). In addition, the P gene also encodes two accessory proteins, V and C. V is generated by RNA editing through the insertion of guanine at position 751 of the P gene nucleotide sequence (Cattaneo et al., 1989). As a result, the N-terminus of V protein has the same amino acid sequence as that of P protein. C protein is translated from another initiation codon (Bellini et al., 1985). A flame shift occurs so that the amino acid sequence of C protein is distinct from those of P and V proteins. V and C proteins have been reported to contribute to virulence and efficient viral replication. V protein has been shown to suppress interferon (IFN) signaling via interaction with Jak1 and STAT1 (Caignard et al., 2009; Caignard et al., 2007; Takeuchi et al., 2003) and to suppress IFN production via interaction with MDA-5, a cytosolic RNA sensor (Nakatsu et al., 2008; Ramachandran and Horvath, 2010). C protein supports virus replication, host cell growth, and apoptosis. It contributes to genome replication (Bankamp et al., 2005) and virus assembly (Devaux and Cattaneo, 2004). It releases the translational inhibition of viral RNA and suppresses IFN induction (Nakatsu et al., 2006; Nakatsu et al., 2008). C protein was also reported to inhibit IFN signaling (Shaffer et al., 2003); however, V protein was shown to be a more potent inhibitor of IFN-inducible gene expression than C protein (Fontana et al., 2008). On the other hand, some reports indicate that C protein has no effect on IFN-α/β or IFN-γ signaling (Nakatsu et al., 2008; Takeuchi et al., 2003).

Two types of MeV strains exist, namely the wild strains and Vero cell-adapted (Vero-adapted) strains (Yanagi et al., 2009). Wild strains infect host cells via the SLAM (CD150) as a receptor. On the other hand, MeV can adapt to SLAM-negative cells, such as Vero cells. The resulting Vero-adapted strains infect cells via CD46, which is expressed in various types of primate cells, as a receptor. Attenuated strains are Vero-adapted type. Strains isolated from clinical specimens using B95a cells or Vero cells with the human SLAM gene (Vero-hSLAM) are wild strains. In cynomolgus and squirrel monkey experimental infection models, the wild strains induce clinical symptoms that resemble human measles (Kobune et al., 1996). An alternative and still unidentified receptor for MeV is suggested to exist on epithelial cells (Takeda, 2008). Cell tropism and pathogenicity are considered to be different between wild strains and Vero-adapted strains (Takeda, 2008).

Our previous study indicated that epithelial cell lines infected with Vero-adapted strains showed growth arrest caused by the induction of interferon regulatory factor-1 (IRF-1) in an IFN-independent manner (Yokota et al., 2004). However, a wild strain showed less

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IRF-1 induction and less growth arrest than Vero-adapted strains. IRF-1 is an IFN-γ-inducible protein (Imanishi et al., 2000; Kroger et al., 2002). IFN-γ induces the activation of the transcription factor, gamma-activated factor (GAF), which is a homodimer of Tyr-phosphorylated STAT1, and the STAT1 is phosphorylated by Jak1 and Jak2 protein kinases which associated with IFN-γ receptor (reviewed in Goodbourn et al., 2000, and Stark et al., 1998). GAF translocates into the nucleus and binds to the gamma activation sequence (GAS) motif of the IFN-γ inducible gene promoters. Our previous data described that the induction of IRF-1 in Vero-adapted MeV-infected epithelial cells occurred in an IFN-γ-independent manner and constitutive phosphorylation of STAT1 and Jak1 is observed (Yokota et al., 2004). In this paper, we show that C protein suppresses the induction of IRF-1 via the inhibition of GAF formation.

Results

Viral protein expression in SiHa cells infected with various strains of MeV

All SiHa cells infected with MeV except for CAM70 showed similar levels of H, P, and V proteins determined by Western blotting (Fig. 1A). SiHa cells infected with CAM70 (SiHa-CAM70) expressed significantly lower levels of these proteins, and H and V proteins of CAM70 had a higher molecular weight than those of other MeV strains. The low levels in SiHa-CAM70 cells were consistent with the lower production of infectious particles reported previously (Yokota et al., 2004). Similar observation of higher molecular weight CAM70 V protein was reported (Fontana et al., 2008). The authors speculated that V protein of CAM70 had received post-transcriptional modifications, such as phosphorylation.

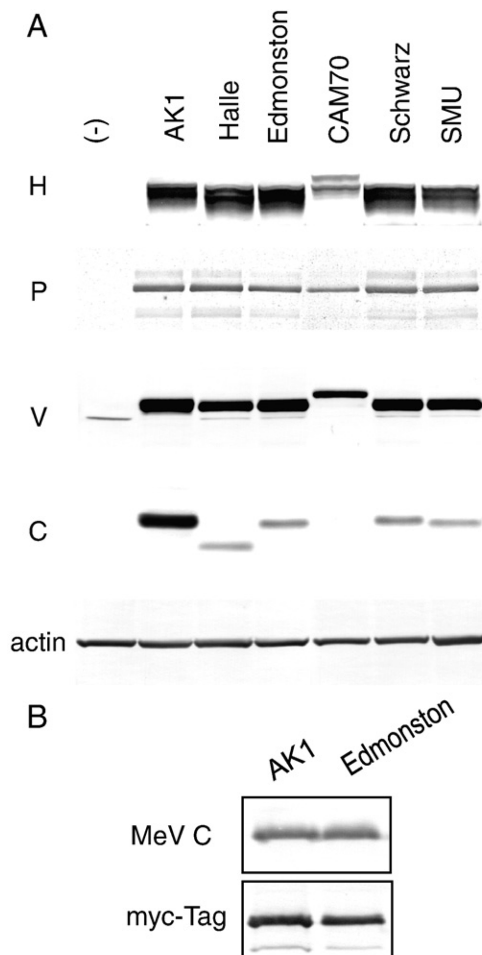

Fig. 1. Viral protein expression in SiHa cells infected with various MeV strains. A) Expression of viral proteins (H, P, V, and C) by Western blotting. Actin was used as a control for protein loading. B) Comparison of anti-C protein antibody reactivity toward C proteins derived from wild strain (AK1) and Vero-adapted strain (Edmonston). Recombinant myc-tagged C proteins were prepared using the rabbit reticulocyte in vitro translation/transcription system. C protein was detected by Western blotting using anti-C antibody and anti-myc-TAG antibody.

C protein expression varied among strains as shown by Western blotting (Fig. 1A). The anti-C antibody used in this study was prepared by immunization with a peptide consisting of amino acid residues no. 20 to 40 of a wild strain, IC-B (Takeuchi et al., 2003). Two amino acid residues in this region are different between the wild and Vero-adapted strains (Table 1). First, we examined the reactivity of anti-C antibody in C proteins derived from the wild strain AK1 and Vero-adapted strain Edmonston. Myc-tagged C protein derived from AK1 and Edmonston were prepared by in vitro translation. Western blotting with anti-C antibody and anti-myc antibody showed similar band intensity (Fig. 1B). This suggested that the anti-C antibody had a similar reactivity to wild strain C protein and Vero-adapted strain C protein.

C protein was not detected in SiHa-CAM70 by Western blotting. A smaller molecular weight C protein was detected in SiHa-Halle cells. Analysis of the nucleotide sequence of MeV P cDNA revealed a point mutation that introduced a stop codon at position of no. 168 of the amino acid sequence of C protein. Therefore, C protein was truncated in SiHa-Halle cells. Other cell lines infected with Halle showed ordinary molecular weight C protein or both the ordinary and truncated form (data not shown). The mutant of Halle strain with the truncation seemed to be selectively proliferated in SiHa cells. SiHa-AK1 cells showed a remarkably high level of C protein compared with cells infected with other MeV strains.

SiHa cells infected with MeV showed growth suppression. The degree of suppression varied among MeV strains (Fig. 2A) as we reported previously (Yokota et al., 2004). Growth suppression and the expression levels of C protein seemed to be inversely correlated (Fig. 2B). Basal expression levels of IFN-γ inducible genes, IRF-1 and CIITA, were determined by real time reverse transcription–PCR (RT–PCR) and Western blotting in MeV-infected SiHa cells (Figs. 2C and 3A). Levels varied among MeV strains and were inversely correlated with C protein levels and cell growth rate. These IRF-1 expressions were independent to IFN produced by the infected SiHa cells as previously reported (Yokota et al., 2004). In addition, IFN-γ-induced IRF-1 expression in SiHa-AK1 cells was significantly suppressed compared with uninfected SiHa cells and SiHa-CAM70 cells (Fig. 3B). These results suggested that SiHa-AK1 cells, which expressed higher levels of C protein, showed suppressed IFN-γ inducible gene expression both at basal and at IFN-γ-induced levels.

C protein suppresses MeV-induced IRF-1 transcription and growth arrest

We examined the effect of C protein expression on cell growth. Upon infection with a recombinant C-deficient MeV mutant derived from wild strain IC-B (ΔC), SiHa cells showed significant growth arrest (Fig. 4A) and IRF-1 upregulation (Fig. 4B). On the other hand, wild-type recombinant virus (wt) did not significantly affect cell growth or the expression of IRF-1. Infection and replication efficacy of the recombinant

Table 1
Differences in C protein amino acid sequences.

Strain	Virus type	Amino acids different from the Edmonston strain
Edmonston	Vero-adapted	–
SMU	Vero-adapted	–
Schwarz	Vero-adapted	M104T
Halle	Vero-adapted	W168Stop
CAM70	Vero-adapted	R13K
AK1	Wild	L25P, S39T, G44R, R78K
IC-B	Wild	L25P, S39T, G44R, R78K

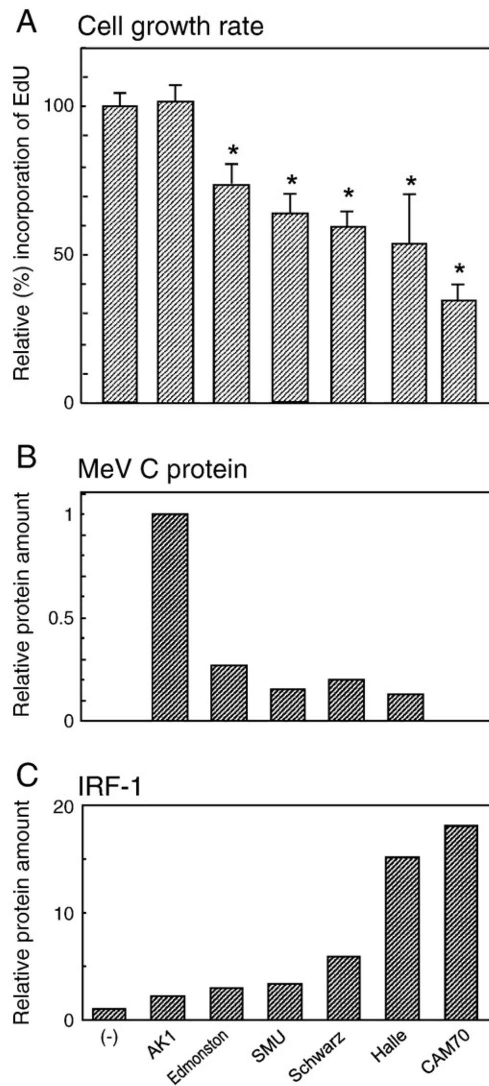

Fig. 2. Cell growth rate and protein expression levels of MeV C protein and IRF-1. A) Growth rate of infected cells. Rate of DNA synthesis was determined by the uptake of 5-ethinyl-2′-deoxyuridine (EdU) into DNA. The experiments were performed in triplicate, and the data were expressed as mean value ± standard deviation. *$P<0.01$ compared to uninfected cells. B) Protein levels of MeV C protein. The data were expressed as relative values to the value obtained from SiHa cells infected with MeV AK1. C) Protein levels of IRF-1. The data were expressed as a relative value to the value obtained from uninfected SiHa cells. The protein expression levels were determined by Western blotting.

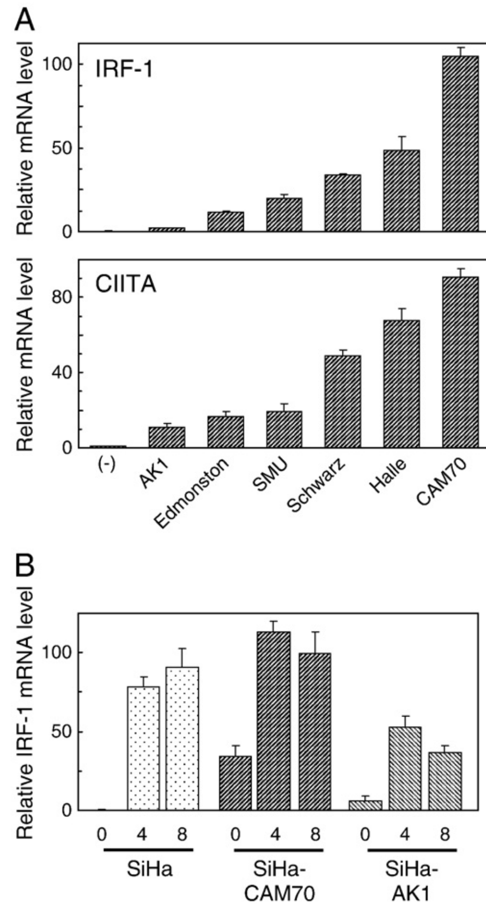

Fig. 3. IFN-γ inducible gene mRNA expression in SiHa cells infected with various MeV strains in presence or absence of IFN-γ treatment. A) mRNA expression levels of IRF-1 and CIITA. Total RNA was isolated from cultured cells under normal condition and assessed by real time RT–PCR. B) IRF-1 induction by IFN-γ in SiHa cells and SiHa cells infected with MeV CAM70 or AK1. Cells were treated with IFN-γ at a concentration of 1000 U/ml. Total RNA was isolated at the time points indicated in the figure and assessed by real time RT–PCR. Each experiment was performed in triplicate. The data (mean value ± standard deviation) are normalized with the levels of GAPDH and expressed relative values to the value obtained from uninfected SiHa cells.

viruses were confirmed by mRNA levels of MeV H gene determined by RT–PCR. The cell growth suppression by the C-deficient MeV mutant was significantly released by the forced expression of C protein by transfection of the plasmid (Fig. 4C), and suppression of IRF-1 induction was also observed by the C protein expression (Fig. 4D).

Transfection of myc-tagged C protein in SiHa-CAM70 cells resulted in the release from growth arrest and downregulation of IRF-1 (Fig. 5). Myc-tagged V protein did not show an effect. Myc-tagged C proteins did not have any significant effect on the growth rate or IRF-1 in uninfected SiHa cells or SiHa-AK1 cells. Data concurred in showing that C protein suppressed the expression of MeV-induced IRF-1, resulting in the release from MeV-induced cell growth arrest.

C protein suppresses dimerization of Tyr-phosphorylated STAT1

We reported previously that MeV AK1 suppressed signaling downstream of IFN-α but not IFN-γ (Yokota et al., 2003). The suppression is indicated to be induced by V protein but not by C protein (Nakatsu et al., 2008; Takeuchi et al., 2003), as discussed later. We examined the IFN-induced phosphorylations of Jak1 and STAT1 in SiHa cells infected with various MeV strains. All MeV strains tested suppressed IFN-α induced phosphorylations of Jak1 and STAT1 (Fig. 6). However, Jak1 was constitutively phosphorylated in MeV-infected SiHa cells, and the levels of Jak1 constitutive phosphorylation correlated positively with growth suppression as described previously (Yokota et al., 2004). IFN-γ induced phosphorylations of STAT1 and Jak1 in MeV-infected SiHa cells were not significantly affected by infection with all the strains tested (Fig. 6).

C protein seemed to suppress MeV-activated IFN-γ signaling pathway at the downstream of STAT1 phosphorylation. IFN-γ signaling includes STAT1 phosphorylation, dimer formation, translocation into nucleus, and interaction with GAS in IFN-γ-inducible gene promoters. STAT1 dimer, namely GAF, formation was determined by native-PAGE/Western blotting analysis. Higher levels of STAT1 dimer were detected in SiHa-CAM70 cells compared to uninfected cells and SiHa-AK1 cells, while the total protein amounts of STAT1 were not significantly different in these cells (Fig. 7A). The STAT1 dimerization in SiHa-CAM70 cells decreased upon expression of C protein by transfection of the plasmid. IFN-induced STAT1 phosphorylation occurred mainly on STAT1α, which is higher molecular weight one (Figs. 6 and 7B). Immunoprecipitation of IFN-γ-treated SiHa-AK1 cell

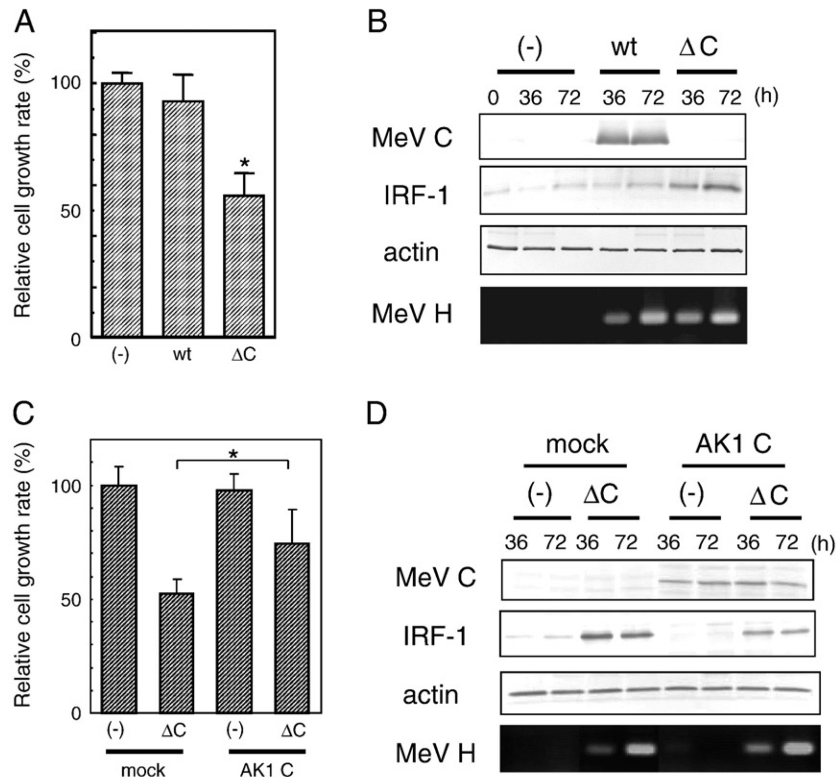

Fig. 4. C-deficient recombinant MeV decreased the growth rate in SiHa cells. A, B) SiHa cells were infected with recombinant viruses, IC323-EGFP (wt), or ΔC-EGFP (ΔC), at a multiplicity of infection of 0.1. A) Cell growth rate. At 72 h post-infection, growth rate was measured by the uptake of 5-ethinyl-2′-deoxyuridine (EdU). (−) indicates uninfected cells. **P*<0.01 compared to uninfected cells. B) Protein expression. At 36 h and 72 h post-infection, cells were harvested, and expression levels of MeV C protein, IRF-1, and actin (as a loading control) were determined by Western blotting. mRNA levels of MeV H were determined by RT–PCR as a control for virus infection. C, D) SiHa cells were transfected with expression plasmid of C protein derived from MeV AK1 and then infected with C-deficient recombinant virus ΔC-EGFP (ΔC) at a multiplicity of infection of 0.1. C) Cell growth rate. At 72 h post-infection, growth rate was measured by the uptake of EdU. Mock indicates transfected with a control plasmid; (−), uninfected. **P*<0.01 compared to uninfected cells. D) Protein expression. Detail experimental condition is the same as panel B.

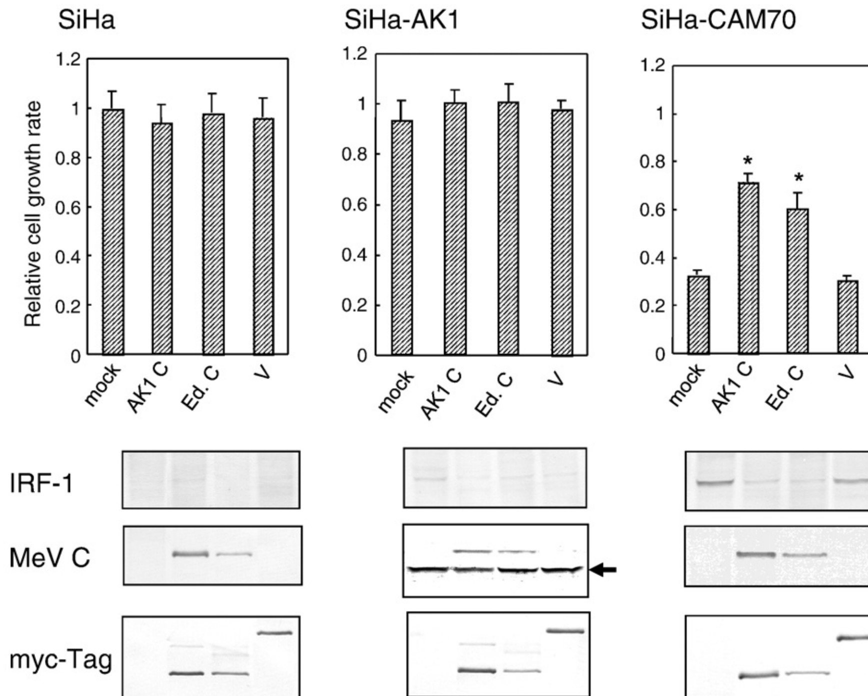

Fig. 5. Effect of MeV C protein on the growth inhibition and IRF-1 upregulation induced by MeV infection in SiHa, SiHa infected with AK1, and SiHa infected with CAM70 cells. Cells were transfected with expression plasmid of myc-tagged C derived from AK1 (AK1 C), myc-tagged C protein derived from Edmonston (Ed. C), or myc-tagged V protein derived from Edmonston (V). After 36 h transfection, cells were applied to the assays. Cell growth rate was determined by the uptake of 5-ethinyl-2′-deoxyuridine. Mock indicates a transfectant of control plasmid. **P*<0.01 compared to mock cells. Protein levels of IRF-1 and MeV C protein were determined by Western blotting. Plasmid-mediated expression of AK1 C, Edmonston (Ed.) C, and Edmonston V proteins was detected by Western blotting with anti-myc-tag antibody. An arrow in SiHa-AK1 panel indicates endogenous C protein.

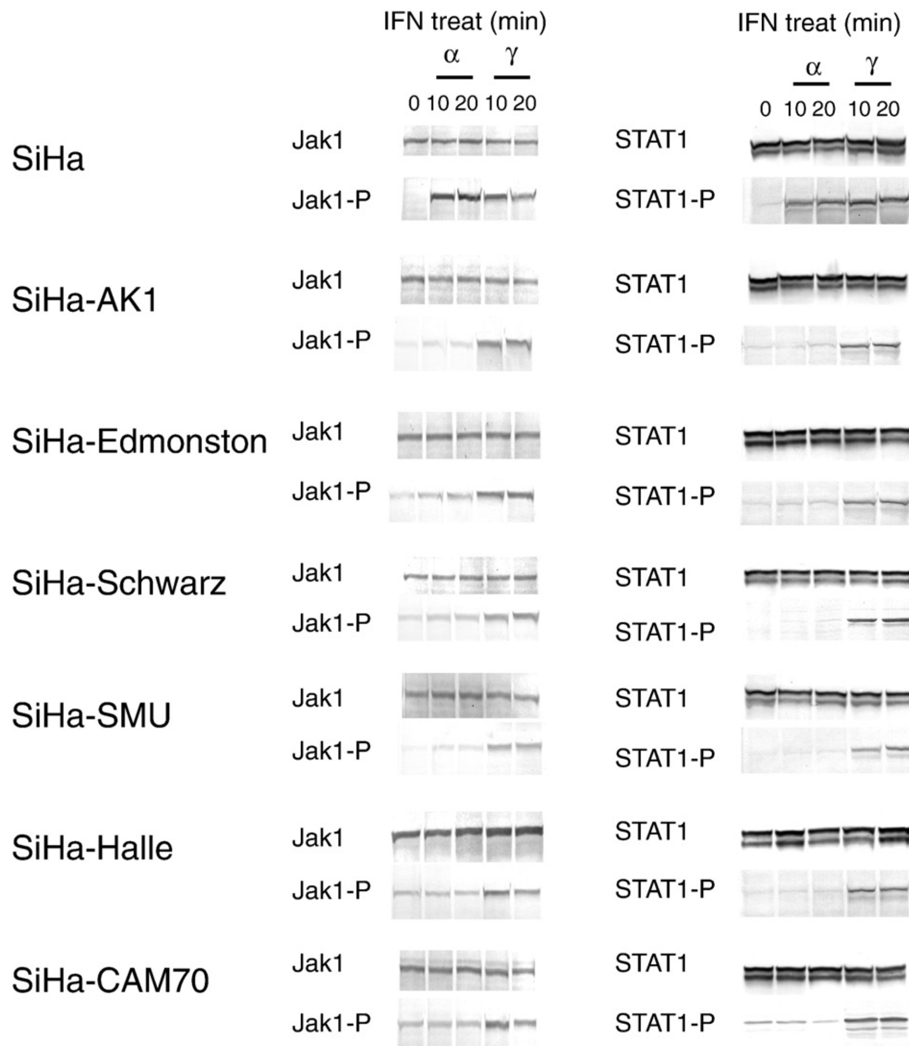

Fig. 6. IFN-α induced and IFN-γ induced phosphorylation of Jak1 and STAT1 in SiHa cells infected with various MeV strains. SiHa and MeV-infected SiHa cells were stimulated with IFN-α or IFN-γ at a concentration of 1000 U/ml. The cells were lysed at the time points indicated in the figure and analyzed by Western blotting using antibodies against STAT1, Tyr-phosphorylated STAT1 (STAT1-P), Jak-1, and Tyr-phosphorylated Jak1 (Jak1-P).

lysate with anti-C protein antibody showed specific co-precipitation of Tyr-phosphorylated STAT1α (Fig. 7B). The results indicated that C protein preferentially interacts with Tyr-phosphorylated STAT1 and suppresses STAT1 dimer formation. We examined localization of STAT1 by fluorescence microscopy. In uninfected SiHa and SiHa-AK1 cells, STAT1 located in cytosol but not in nucleus (Fig. 7C). In SiHa-CAM70 cells, STAT1 located in both cytosol and nucleus. IFN-γ-treatment translocated STAT1 into nucleus. However, in IFN-γ-treated SiHa-AK1 cells, a part of STAT1 retained cytosol compared to uninfected SiHa cells and SiHa-CAM70 cells. The results suggested that STAT1 tended to retain in the nucleus in SiHa-CAM70 cells and in the cytosol in SiHa-AK1 cells.

We examined the effect of C protein on GAS-dependent transcription by luciferase reporter gene assay using tandem repeats of GAS as an enhancer element (Fig. 8A) and binding of GAF to GAS by ELDIA (Fig. 8B). SiHa-CAM70 cells showed constitutive activation of GAS-dependent transcription and GAF binding to GAS, and the transcription and the binding were inhibited by forced expression of C proteins by the transfection of plasmids. The wild strain (AK1) and Vero-adapted strain (Edmonston) C proteins showed the similar inhibitory activities and expressed at similar levels in SiHa-CAM70 cells (Fig. 8C). The data suggested that the two C proteins inhibit GAS-dependent transcription to the similar extent.

Effect of C and V proteins on IFN-α and IFN-γ signaling

We examined IFN-α- and IFN-γ-induced IFN stimulating gene expression in SiHa cells transfected with expression plasmid of C protein or V protein. SiHa cells expressing V protein markedly suppressed IFN-α-induced MxA (Fig. 9A) and did not alter IFN-γ-induced IRF-1 (Fig. 9B). SiHa cells transfected with C protein only slightly suppressed both IFN-α-induced MxA and IFN-γ-induced IRF-1. These results suggested that V protein strongly suppressed IFN-α/β signaling. C protein seemed to suppress both IFN-α/β and IFN-γ signaling, but the extent of suppression was very weak.

Discussion

MeV accessory proteins, V and C, are required for the optimal growth of the virus. V protein counteracts the IFN-α/β signaling pathway via interaction with STAT1 and Jak1 (Caignard et al., 2009; Caignard et al., 2007; Ohno et al., 2004; Takeuchi et al., 2003). C protein contributes to maintaining virus replication and host cell survival. Toth et al. (2009) reported that C protein suppresses the apoptosis of virus-infected cells through the suppression of PKR activity. Nakatsu et al. (2006; 2008) reported that the translational inhibition of virus proteins was released by C protein. C protein also inhibits IFN-β production (McAllister et al.,

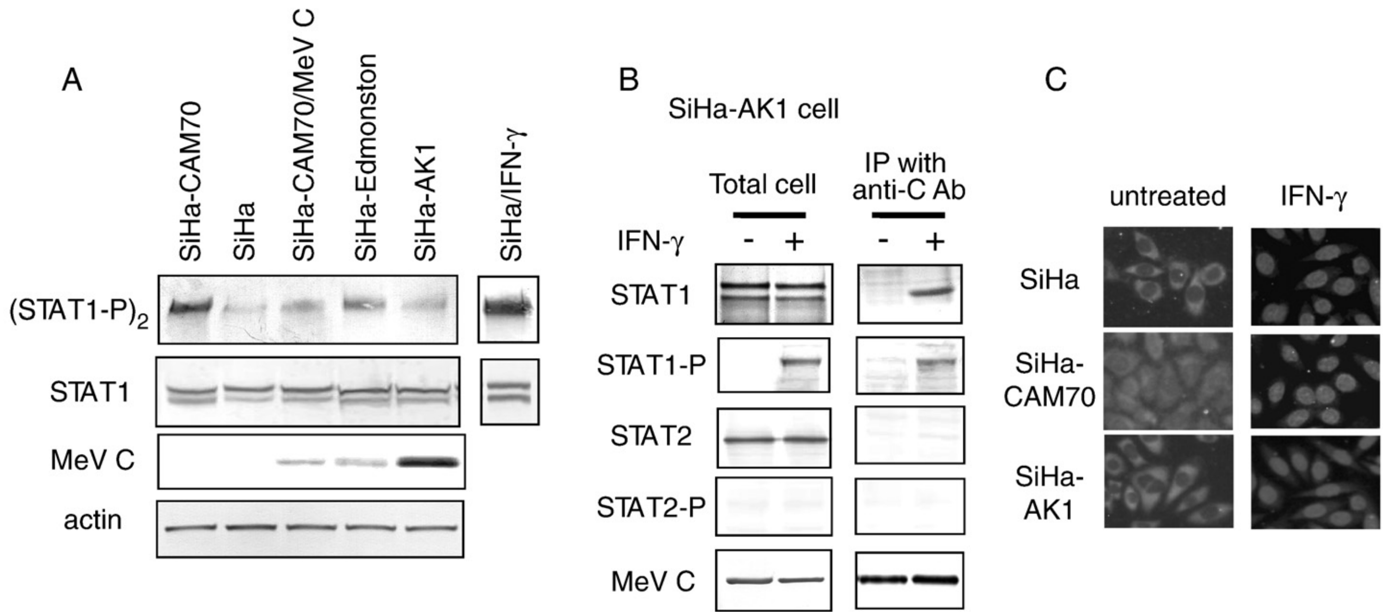

Fig. 7. MeV C protein interacts with Tyr-phosphorylated STAT1 and suppresses dimer formation. A) Tyr-phosphorylated STAT1 dimerization was determined by native PAGE. Uninfected SiHa, SiHa infected with various MeV strains, and SiHa-CAM70 transfected with C protein (derived from AK1) expression plasmid were lysed and assessed by native PAGE and Western blotting with anti-STAT1 antibody. Protein expression levels of STAT1, MeV C, and actin (as a loading control) were determined by SDS–PAGE/Western blotting. SiHa cells treated with IFN-γ (at 1000 U/ml for 30 min) were analyzed as a control for STAT1 dimerization. B) Interaction of Tyr-phosphorylated STAT1 and MeV C protein. SiHa-AK1 cells were treated with IFN-γ at 1000 U/ml for 30 min and lysed. Immunoprecipitation was carried out with anti-C protein antibody. Total cell lysates and immunoprecipitates were analyzed by Western blotting with antibodies against STAT1, Tyr-phosphorylated STAT1 (STAT1-P), STAT2, Tyr-phosphorylated STAT2 (STAT2-P), and MeV C protein. C) Immunofluorescence staining STAT1 in SiHa cells and SiHa cell infected with CAM70 or AK1 in the presence or absence of IFN-γ treatment (1000 U/ml for 2 h).

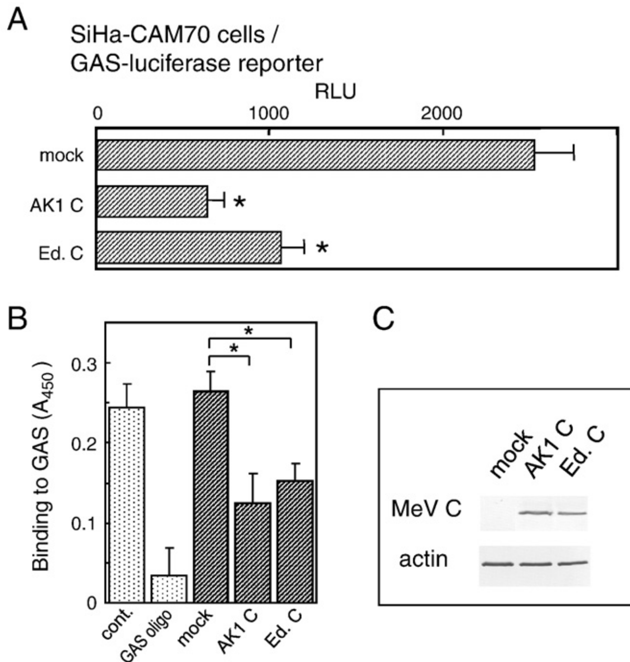

Fig. 8. Effect of MeV C protein on GAF formation in SiHa cells infected with CAM70. SiHa-CAM70 cells were transfected with GAS-luciferase plasmid and phRL-TK, together with expression plasmid encoding MeV C protein derived from AK1 (AK1 C) or Edmonston (Ed. C) or control plasmid (mock). After 36 h post-transfection, cells were lysed, and then GAF activities were assayed. $P<0.01$ compared to mock cells. A) GAS-luciferase reporter gene assay. Cell lysate was assessed by dual luciferase assay system as described in text. B) ELDIA using the GAS oligonucleotide as a coated target. Nuclear extract was prepared and assessed to ELDIA. Lysate from untransfected SiHa-CAM70 cells was applied in the absence (cont.) or in the presence of GAS oligonucleotide as a competitor (GAS oligo) as a control experiment. C) Expression of C protein in the transfectants was determined by Western blotting. Actin was determined as a control.

2010; Nakatsu et al., 2008) by the suppression of PKR, which is a mediator of IFN-β induction (McAllister et al., 2010). These events should lead to efficient virus replication. In the present study, we showed that C protein inhibited IFN-γ signaling, which led to the release

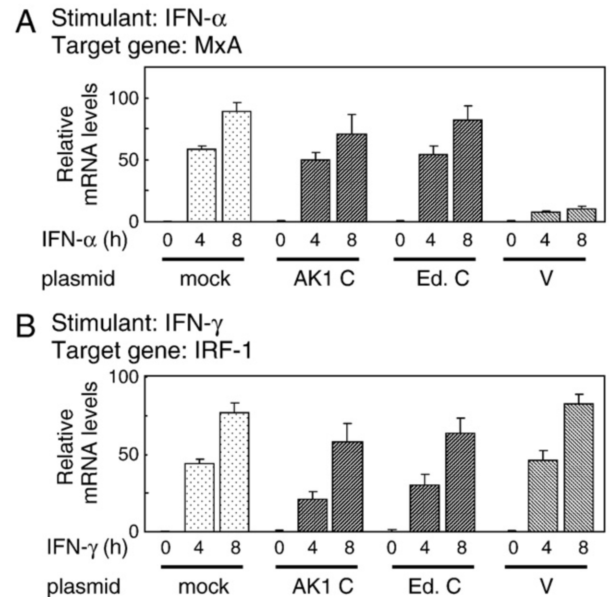

Fig. 9. Effect of MeV C and V proteins on the expression of IFN-α-induced MxA and IFN-γ-induced IRF-1 in SiHa cells. Cells were transfected with expression plasmid of myc-tagged C derived from AK1 (AK1 C), myc-tagged C protein derived from Edmonston (Ed. C) or myc-tagged V protein derived from Edmonston (V), or control plasmid (mock). After 36 h transfection, the transfectants were treated with IFN-α (1000 IU/ml) (A) or IFN-γ (1000 U/ml) (B). At a time indicated in the figure, total mRNA was extracted and assessed by real time RT–PCR. Each experiment was performed in triplicate. The data were normalized with the data for GAPDH and expressed as mean value ± standard deviation. Protein expression levels of C and V proteins were described in Fig. 5.

of host cells from growth arrest through the inhibition of virus-induced IRF-1 expression. C protein interacted with Tyr-phosphorylated STAT1 and suppressed the formation of STAT1 dimer, GAF. Other IFN-γ inducible gene expressions were also suppressed by C protein. We indicated that CIITA, which is an MHC class II transactivator, showed a reduced expression level in SiHa-AK1 cells compared to SiHa cells infected with other MeV strains. If suppression of CIITA expression by MeV infection occurs in the antigen presenting cells, C protein may affect antigen presentation. We observed decreased CIITA expression in human monocytic cell line U937 by MeV infection (data not shown). The ability of C protein to inhibit IFN signaling is still controversial. Takeuchi et al. (2003) reported that C protein affects neither IFN-α/β nor IFN-γ signaling, whereas V protein suppresses IFN-α/β signaling. Rota and his colleagues reported that both C and V proteins suppress IFN-α/β and IFN-γ signaling (Shaffer et al., 2003). V protein was shown to be a more potent inhibitor of IFN-signaling than C protein (Fontana et al., 2008), and C protein inhibited IFN-γ signaling to a lesser degree than IFN-α/β signaling (Shaffer et al., 2003). Consistent with these studies, we observed that V protein was a potent inhibitor of IFN-α signaling, but C protein was a lesser inhibitor. On the other hand, V protein did not affect IFN-γ signaling, whereas C protein partly suppressed IFN-γ signaling. Our previous study shows that both V and C proteins interact with IFN-α receptor complex, and we speculated that both V and C proteins contributed to suppression of IFN-α/β signaling in MeV-infected epithelial cells (Yokota et al., 2003). However, the present study indicated that V protein, but not C protein, is a major contributor for the suppression of IFN-α/β signaling.

In the previous study, we find that MeV infection induces IRF-1 independent to IFN-γ and cell growth arrest (Yokota et al., 2004). MeV poorly replicates in the growth arrest cells so we consider that the induction of IRF-1 is an antiviral strategy of host cells. The extent of induction varies among MeV strains. CAM70 showed most potent IRF-1 induction in SiHa cells compared to other MeV strains. The IFN-γ-independent induction of IRF-1 is controlled by GAF, as well as IFN-γ stimulation; however, it has been unclear how GAF has been activated. We speculate that the site of action of MeV is upstream of Jak1-IFN-γ-receptor complex because constitutive Jak1 phosphorylation occurred in SiHa cells infected with MeV, especially CAM70 (Fig. 6). We propose that C protein partially inhibits IFN-γ-independent induction of IRF-1 and IFN-γ signaling but not IFN-α/β signaling. On the other hand, C protein derived from wild strains and Vero-adapted attenuated strains showed similar inhibition of IFN-γ signaling, when C proteins were expressed by transfection of expression plasmid (Figs. 4D, 5, and 8C). Our results are consistent with those published by Fontana et al. (2008) showing similar IFN inhibition by C proteins derived from wild strains and from Vero-adapted attenuated strains. However, the expression levels of C protein in wild strain-infected cells were more potent than in Vero-adapted strain-infected cells (Figs. 1 and 2). Therefore, wild strains were more potent suppressors of IFN-γ signaling.

The molecular mechanisms underlying the higher expression level of C protein in wild-strain-infected cells than in Vero-adapted-strain-infected cells are unknown. The amino acid sequence of wild-type strain AK1 and IC-B differs from that of the Edmonston strain by four residues (Table 1). Each residue could contribute to the stability of C protein in host cells. Furthermore, no C protein was detected in SiHa-CAM70. Only one amino acid differs between CAM70 and Edmonston, and the reason for the lack of detectable C protein is unclear. In a preliminary experiment, the amount of C protein in Vero-adapted strain-infected SiHa cells, other than SiHa-CAM70, was increased upon exposure to the proteasome inhibitor lactacystin (data not shown). On the other hand, exogenous C protein expression using plasmid showed lower levels than C protein in MeV-infected cells, especially wild strain-infected ones. The C protein expression levels seemed to relate with intensity of IFN-γ signaling suppression. The higher expression levels of C protein may need infection of virus and thus may require other viral factors. The unknown viral factors, such as additional viral proteins, are needed to be investigated. Nishie et al. (2007) reported that transfected FLAG-tagged C protein accumulated exclusively in the nucleus during the early stages of MeV infection and in the cytoplasm during the late stage of infection. Nakatsu et al. (2008) reported that C protein localized exclusively in the cytoplasm upon MeV infection. These results suggest that the localization of C protein varies depending on whether it is expressed alone or whether it is expressed during viral infection. In addition, we have not identified any viral factors capable of activating IFN-γ signal transduction independently of IFN-γ stimulation. Further studies are needed to clarify these points.

Materials and methods

Cells and viruses

SiHa cells, MeV strains, and SiHa cells persistently infected with MeV were described previously (Yokota et al., 2004; Yokota et al., 2003). SiHa cell is a human uterine cervix carcinoma cell line. Strain AK1 is a wild strain, and the other 5 strains are Vero-adapted strains. Recombinant viruses (Nakatsu et al., 2006) were kindly provided by Professor Yusuke Yanagi (Kyushu University, Fukuoka, Japan) and Dr. Makoto Takeda (National Institute of Infectious Diseases, Tokyo, Japan). The recombinant wild MeV strain IC323-EGFP was derived from the plasmid p(+)MV323-EGFP and encodes the full-length genome of the wild MeV strain IC-B and the cDNA of green fluorescence protein (GFP). A C-defective recombinant MeV strain derived from strain IC-B (ΔC-EGFP) was derived from the plasmid p(+)MV ΔC-EGFP. Vero-hSLAM cells, which were kindly provided by Professor Yusuke Yanagi, were used for propagation and titration of the recombinant viruses, as described elsewhere (Nakatsu et al., 2006).

Expression plasmids and transfection into mammalian cells

Expression plasmids of myc-tagged V and myc-tagged C were prepared as follows. The cDNA of MeV P was prepared by RT–PCR using RNA from SiHa cells infected with AK1 or Edmonston. The MeV V and MeV C cDNAs (originated from AK1 or Edmonston) were prepared from pTARGET plasmid (Promega, Madison, WI) carrying the P cDNA as described previously (Yokota et al., 2008). The MeV V and MeV C cDNAs were inserted into pTARGET plasmids, after which XhoI-SalI fragments of these plasmids were inserted into the XhoI-cut pCMV-MYC vector (Clontech, Mountain View, CA).

Plasmids were transfected into SiHa and MeV-infected SiHa cells using Fugene 6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Cells were assayed 36 h after transfection.

In vitro translation

Viral proteins were prepared by in vitro translation using TNT Quick Coupled Transcription/Translation System (Promega) using an expression plasmid.

Antibodies

Rabbit anti-MeV C protein and rabbit anti-MeV V protein antibodies (Takeuchi et al., 2003) were kindly provided by Dr. Kaoru Takeuchi (Tsukuba University, Tsukuba, Japan). Mouse anti-MeV H protein monoclonal antibody was purchased from Chemicon (Temecula, CA). Mouse anti-MeV P protein monoclonal antibody (clone 49–21) was from Argene (Varilhes, France). Rabbit anti-IRF-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-STAT-2 (Y689) antibody was purchased from R&D systems (Minneapolis, MN). Mouse anti-myc-TAG monoclonal antibody was from Clontech. The other antibodies were as described previously (Yokota et al., 2003).

Western blotting

Cells were lysed with 1% Nonidet P-40, 120 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and 20 mM HEPES–NaOH (pH 7.5) as previously described (Yokota et al., 2001b). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), native PAGE, and Western blotting were carried out as previously described (Yokota et al., 2001a; Yokota et al., 2001b). Alkaline phosphatase-labeled goat anti-mouse or anti-rabbit immunoglobulin antibodies as secondary antibodies were purchased from BioSource International (Camarillo, CA). Specific binding was detected by using tetrazolium bromochloroindolylphosphate–nitro blue tetrazolium as a developing substrate. The resulting membrane was scanned using a flatbed scanner. The bands were quantified using the public domain Image J program (US National Institutes of Health, Bethesda, MD).

Real time RT–PCR

Total cellular RNA was isolated using the QuickGene-800 nucleic acid isolation system (Fuji Film, Tokyo, Japan) and QuickGene RNA cultured cell HC kit (Fuji Film). The cDNA was prepared from total RNA with a reverse transcription system kit (Promega, Madison, WI) using random primers (GE Healthcare Bioscience, Piscataway, NJ). Real time PCR was performed using the cDNA as templates with TaqMan® Gene Expression Master Mix system and Applied Biosystems 7500 real time PCR system (Applied Biosystems, Foster City, CA). The primer sets used were IRF-1 (Hs00971960 m1), CIITA (Hs00172094 m1), MxA (Hs00895608 m1), and GAPDH (Hs03929097 g1) (Applied Biosystems). The mRNA levels were normalized with the mRNA levels of GAPDH.

Cell proliferation assay

Cell proliferation was measured by the uptake of 5-ethinyl-2′-deoxyuridine (EdU) into DNA, using a Click-iT EdU microplate assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Luciferase reporter gene assay

GAS-luciferase reporter plasmid (PathDetect in Vivo Signal Transduction Pathway cis-Reporting Systems) was purchased from Stratagene (La Jolla, CA). The plasmid contains $(AGTTTCATATTACTCTAAATC)_4$ upstream of the luciferase gene as the enhancer element. The cells were plated in 96-well microplates. The plasmid mixtures were subsequently transfected using SuperFect reagent (Qiagen) according to the manufacturer's instructions. The plasmid mixtures contain C protein expression plasmid or control empty pTARGET plasmid (0.04 μg/well) and GAS-luciferase plasmid (0.02 μg/well), in addition to 0.0025 μg/well of phRL-TK (Promega) for normalization. Thirty-six hours after transfection, luciferase activity was measured in cell lysates by using the dual-luciferase reporter assay system (Promega) according to manufacturer's instructions.

Enzyme-linked DNA–protein interaction assay (ELDIA)

ELDIA was performed as described previously (Yokota et al., 2004). Briefly, nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). A biotin-labeled double stranded GAS oligonucleotide (5′-GCCTGATTTCCCCGAAATGACGGC-3′) derived from IRF-1 promoter sequence was immobilized on a streptavidin-coated microtiter plate (Pierce). Nuclear extract, a mouse anti-STAT1 monoclonal antibody (BD Bioscience, Franklin Lakes, NJ) and a horseradish-labeled goat antimouse Ig's antibody (Biosource) were subsequently dispensed into the microplate and incubated. Binding GAF to GAS was detected by developing by 3,3′,5,5′-tetramethylbenzidine solution (Biosource). The double stranded oligonucleotide (5′-GCCTGATTTCCCCGAAATGACGGC-3′) was used as a competitor for confirmation of the specific binding.

Immunoprecipitation

Immunoprecipitation was carried out as described previously (Yokota et al., 2003). Cellular proteins were solubilized in RIPA buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 100 mM NaCl, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 mTIU/ml aprotinin, 1 mM Na_3VO_4, 1 mM NaF, and 50 mM HEPES–NaOH, pH 7.5). The cell lysate and 5 μg of antibody were mixed and incubated at 4 °C for 1 h. Protein G-Sepharose 4B (GE Healthcare, Uppsala, Sweden) was added to the mixture and incubated at 4 °C overnight on a rotator. The resin was harvested by centrifugation and washed in the RIPA buffer 4 times. The bound material was solubilized by treatment with SDS–PAGE sample buffer at 100 °C for 5 min, and the supernatant was analyzed by Western blotting as described above.

Immunofluorescence microscopy

Cells cultured on a coverslip were fixed with methanol at room temperature for 5 min and then blocked with PBS containing 2% goat serum. The specimens were incubated with mouse anti-STAT1 monoclonal antibody (Transduction, Lexington, KY) at 37 °C for 1 h and then with fluorescence-labeled goat anti-mouse Ig's antibodies (Biosource) at 37 °C for 1 h. Fluorescence microscopy was carried out with an Olympus IX71 system (Olympus, Tokyo, Japan).

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