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Measles virus suppresses interferon-α signaling pathway: suppression of Jak1 phosphorylation and association of viral accessory proteins, C and V, with interferon-α receptor complex

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Abstract

To establish infections, viruses use various strategies to suppress the host defense mechanism, such as interferon (IFN)-induced antiviral state. We found that cells infected with a wild strain of measles virus (MeV) displayed nearly complete suppression of IFN-α-induced antiviral state, but not IFN-γ-induced state. This phenomenon is due to the suppression of IFN-α-inducible gene expression at a transcriptional level. In the IFN-α signal transduction pathway, Jak1 phosphorylation induced by IFN-α is dramatically suppressed in MeV-infected cells; however, phosphorylation induced by IFN-γ is not. We performed immunoprecipitation experiments using antibodies against type 1 IFN receptor chain 1 (INFAR1) and antibody against RACK1, which is reported to be a scaffold protein interacting with type I IFN receptor chain 2 and STAT1. These experiments indicated that IFNAR1 forms a complex containing the MeV-accessory proteins C and V, RACK1, and STAT1 in MeV-infected cells but not in uninfected cells. Composition of this complex in the infected cells altered little by IFN-α treatment. These results indicate that MeV suppresses the IFN-α, but not IFN-γ, signaling pathway by inhibition of Jak1 phosphorylation. Our data suggest that functional disorder of the type I IFN receptor complex is due to "freezing" of the receptor through its association with the C and/or V proteins of MeV.

Keywords: Measles virus; Type I interferon; JAK/STAT pathway; Accessory proteins; Jak1; Interferon receptor

Introduction

Cells are provided with various defense mechanisms against viral infection. The most important of these is the establishment of an antiviral state induced by interferon (IFN), which also has multiple functions to the cell (cell-growth regulation, cell differentiation, and immune regulation). The antiviral activity of IFN is due to IFN-inducible proteins such as 2′,5′-oligoadenylate synthetase (2-5AS), dsRNA-activated protein kinase (PKR), and MxA protein (Fujii, 1994; Samuel, 1991; Sen and Ransohoff, 1993). The expression of these proteins is induced by IFN upon activation of signal transduction pathway after IFN binds to certain specific cell-surface receptors. The type I IFN, namely IFN-α/β, receptor consists of two polypeptide chains: human type I IFN receptor chain 1 (IFNAR1/IFN-αR) and human type I IFN receptor chain 2 (IFNAR2.2/IFNAR2c/IFN-αRβL) (Mogensen et al., 1999). The intracellular IFN signal transduction pathway consists of Janus protein kinases (JAKs) and signal transducer and activator of transcription (STAT) family proteins, as well as numerous other cytokines (Stark et al., 1998). JAKs interact with

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cytoplasmic domain of IFN receptors. The type I IFN receptor associates with Jak1 and Tyk2, and the IFN-γ receptor associates with Jak1 and Jak2. When type I IFN binds to the specific receptor, tyrosine residues on the cytoplasmic domain of IFNAR2.2 chain are phosphorylated (Wagner et al., 2002). As a result, JAKs are activated by phosphorylation, and activated JAKs phosphorylate STATs, a component of transcriptional factors that binds to IFN-responsive promoter elements. IFN-α-responsive element, called the IFN-stimulated response element (ISRE), activated by binding of the transcriptional factor ISGF3, which consists of phosphorylated STAT1, phosphorylated STAT2 and IRF-9/p48/ISGF3γ. IFN-γ-responsive element, called the γ activation sequence (GAS), activated by GAF, a homodimer of phosphorylated STAT1 (Goodbourn et al., 2000; Sen and Ransohoff, 1993).

Most DNA and RNA viruses have evolved the ability to suppress the functions of these antiviral proteins to resist the antiviral defense provided by IFN (Fujii, 1994; Garcia-Sastre, 2001; Goodbourn et al., 2000). Furthermore, some viruses have the capacity to block the IFN-mediated activation of the JAK/STAT signal transduction pathways using several strategies such as the following: (i) suppression of the phosphorylation of STATs and JAKs, (ii) inactivation of components of the JAK/STAT pathway, and (iii) reduction of the constitutive or basal levels of particular molecules that contribute to the JAK/STAT pathway (Garcia-Sastre, 2001; Goodbourn et al., 2000; Joseph and Look, 2001; Najarro et al., 2001; Tan and Katze, 2001; Yokota et al., 2001).

Viruses belonging to family *Paramyxoviridae* block the IFN signal transduction pathway using a variety of mechanisms (Gotoh et al., 2001; Young et al., 2000). Sendai virus (SeV) and human parainfluenza virus type 3 (hPIV3), both belonging to genus *respirovirus*, reduce the levels of phosphorylation of STAT1 (Gao et al., 2001; Garcin et al., 1999; Kato et al., 2001; Komatsu et al., 2000). Viruses belonging to genus rubulavirus dramatically decrease the basal levels of STAT proteins. Simian virus 5 (SV5) and mumps virus (MV) reduce STAT1 protein levels (Didcock et al., 1999; Fujii et al., 1999; Kubota et al., 2001; Yokosawa et al., 1998), and parainfluenza virus type 2 (hPIV2) reduce STAT2 protein levels (Nishio et al., 2001; Parisien et al., 2001). Inhibition of the IFN-signaling pathway caused by these viruses is shown to be mediated through viral accessory proteins, such as V and C (Didcock et al., 1999; Garcin et al., 1999; Kato et al., 2001; Kubota et al., 2001; Nishino et al., 2001; Parisien et al., 2001). In viruses belonging to genus *pneumovirus*, such as human respiratory syncytial virus (hRSV), these events are not found, although hRSV is able to circumvent the IFN response (Young et al., 2000). Therefore, hRSV may have an alternative strategy. Viruses belonging to genus *morbillivirus* should also suppress the IFN response using an unknown strategy for suppression of IFN-α action.

Measles virus (MeV) is a member of genus *morbillivirus*. Influence of MeV on IFN signaling pathway has been investigated using several classical (laboratory-adapted) strains and vaccine strains of MeV. Suppression of IFN-induced 2-5AS was shown in persistent infection of MeV (Fujii et al., 1988, 1990). The events were shown to be dependent on combinations of host cell types and virus strains. For example, 2-5AS activity is significantly induced by infection with Edmonston and CAM-70 strains in K562 cells, but merely induced by infection with AIK-C and Hälle strains (Fujii et al., 1988). Induction of 2-5AS by IFN occurs mainly through activation of JAKs and STATs, as well as other IFN-inducible genes. Several lines of evidence reveal that MeV has the potential to block or suppress the IFN response (Fujii et al., 1988, 1990). On the other hand, wild strains of MeV isolated from the throat swabs of patients with acute measles using marmoset cell line B95a (Saito et al., 1992, 1994) were not able to propagate in monkey kidney cell lines such as Vero cells. These clinically isolated wild strains are found to have a hemagglutinin (HA)-type distinct from that of classical strains such as the Edmonston strain (Saito et al., 1994, 1995). In the present study, we show that a wild strain of MeV suppresses the IFN response, specifically through induction of IFN-inducible genes, fluctuation of IFN-induced antiviral activity, and inhibition of the JAK/STAT signaling pathway. Furthermore, we discuss the molecular mechanisms of this suppression.

Results

AK-1 infection in human cell lines

Various cell lines were infected with a MeV wild strain AK-1 and cultured for 14 days without any CPE. Viral load increased in the infected cell lines K562, SiHa, CaSki, SK-N-AS, and P3HR-1 as revealed by detecting HA mRNA, and the HA mRNA was confirmed to be AK-1-derived by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (data not shown) in the manner described previously (Saito et al., 1995). On the other hand, AK-1 did not grow in U937 or Jurkat cells. MeV infection in five cell lines was confirmed by expression of MeV C and V proteins by using Western blotting, and cells tested were positive for MeV antigens using fluorescein isothiocyanate (FITC)-labeled rabbit anti-MeV antiserum (data not shown). Infected cells produced progeny virus into culture fluids, which induced significant syncytia formation in B95a cells. These cell lines were confirmed to carry no EB virus genome derived from AK-1 propagating cell B95a by EBER transcripts that were not detectable by PCR (data not shown). We also established cell lines infected with other wild-type strains, AK-2, AK-3, AK-4, AK-5, and AK-6, as above.

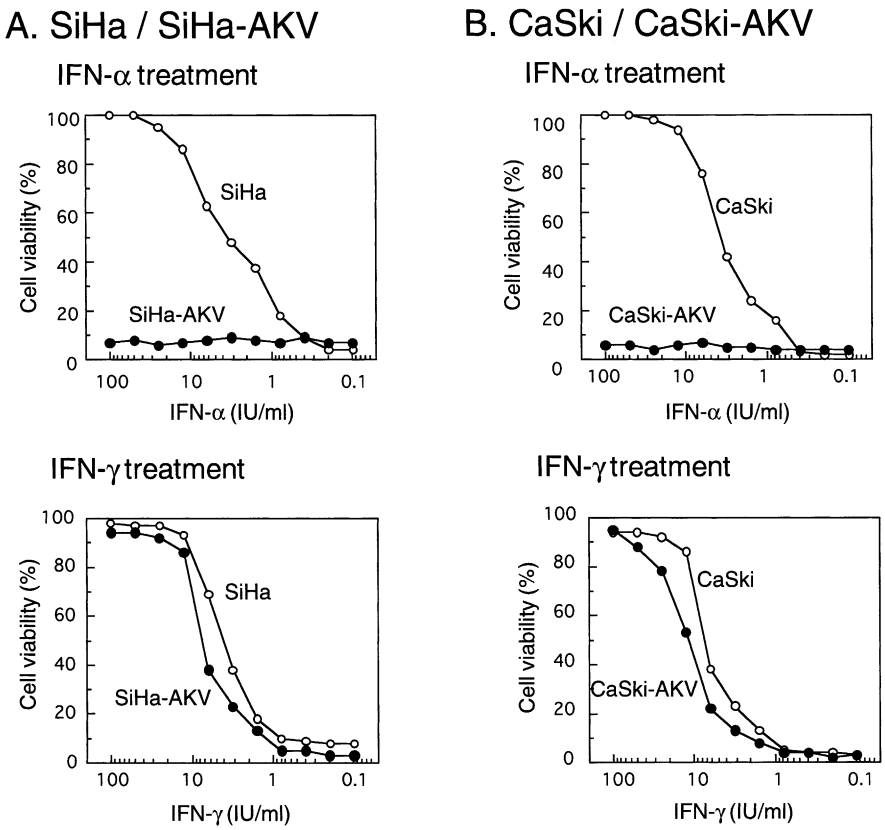

Fig. 1. Fluctuation of IFN-induced antiviral activity in cells with strain AK-1 of MeV. (A) SiHa cells (○) and AK-1-infected SiHa (SiHa-AKV) cells (●). (B) CaSki cells (○) and AK-1-infected CaSki (CaSki-AKV) cells (●). The cells were treated with various concentrations of IFN-α (top) or IFN-γ (bottom) for 24 h at 37°C. Thereafter, VSV was challenged at an m.o.i. of 1.0. After cultivation, cell viability was determined by a dye-binding assay using gentian violet.

AK-1-infected cells are resistant to IFN-α, but not IFN-γ

We examined the IFN-induced antiviral activity in uninfected and AK-1-infected cells by determining CPE formation by VSV. AK-1-infected SiHa cells and AK-1-infected CaSki cells showed nearly complete suppression of IFN-α-induced resistance to VSV compared to uninfected cells (Fig. 1). On the other hand, IFN-γ-induced resistance to VSV did not change in AK-1-infected cells compared to uninfected cells.

We also determined efficacy of VSV replication in uninfected and AK-1-infected cells (Table 1). SiHa and CaSki cells showed a similar decrease in yields of VSV (10^5 order) in the culture supernatant after treatment with both IFN-α and IFN-γ, although SiHa-AKV and CaSki-AKV displayed similar yields of VSV between IFN-α-treated and untreated cells. IFN-γ-induced decreased yields of VSV were not significantly affected by infection with AK-1. P3HR-1 also showed that IFN-α induced suppression of VSV replication was clearly diminished by AK-1 infection. Because significant IFN-γ-induced antiviral activity was not observed in P3HR-1 cells, the cells are predicted to be unresponsive to IFN-γ.

These results suggest that IFN-α-, but not IFN-γ-, induced antiviral activity is suppressed by infection with the

Table 1
Efficacy of VSV replication after treatment with IFN in MeV strain AK-1-infected SiHa, CaSki, and P3HR-1 cells

Cells	IFN	Yield of VSV (PFU/ml)
SiHa	—	2.4×10^8
	α	3.2×10^3
	γ	4.4×10^3
SiHa-AKV	—	1.7×10^8
	α	2.4×10^8
	γ	2.8×10^4
CaSki	—	1.7×10^8
	α	2.0×10^3
	γ	2.6×10^3
CaSki-AKV	—	1.1×10^8
	α	9.2×10^7
	γ	3.8×10^4
P3HR-1	—	1.5×10^8
	α	1.0×10^3
	γ	4.8×10^7
P3HR-1-AKV	—	6.7×10^7
	α	2.2×10^8
	γ	8.6×10^6

Note. Cells were treated with IFN-α or IFN-γ at a concentration of 10^3 IU/ml for 24 h at 37°C and then challenged with VSV. Virus titer in the resulting supernatant was determined by a plaque-forming assay using Vero cells as indicator cells.

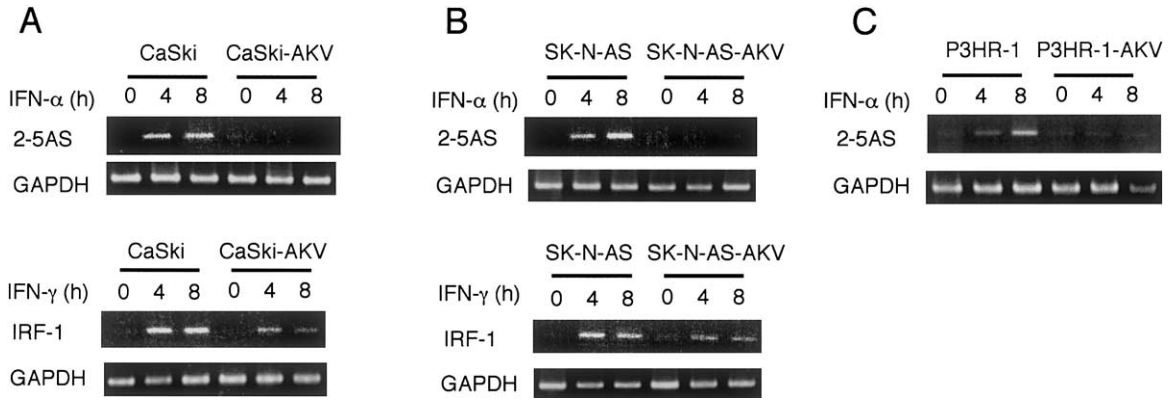

Fig. 2. mRNA levels of IFN-inducible genes, 2-5AS, and IRF-1 in cells infected with MeV strain AK-1. Targets used were 40 kDa 2-5AS and IRF-1 as an IFN-α-inducible gene and an IFN-γ-inducible gene, respectively. (A) CaSki and CaSki-AKV cells. (B) SK-N-AS and SK-N-AS-AKV cells. (C) P3HR-1 and P3HR-1-AKV cells. GAPDH used as a control.

AK-1 strain of MeV. Similar results were obtained in cells infected with other MeV wild-type strains (data not shown).

Effect of MeV infection on induction of IFN-inducible antiviral genes

We examined the expression of IFN-inducible genes in AK-1-infected and uninfected cells at mRNA level as determined by RT-PCR (Fig. 2). The induction of 2-5AS mRNA by IFN-α was inhibited in the AK-1-infected cells tested, such as CaSki-AKV, SK-N-AS-AKV, and P3HR-1-AKV, compared with uninfected controls. Induction of IRF-1 mRNA by IFN-γ in CaSki-AKV and SK-N-AS-AKV cells was detected with a slight reduction compared to the uninfected controls. IRF-1 mRNA was not induced by IFN-γ in P3HR-1 cells (data not shown). Consistent with the results of antiviral activity (Table 1), P3HR-1 cells have no or a low response to IFN-γ.

Furthermore, we examined the effect of MeV infection on IFN-inducible gene expression by a luciferase reporter gene assay. To examine the response to IFN-α, five tandem repeats of ISRE were used as enhancer elements of the firefly luciferase gene. We used a dual luciferase assay system using the *Renilla* luciferase gene under the control of the HSV thymidine kinase promoter as a control for transfection efficacy. Treatment with 1000 IU/ml IFN-α for 24 h increased firefly luciferase activity about 8- and 13-fold in SiHa and CaSki cells, respectively, as compared to the untreated control. In the AK-1-infected cells (SiHa-AKV and CaSki-AKV), IFN-α-induced reporter activity was 1.7- and 2.2-fold, respectively, compared to the untreated control (Fig. 3). Induction of reporter gene expression by IFN-α was dramatically suppressed in AK-1-infected cell lines. To examine IFN-γ-induced gene expression, four tandem repeats of the GAS element were used as an enhancer element for the firefly luciferase gene with no significant difference

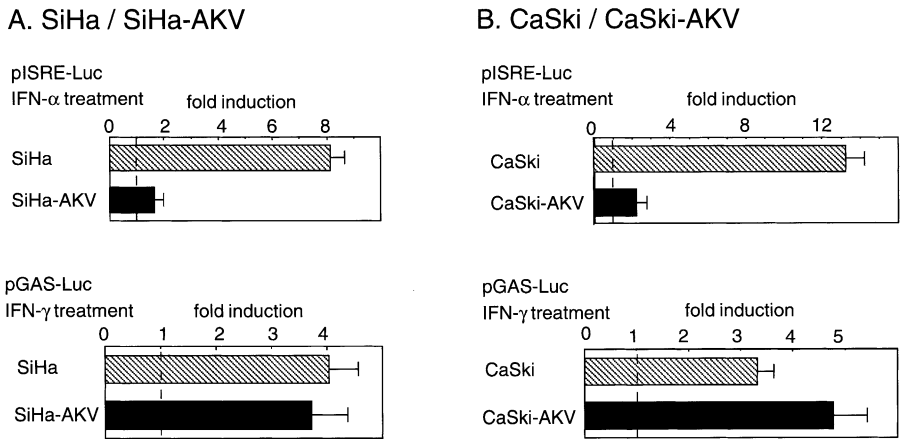

Fig. 3. Effect of MeV strain AK-1 infection on induction of IFN-inducible genes as determined by a reporter gene assay in SiHa and CaSki cells. Cells with or without AK-1 infection were transfected with reporter plasmids containing luciferase reporter genes linked to IFN-responsive enhancers, pIRSE-Luc for IFN-α stimulation and pGAS-Luc for IFN-γ stimulation, plus pRL-TK as an internal reference for transfection efficacy. The transiently expressed transfectants were treated with 1000 IU/ml IFN-α or IFN-γ for 24 h. The cell lysates were assayed for luciferase activity. All experiments were performed in quadruplicate. The results are expressed as fold induction relative to the values obtained from experiments without IFN treatment.

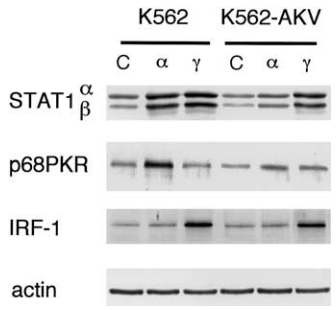

Fig. 4. Effect of MeV strain AK-1 infection on induction of protein levels of IFN-inducible STAT1, p68PKR, and IRF-1 in K562 and AK-1-infected K562 cells. K562 and K562-AKV cells were treated with IFN-α or IFN-γ at 1000 IU/ml at 37°C for 24 h. The cell lysates (50 or 5 μg/lane) were applied to SDS–PAGE on a 7.5 or 10% acrylamide gel and analyzed by Western blotting. C: control (no treatment); α: IFN-α treated; γ: IFN-γ treated.

in induction of reporter activity by IFN-γ treatment between SiHa and SiHa-AKV cells. In the case of CaSki, induction by IFN-γ was slightly enhanced by AK-1 infection (Fig. 3).

Consistent results were observed in protein levels of the IFN-inducible genes. We determined protein levels of STAT1 (inducible by both IFN-α and IFN-γ), p68PKR (IFN-α-inducible), and IRF-1 (IFN-γ inducible) in K562 and K562-AKV cells (Fig. 4) IFN-α-induced upregulation of STAT1 and p68PKR was severely decreased in K562-AKV. On the other hand, IFN-γ-induced upregulation of STAT1 and IRF-1 protein levels were not altered by AK-1 infection.

The results indicate that the inhibition of IFN-α-induced antiviral activity by MeV infection is due to suppressed expression of IFN-inducible genes in response to IFN-α.

Basal protein levels of STATs and IRF-9 in MeV-infected cells

To determine the suppression mechanism of IFN-inducible gene expression by MeV infection, we first used Western blotting to investigate changes in protein levels of STAT1, STAT2, and IRF-9/p48, all of which contribute to the IFN-α intracellular signaling pathway in cells with or without MeV infection (Fig. 5). Varied patterns were observed among cell lines. For example, STAT2 and IRF-9 increased in AK-1-infected CaSki cells. STAT1 and IRF-9 increased in AK-1-infected P3HR-1 cells. On the other hand, STAT1 decreased in AK-1-infected SK-N-AS cells. Protein levels of JAKs did not show significant differences between uninfected and AK-1-infected cells of SiHa and CaSki (Fig. 6). These results indicate that changes in protein levels of STAT1, STAT2, IRF-9, and JAKs do not contribute to suppression of IFN-induced antiviral activity.

Suppression of IFN-α-induced JAK phosphorylation in MeV-infected cells

We investigated the suppression of IFN-α-induced events in AK-1-infected cells as it relates to the JAK/STAT pathway level. Figure 7 shows activation (namely phosphorylation status) of STAT1 and JAKs in SiHa and SiHa-AKV cells during treatment with IFN-α or IFN-γ. In SiHa-AKV cells, IFN-α-induced phosphorylation of STAT1 and Jak1 was dramatically suppressed more than 95% at 20 min treatment compared with the uninfected control (Fig. 6A). On the other hand, IFN-α-induced Tyk2 phosphorylation, and IFN-γ-induced phosphorylation of STAT1, Jak1, and Jak2, did not show significant changes following AK-1 infection. Similar results were observed in CaSki and CaSki-AKV cells. IFN-α-induced phosphorylation of STAT1 and Jak1 inhibited almost completely and 84% at 10 min treatment, respectively (Fig. 6B). Although IFN-α-induced phosphorylation of STAT1 and Jak1 was clearly inhibited in CaSki-AKV cells, IFN-γ-induced phosphorylation was not. Interestingly, IFN-γ-induced phosphorylation of Jak1 and Jak2 was prolonged and enhanced 20 min after induction in CaSki-AKV cells compared to uninfected cells (Fig. 6B). This result may be consistent with the finding that inducibility of reporter gene expression under the GAS promoter by IFN-γ is slightly enhanced in CaSki-AKV cells compared to CaSki cells (Fig. 3).

Viral proteins, C and V, interact with type I IFN receptor

Suppression mechanism of the IFN-signaling pathway by MeV is very interesting, because IFN-α-induced Jak1 phosphorylation, but not IFN-γ-induced phosphorylation, is specifically suppressed. These results suggest that MeV interferes with the IFN-α-signaling pathway upstream of the Jak1 phosphorylation step. Thus, we tried to determine the components of the type I IFN receptor (IFNAR) complex in uninfected and AK-1-infected cells by immunoprecipitation analysis (Fig. 7). First, we performed immunoprecipitation using the anti-IFNAR1 antibody. The immunoprecipitates obtained from SiHa-AKV cell lysate contained viral proteins C and V, STAT1, and receptor for activated C kinase β (RACK1), together with IFNAR1. The other viral proteins are not detected in the precipitates as shown by im-

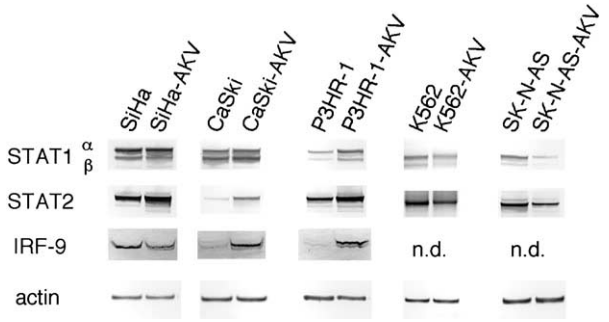

Fig. 5. Fluctuation of basal protein expression levels of STAT1, STAT2, and IRF-9 in MeV strain AK-1-infected cells. Cultured cells were collected and lysed. The lysates (50 or 5 μg/lane) were applied to SDS–PAGE on a 7.5% (for STAT1 and STAT2) or 12.5% (for IRF-9 and actin) acrylamide gel and analyzed by Western blotting. n.d.: not detectable.

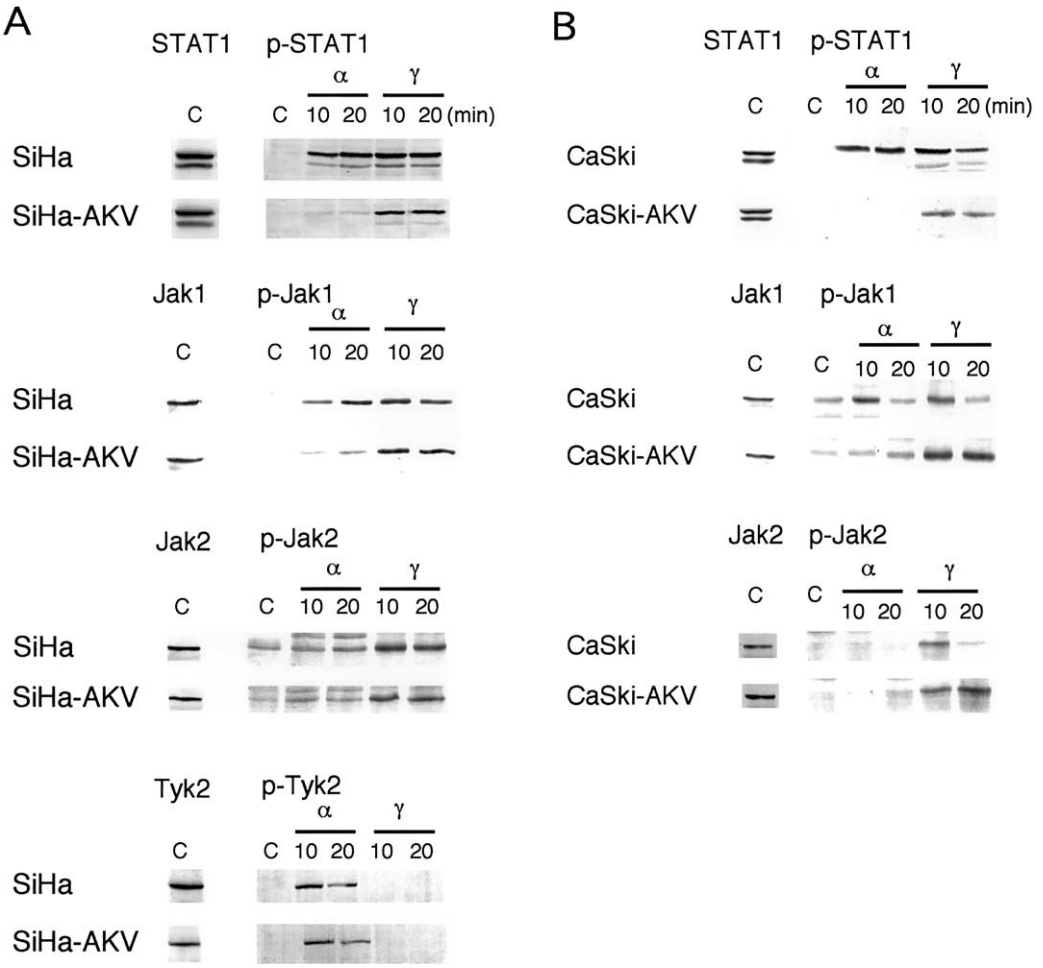

Fig. 6. Effect on IFN-induced phosphorylation of STAT1 and JAKs by MeV strain AK-1 infection in SiHa (A) and CasKi (B) cells. SiHa, SiHa-AKV, CasKi, and CasKi-AKV cells were treated with IFN-α or IFN-γ at 1000 IU/ml at 37°C for 10 or 20 min. The cell lysates (50 μg/lane) were applied to SDS–PAGE on a 7.5% polyacrylamide gel and analyzed by Western blotting. Protein levels of STAT1 and JAKs in the untreated control are presented as a representative result. Protein levels did not change during IFN treatment (data not shown). C: control experiment without IFN treatment; α: IFN-α treatment; γ: IFN-γ treatment.

munoblotting with anti-MeV M and anti-MeV N (lowest panels of Fig. 7). We also examined immunoprecipitation using anti-IFN-γ receptor (IFN-γRα) antibody. Any of these MeV proteins did not coprecipitate with the anti-IFN-γRα antibody (data not shown). This indicated that coprecipitation of these MeV proteins with anti-IFNAR1 antibody was due to formation of a specific complex with IFNAR. The components of this interaction did not significantly change after IFN-α treatment for 20 min; however, the amounts of STAT1 and MeV C and V proteins coprecipitated with IFNAR1 were reduced (Fig. 7B). Furthermore, phosphorylated STAT1 was not detected in the precipitates even after IFN treatment by Western blotting (data not shown). Interestingly, precipitated IFNAR1 by anti-IFNAR1 antibody slightly increased at 10 min IFN treatment in both uninfected and infected cells. The reason was obscure; however, the result was reproducible.

RACK1 is a member of G protein family and acts as a scaffold protein. It has been reported that RACK1 interacts with many proteins, including IFNAR2.2 and inactivated (unphosphorylated) form of STAT1 (Croze et al., 2000; Usacheva et al., 2001). On the other hand, STAT1 protein cannot be detected in the immunoprecipitate obtained from SiHa cells (Fig. 7B). Next, we performed immunoprecipitation using the anti-RACK1 antibody (Fig. 7C). In uninfected SiHa cells, IFNAR1 and STAT1 were coprecipitated with RACK1. IFNAR1 and STAT1 precipitated by anti-RACK1 antibody seemed to interact with independent RACK1 molecules and did not exist on the same complex, as shown in Fig. 8, because STAT1 did not coprecipitated with IFNAR1 (Fig. 7B). Furthermore, the coprecipitated STAT1 with RACK1 diminished following IFN-α treatment. This result is consistent with the report that RACK1 interacts with the resting form of STAT1 but not with its activated (phosphorylated) form (Croze et al., 2000). Interestingly, larger amounts of IFNAR1 were found in precipitates obtained from SiHa-AKV than in precipitates obtained from SiHa. It seems that the IFNAR1 complex

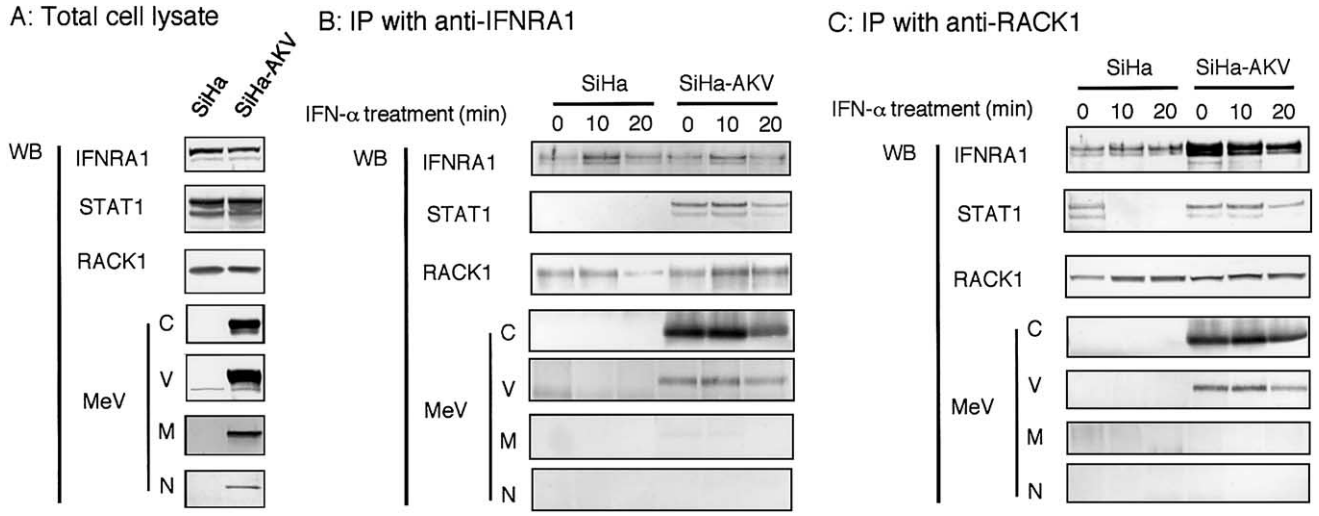

Fig. 7. Immunoprecipitation analysis of type I IFN receptor complex in SiHa and MeV strain AK-1-infected SiHa cells by using anti-IFNAR1 antibody (B) and anti-RACK1 antibody (C). SiHa and SiHa-AKV cells were treated with IFN-α at 1000 IU/ml for 10 or 20 min. IFNAR1- and RACK1-interacting proteins were precipitated from cell lysates using the relevant antibodies. The precipitates were analyzed by Western blotting (WB) using antibodies indicated on the left side of the column. As a control, Western blots of the total cell lysates are shown in (A).

containing RACK1 and MeV C and V is very tight and large in the infected SiHa cells, so anti-RACK1 antibody can precipitate it with high avidity. This speculation is supported by the fact that this complex was detected under higher stringency condition using a RIPA buffer containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.02% Sodium dodecyl sulfate (SDS), although the interactions of STAT1, RACK1, and IFNAR1 in uninfected cells were dramatically reduced (data not shown). Proteins C and V, but not other MeV proteins, were found to interact with the IFNAR1-STAT1-RACK1 complex in SiHa-AKV. This interaction was not drastically changed by IFN-α; however, the amount of STAT1, RACK1, and MeV proteins tended to decrease following IFN-α treatment for 20 min. Similar results were observed in CaSki and CaSki-AKV cells (data not shown). These lines of evidence suggest that the IFNAR-STAT1-RACK1 complex interacts with MeV C and V proteins and this complex is stabilized in AK-1-infected cells.

We also performed immunoprecipitation using anti-C and anti-V antibodies. Both antibodies can precipitate only a small part of proteins in cell lysates, and therefore neither

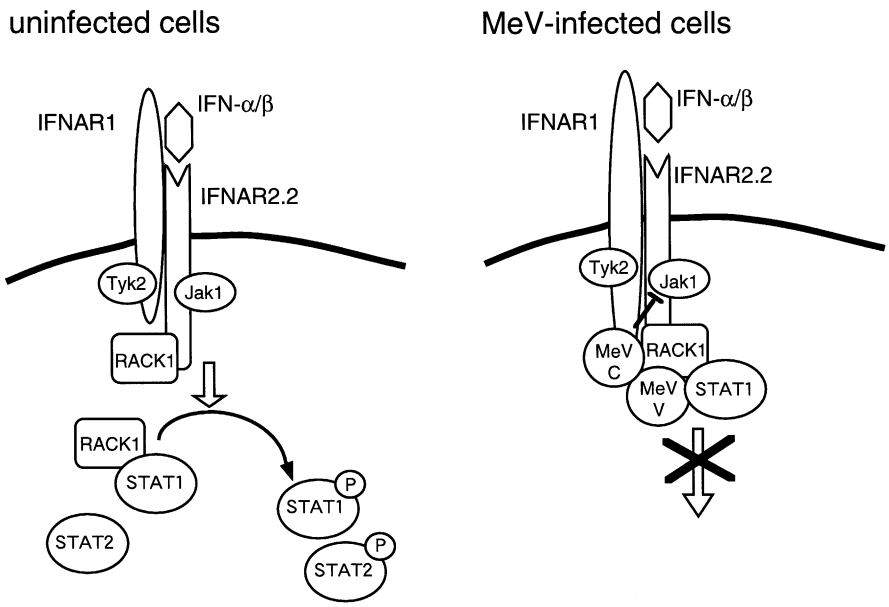

Fig. 8. Proposed scheme for suppression of IFN-α signaling pathway by MeV. In MeV-infected cells, IFN-α receptor complex is "frozen" by the association of viral accessory proteins, C and V, and consequently IFN-α signal is not transduced.

IFNAR1, STAT1, nor RACK1 were detected in the immunoprecipitates. This may be due to the fact that the anti-C and anti-V antibody-binding sites are also involved in interaction with the IFNAR-STAT1-RACK1 complex.

Discussion

In the present study, we show the suppression of an innate immune response, IFN-induced antiviral activity, following MeV infection. We used the MeV wild strains, AK-1, AK-2, AK-3, AK-4, AK-5, and AK-6 (Saito et al., 1992, 1994) to infect various types of cells, including epitheloid carcinoma cells (SiHa and CaSki), lymphoid cells of B cell origin (P3HR-1), chronic myelogenous leukemia cells (K562), and neuroblastoma cells (SK-N-AS). MeV infection of these cells commonly resulted in inhibition of IFN-α-induced antiviral activity (Figs. 1, 2, and Table 1). We examined the inhibition mechanism step by step. IFN-α-induced transcriptional activation of ISRE, an IFN-α-responsive element, was not observed by a reporter gene assay and activation of STAT1, a component of the transcriptional factor ISGF3, was not induced by IFN-α. Numerous viruses of genus rubulavirus, a genus of *Paramyxoviridae*, share the ability to dramatically decrease basal levels of STAT proteins due to the effects of their V protein (Didcock et al., 1999; Kubota et al., 2001; Nishio et al., 2001; Parisien et al., 2001; Yokosawa et al., 1998). MeV (of the genus *morbillivirus*) changed basal protein levels of STAT1, STAT2, and IRF-9 in some cases (Fig. 5); however, there is no correlation between basal levels of these proteins and the ability of the virus to inhibit the innate antiviral activity of cells. Inhibition of antiviral activity by MeV was shown to be due to suppression Jak1 phosphorylation, which was induced by IFN-α, but not by IFN-γ. So we believed that the effect on IFN-α signaling was not mediated by a direct interaction with Jak1. Therefore, we examined proteins associated with the type I IFN receptor by immunoprecipitation analysis (Fig. 7). We demonstrated that the scaffold protein RACK1 is an important component of the type I IFN receptor complex. RACK1 is a 36,000 Da protein composed of seven WD motif, which consists of about 40 amino acid residues terminated with tryptophan and aspartic acid residues, repeats that resemble the structure of the β subunit of G protein (Mochly-Rosen et al., 1995; Neer and Smith, 1996; Ron et al., 1994). RACK1 is considered a scaffold protein. It has been reported to interact with many cellular proteins, including protein kinase Cβ (Mochly-Rosen et al., 1995), Src (Chang et al., 2001), common β subunit of GMCSF/IL-3/IL-5 receptors (Geijsen et al., 1999), insulin-like growth factor-I receptor (Hermanto et al., 2002), β-integrins (Liliental and Chang, 1998), and protein tyrosine phosphatase μ (Mourton et al., 2001). RACK1 is believed to modulate cellular signaling pathways, cell migration, and cell-to-cell-contact. Interestingly, RACK1 also interacts with IFNAR2.2, type I IFN receptor chain 2 (Croze et al., 2000), and unphosphorylated STAT1 (Usacheva et al., 2001); it is required for recruitment and activation of IFN-α-induced STAT1 activation and transduction of IFN-α signaling. In addition, it can associate with various viral proteins, including adenovirus E1A protein (Sang et al., 2001), Epstein–Barr virus BZLF1 protein (Baumann et al., 2000), human immunodeficiency virus-1 Nef protein (Gallina et al., 2001), and influenza A virus M1 protein (Reinhardt and Wolff, 2000). More recently, by using yeast two-hybrid system, we found that V protein of mumps virus interacted with RACK1 (Kubota et al., 2002). Our immunoprecipitation study (Fig. 7) is explained by showing that RACK1 interacts with either type I IFN receptor complex and/or STAT1 and that it releases activated STAT1 from the receptor following IFN-α treatment in uninfected cells. This is consistent with previous observations (Croze et al., 2000). On the other hand, IFNAR1/IFNAR2.2, RACK1, STAT1, and MeV proteins V and C formed tight complexes in MeV-infected cells. The complexes were only slightly altered by IFN-α treatment for 20 min (Fig. 7). We propose that type I IFN complex, which contains IFNAR1, IFNAR2.2, RACK1, and STAT1, is "frozen" by association with the MeV accessory proteins C and/or V (Fig. 8). The C and/or V proteins should contribute to the suppression of IFN-α signal pathway; however, it is still unclear whether C and V have a direct or indirect role in the function of type I IFN receptor. For example, it is not known whether the C and/or V proteins play a role in the phosphorylation of Jak1 or the phosphorylation of IFNAR. MeV C and V proteins were reported to interact some cellular proteins of host cells and considered to alter somewhat host cellular responses (Lin et al., 1998; Liston et al., 1995). However, these interacting proteins were not identified except one of the V interacting proteins is damage-specific DNA-binding protein (Lin et al., 1998), the relationship between these proteins and our results have not been defined.

Contribution of C and V proteins on MeV pathogenicity has been studied using defective recombinant viruses generated by a reverse genetics system. Both the C-defective virus and the V-defective virus multiply in Vero cells without obvious impairment (Radecke and Billeter, 1996; Schneider et al., 1997); however, the C-defective virus replicates with severely reduced efficacy in peripheral blood mononuclear cells (Escoffier et al., 1999). Compared to the parent MeV and other mutants, the V-defective virus shows milder clinical symptoms and a lower mortality rate in a human CD46-transgenic mouse model (Patterson et al., 2000) and prolonged thymocyte survival in a mouse-engrafted human thymus/liver implants model (Valsamakis et al., 1998). Mrkic et al. (2000) reported that the C- or V-defective MeV mutant showed less pathogenicity than the parent MeV in genetically engineered mice defective for the IFNAR and expressing human CD46. The results suggest that there is a mechanism other than the counteracting IFN system in pathogenicity of C and V proteins. However,

it is also possible that mutant strains of MeV with high sensitivity to IFN show reduced pathogenicity compared to parent strains of MeV, which has the ability to suppress the IFN signaling pathway. Furthermore, the results described above were obtained from experiments using C- or V-deficient mutants generated from c-DNA based on a classical strain, Edmonston. It is, therefore, necessary to investigate the pathogenicity of mutant strain derived from other MeV strains, such as AK-1.

Similar to other viruses, MeV induces IFN production via activation of transcription factors, such as STATs and NF-κB, which also contribute to the expression of various cytokines and inflammatory mediators. IFN-α/β plays an important role in the establishment of an antiviral state. Recently, a virus-induced IFN production system was clearly described (Mamane et al., 1999; Sato et al., 2000). Briefly, when virus or dsRNA attacks cells, small amounts of IFN-β and a specific subtype of IFN-α is induced by transcriptional activation contributing the transcription factors, including IRF-3, ATF2/c-*jun*, and NF-κB. The primarily produced IFN acts in a positive feedback mechanism to induce IRF-7 via the JAK/STAT pathway, namely contribution of ISGF3. Consequently, large amounts of IFN-α/β containing various subtypes of IFN-α are produced by the contribution of the IRF-3/IRF-7 complex in cooperation with ATF2/c-*jun* and NF-κB. MeV strain AK-1 should inhibit the latter system, namely, large amounts of IFN production via the JAK/STAT pathway. Naniche et al. (2000) reported that wild strains of MeV downregulate IFN production in peripheral blood lymphocytes, unlike Vero cell-adapted virus. On the other hand, Helin et al. (2001) reported that a Vero cell-grown MeV strain can activate NF-κB and STATs and induce higher production of IFN-α/β compared to virulent MeV strains. It is very interesting what factor(s) contributes to the ability, namely suppression of IFN-α/β production, by wild strains of MeV. However, Naniche et al. (2000) stated that the suppression of IFN-α/β production does not seem to be due to a block in IFN signaling pathway because the levels of STAT1 protein, an IFN-inducible protein, are increased in response to a viral infection to comparable levels observed after IFN treatment. The suppression of IFN-α signal transduction pathway by wild strains of MeV, that is our finding in the present study, should allow for the suppression of IFN-α/β production. It may enable the wild MeV strain AK-1 easy transition to persistent infection. In fact, we observed that strain-AK1-infected cells produce undetectable or very low amounts of IFN-α/β (data not shown).

Materials and methods

Cells and viruses

Human cell lines P3HR-1 (lymphoid cells of B cell origin), K562 (chronic myelogenous leukemia cells), SK-N-AS (neuroblastoma cells), SiHa, CaSki (epitheloid carcinoma cells), U937 (monocytic lymphoma), Jurkat (T cell), and marmoset cell line B95a were routinely cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The wild-type strains of MeV (AK-1, AK-2, AK-3, AK-4, AK-5, and AK-6) were isolated from throat swabs of patients with clinical measles using B95a cells (Saito et al., 1992, 1994, 1995). The viruses used in this study was propagated in B95a cells.

Reagents

IFN-α and IFN-γ were purchased from Serotec (Oxford, U.K.) and Genzyme (Cambridge, MA), respectively. Both were used at a final concentration of 1000 IU/ml. Rabbit polyclonal antibodies against STAT1 p84/p91 (E-23), STAT2 (C-20), Jak2 (M-126), IRF-1 (C-20), RACK1 (H-187), IFNAR1/IFN-αR (R-100), and IFN-γRα (C20), and mouse monoclonal antibody against IFNR1 (H-11) and IFN-γRα (GIR-94), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against Jak1 and Tyk2 were acquired from Upstate Biotechnology (Lake Placid, NY). Rabbit antibodies against $Tyr^{1022/1023}$-phosphorylated Jak1 and $Tyr^{1007/1008}$-phosphorylated Jak2 were acquired from BioSource International (Camarillo, CA). Rabbit antibodies against Tyr^{701}-phophorylated STAT1 and $Tyr^{1054/1055}$-phosphorylated Tyk2 were acquired from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibodies against IRF-9/p48 (clone 6), p68PKR (clone 13), and RACK1 (clone 20) were acquired-from Transduction Laboratories (San Diego, CA). Mouse monoclonal antibodies against actin (C4), MeV M protein, and MeV N protein were acquired from Chemicon (Tumecula, CA). Rabbit anti-MeV C protein and anti-MeV V protein antibodies were kindly donated by Drs. Atsushi Kato and Kaoru Takeuchi (National Institute of Infectious Diseases, Tokyo, Japan).

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, and the lysates were centrifuged at 10,000 *g* for 10 min at 4°C. The supernatants were recovered, and the protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting were carried out as described previously (Yokosawa et al., 1998; Yokota et al., 2001). Alkaline phosphatase labeled goat anti-mouse or rabbit Ig antibody (BioSource International) and bromochloroindolyl phosphate/nitroblue tetrazolium were used as a secondary antibody and an enzyme substrate, respectively.

RT-PCR and PCR-RFLP

Total RNA was isolated from cells using Isogene (Nippon Gene, Toyama, Japan) and then treated with DNase (Message clean kit, Gen Hunter, Brookline, MA).

The infection of MeV strain AK-1 was confirmed by detection of viral HA expression using RT-PCR (Saito et al., 1995). A 344-bp fragment (nucleotide 1068 to 1416) was amplified using rTth polymerase (Toyobo, Osaka, Japan). For identification of the wild-strain AK-1, the RT-PCR fragment was digested with *Sau*3A1 and the fragmentation pattern was analyzed by 2.5% agarose electrophoresis.

Transcriptional levels of the 40-kDa isozyme of 2-5AS and IRF-1 induced by treatment with IFN-α and IFN-γ, respectively, were determined by RT-PCR analysis using an appropriate primer set in the same manner as described previously (Fujii et al., 1996, 1999). A 266-bp fragment (nucleotide 1039 to 1304) was amplified for detecting mRNA of 40 kDa 2-5AS. A 299-bp fragment (nucleotide 874 to 1172) was amplified for detecting mRNA of IRF-1. A 450-kb fragment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA amplified by primer set, 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse) and 5′-ACCACAGTCCATGCCATCAC-3′ (forward), was used as a control.

Induction of an antiviral state following IFN treatment

Antiviral state induced by IFN was examined by two methods: cytopathic effect (CPE) caused by vesicular stomatitis virus (VSV) infection and reduction of VSV replication. CPE assay was carried out as follows: Uninfected and AK-1-infected cells were cultured in 96-well microtiter plates in the presence of various concentrations of IFN-α or IFN-γ for 24 h and then challenged with 0.1 ml VSV (2×10^4 PFU/ml). The cells were stained with gentian violet when a cytopathic effect was obvious. After dissolving dye bound to viable cells with methylcellosolve, the absorbance at 550 nm was measured. VSV reduction assay was carried out as follows: Cells cultured at approximately 80% confluency were treated with 1000 IU/ml of IFN-α or IFN-γ for 24 h and then challenged with VSV at an m.o.i. of 1.0 at room temperature. Virus titer in the culture fluids at 24 and 48 h after infection was determined using Vero cells as an indicator.

Luciferase reporter gene assay

Expression of IFN-inducible genes was determined by a dual luciferase reporter assay by transiently transfecting reporter plasmids containing either the ISRE or the GAS enhancer element. The firefly luciferase reporter plasmids, pISRE-Luc and pGAS-Luc (PathDetect *in Vivo* Signal Transduction Pathway *cis*-Reporting Systems), were purchased from Stratagene (La Jolla, CA). Cells were plated onto a 24-well plate at approximately 30% confluency the day before transfection. Reporter plasmids (1 μg) and reference plasmids (0.1 μg of pRL-TK vector, harboring HSV thymidine kinase promoter just upstream of *Renilla* luciferase gene, Promega, Madison, WI) were mixed and transfected into cells using Superfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After cultivation for 24 h, IFN-α (for pISRE-Luc transfectants) or IFN-γ (for pGAS-Luc transfectants) was added at a final concentration of 1000 IU/ml, and cells were incubated for 24 h. The cells were lysed, and the firefly luciferase and *Renilla* luciferase activities in the lysate were measured using Dual-Luciferase Reporter Assay System (Promega). The experiments were performed in quadruplicate. Reporter activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity. The results are expressed as fold induction, which is the value relative to the value of control experiments without IFN treatment.

Immunoprecipitation analysis

Cellular proteins were solubilized by 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 antibody were mixed and incubated at 4°C for 1 h. Protein A–Sepharose 4B (Amersham Biosciences AB, Uppsala, Sweden) was added to the mixture and incubated at 4°C overnight on a rotator. The resin was washed with the RIPA buffer four times and then the bound materials were solubilized by boiling with SDS–PAGE sample buffer and applied to SDS–PAGE, followed by Western blotting analysis as described above.

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