Biochemical and Biophysical Research Communications **283,** 255–259 (2001)
doi:10.1006/bbrc.2001.4764, available online at http://www.idealibrary.com on IDEAL®

C Terminal CYS-RICH Region of Mumps Virus Structural V Protein Correlates with Block of Interferon α and γ Signal Transduction Pathway through Decrease of STAT 1-α

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Received March 29, 2001

It has been reported that interferon (IFN)-alpha/gamma signal transduction pathway is blocked in several cell lines persistently infected with mumps virus (MV) through decrease of STAT-1α. Expression of the MV structural V protein (MV-V) or C terminal CYS-RICH region of the V protein (MV-Vsp) inhibited the establishment of the antivirus state induced by IFN, but not by expression of the MV-P protein. Suppression of IFN-induced STAT-1α, STAT-2, and IRF-9 (p48) induction was also recognized in the cells transfected with expression vector of the MV-V (pTM-V) or MV-Vsp (pTM-Vsp) protein, eventhough it was in the absence of the other virus protein. It is supposed that the cystein-rich domain of V protein (Vsp) is involved in the suppression of the IFN signal transduction pathway.

Key Words: **interferon; mumps virus; V protein; STAT-1.**

In recent years, studies on a correlation between viral pathogenesis and antiviral activity of IFN revealed that most DNA and RNA viruses are able to block the IFN signal transduction pathway by various distinct mechanisms or strategies, and to promote their replication in spite of the presence of IFN (1–8). In the family paramyxoviridae, there was a specific reduction in the level of serine 727 (S-727)-phosphorylated forms of STAT-1α in sendai virus (SeV) and human parainfluenza virus type 3 (hPIV3) infected cells (6, 7). On the other hand, a drastic decrease in constitutive level of STAT-1α and STAT-2 was found in simian virus 5 (SV5) and hPIV2 infected cells, respectively (7). Therefore, depletion of STAT proteins and inhibition of STAT-phosphorylation in these viruses infected cells contributed to the subsequent failure in ISGF-3 and/or GAF transcription complexes which are essential for IFN signaling pathway.

Virus proteins involved in the block of IFN signaling were examined in SeV and SV5. The results indicated that the C protein of SeV and the V protein of SV5 counteract the IFN-mediated induction of an antiviral state (2, 4, 6). Didcock *et al.* reported that expression of the SV5 structural protein, in the absence of other virus protein, inhibited IFN signaling and induced the degradation of STAT-1 (2).

In addition to these viruses, it has been noted that mumps virus inhibits signal transduction pathways of both IFN-α and IFN-γ through a decrease in the constitutive level of STAT-1α (3, 8, 9). A decrease in STAT-1α results in the failure of ISGF-3 complex formation, and brings about dysfunction of the IFN signaling pathway. Mumps virus and SV5 belong to the same genus of Rublavirus of the family paramyxoviridae based on gene map similarity and antigenic cross reactivity. Therefore, it is expected that the dysfunction of IFN signaling pathway observed in SV5 structure V protein is also demonstrated in cells transfected with expression vector of MV structure V protein.

MATERIALS AND METHODS

Construction of expression vector and establishment of stable transformant. To construct pTM-V, pTM-P, and pTM-Vsp expression vectors, the cDNAs were prepared from viral mRNA corresponding to V and P proteins of MV. Total RNAs were isolated from FL cells infected with Torii strain of mumps virus using Isogene (Nippon Gene, Toyama, Japan) in accordance with the manufacturer's protocol, and then were treated with DNase to digest the contaminated DNA. The cDNA was prepared by RT-PCR method using rTth polymerase (Perkin Elmer, Branchburg, NJ) and appropriate primer set for the target mRNA of P (MPPV-1/MPPR-1), V (MPPV-1/MPVR), or 72 amino acid residues of C terminal region containing CYS-RICH domain (amino acid residues 188–220; WCNPICSPITAAARFHSCK-

Abbreviations used: GAF, IFN-γ activated factor; IFN, interferon; IRF, interferon regulatory factor; ISGF3, IFN-stimulated gene factor-3; STAT, signal transducers and activators of transcription.

0006-291X/01 $35.00

TABLE 1

Expression Vectors and Cloned Transfectant Cell Lines

Primer	5′-3′	Position[a]	cDNA	Amino acids residues	Expression vector	Cell lines	Isolated clones	Reduction of IFN-signaling
MPPU1 (sense)	TTTCCGGGCAAGCCATGGAC	1965–1984	V	Full length, 224 a.a.	pTM-V	FL/V	6	6
MPVR (anti-sense)	CTAAGGAGGTCCATAATCTC	2653–2634						
VspU2 (sense)	TTATGAGGGGGGCCGGGAGC	2433–2453	Vsp	C terminal region, 72 a.a.	pTM-Vsp	FL/Vsp	16	16
MPVR (anti-sense)	CTAAGGAGGTCCATAATCTC	2653–2634						
MPPU1 (sense)	TTTCCGGGCAAGCCATGGAC	1965–1984	P	Full length, 391 a.a.	pTM-P	FL/P	10	0
MPPR (anti-sense)	GGTGAGTTCATATGGCGCTC	3159–3140						

[a] Nucleotide positions were referred from mumps virus cDNA sequence of the genomic RNA (11).

CGNCPAKCDQCEDR) of V (Vsp) (VspV-2/MPVR) (Table 1). The resultant of each cDNA was inserted into pTargeT vectors (Promega Corp. Madison, WI). Monolayer of FL cells grown to 50 to 70% confluence on 60 mm diameter culture dish was transfected with 0.5 μg of vector DNA and 2 μl of Lipofectamine (Life Technologies Inc.) according to the manufacturer's instructions. The transfected cells were cultured with G418 to obtain stable transformants. Six, sixteen, and ten clones were isolated from FL cells transfected with pTM-V, pTM-Vsp, and pTM-P expression vectors, respectively (Table 1). Production of V and Vsp (C terminal CYS-RICH region) were confirmed by Western blot analysis using an anti MV-V monoclonal antibody which recognizes C terminal region of V protein. The antibody was provided by Dr. Kato and Dr. Takeuchi (National Institute of Infectious Diseases, Japan). Expression of the P gene was examined by RT-PCR method in the same manner as described above. All isolated clones were significantly positive for character of expression for V, Vsp, or P gene (data not shown).

Induction of antiviral state by IFN. Induction of the antiviral state by IFN was examined in the same manner as described previously (10). Briefly, FL, FL/P, FL/V, and FL/Vsp cells (4×10^4 cells/0.1 ml) were inoculated into each well of microplate (96 wells). After cultivation for 24 h, the culture fluid of the microplate was discarded, and then 0.1 ml of the indicated titer of IFN-α (Serotec, Oxford, UK) or IFN-γ (Genzyme/Techne, Cambridge, MA) was inoculated into the well and further incubated for 24 h at 37°C. Thereafter, the culture medium containing IFN was discarded and challenged with 0.1 ml of vesicular stomatitis virus (VSV; 2×10^4 PFU/ml). When cytopathic effect induced by VSV was obvious, the cells were washed and stained with gentian violet solution for 5 min. The dye binding to the cells was dissolved with methyl-cellosolve for 3 h, and its optical density was measured at a 550 nm. Percentage of live cells was calculated by the optical density. Furthermore, IFN-induced antiviral state was determined by reduction of VSV replication in cells transiently transfected with expression vector of pTM-V, pTM-Vsp, or pTM-P. FL cells were plated at approximately 30–40% confluency the day before transfection. The expression plasmids (10 μg) were transfected into the FL cells using Superfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instruction manual. After cultivation for 36 h, the transfectants were treated with 10^3 IU/ml of IFN-α for 18 h. The culture medium was discarded and challenged with VSV at a multiplicity of infection (MOI) of 0.5 at room temperature. Virus titer in the culture fluid at 18 h after infection was determined by using Vero cells. Expression of the target transcripts (V, Vsp, or P mRNA) was almost same level among the transiently expression cells of FL/pTM-P, FL/pTM-V, and FL/pTM-Vsp (data not shown). Stable clones of FL/P, FL/V, and FL/Vsp were cultured with 10^3 IU/ml of IFN-α for 24 h, and then the cells were infected with VSV in the same manner described above.

Western blot analysis of the three components for ISGF-3 complex. FL, FLMT, and stable transfectant (FL/V, FL/Vsp, and FL/P) cells were treated with 10^3 IU/ml of IFN-α or IFN-γ for 24 h. On the other hand, experiments of transiently expression system were performed to confirm the effect of Vsp on reduction of IFN-inducible gene products. FL cells were transfected with pTM-Vsp expression plasmid. After incubation for 36 h, the transfectant cells were treated with 10 IU/ml of IFN-α or IFN-γ for 24 h. The cell pellets from stable or transiently expression system were lysed by lysis buffer and analyzed by Western blot technique using appropriate antibodies including anti-PKR (Transduction Lab. Lexington, KY) and anti-IRF-1 (Santa Cruz Biotech, CA) antibodies as described previously (3, 8).

Enzyme assay. 2′,5′-oligoadenylate synthetase (2-5AS) activity in cells was measured by the methods of liquid phase reaction. Cells were treated with 10^3 IU/ml of IFN-α for 24 h, and then cell packs were lysed by a lysis buffer at room temperature for 10 min. The enzyme activity in supernatant was assayed using polyinosinate-cytidylate and [^{3}H]ATP in the same manner as described previously (10).

RESULTS AND DISCUSSION

Antiviral State Is Not Induced in Cells Expressing V Protein

Establishment of antiviral state induced by IFN-α or IFN-γ was examined in these cloned cell lines in the same manner as described previously (10). All clones of FL/V and FL/Vsp series were completely resistant to IFN-α or IFN-γ treatment, and antiviral activity of IFN was not recognized at all (Fig. 1). Reduction of IFN sensitivity in these clones was demonstrated in the presence of 10^3 IU/ml of IFN-α or IFN-γ (data not shown). These results were supported by suppression of IFN-induced 2′,5′-oligoadenylate synthetase (2-5AS) induction (Table 2). However, IFN-mediated antiviral activity in all clones of FL/P series was comparable to that of FL cells in Fig. 1 (data not shown) and virus production of VSV was significantly reduced by treatment with IFN (Table 2). Therefore, it is proposed that MV structural V protein or CYS-RICH region of V protein correlates with the dysfunction of IFN signal transduction pathway. Furthermore, in transiently expressing system, virus yield of VSV was not inhibited by IFNs in cells expressing V or Vsp protein (Table 2). Same results of the viral replication were also obtained in each stable clone (Table 2). No re-

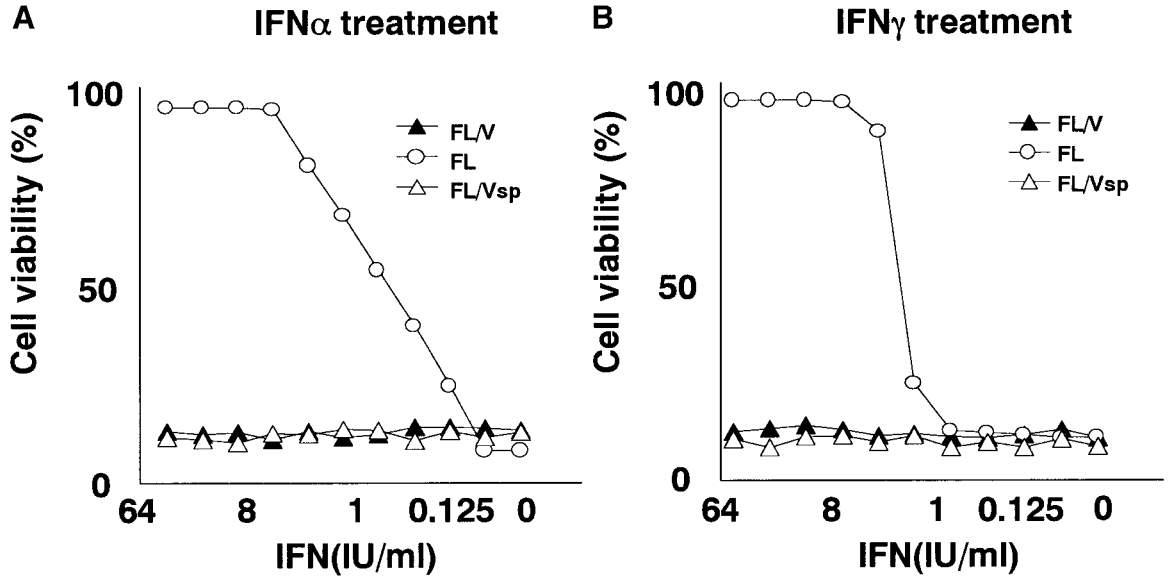

FIG. 1. Sensitivity of cloned cells to antiviral activity of IFN. After cultivation of cells for 24 h, the cells were treated with the indicated titer of IFN-α (A) or IFN-γ (B) and further incubated for 24 h at 37°C. Thereafter, the culture medium was discarded and challenged with vesicular stomatitis virus (VSV). When the cytopathic effect induced by VSV was obvious, the cells were stained with gentian violet solution for 5 min. The dye binding to the cells was dissolved and its optical density was measured.

duction of yield after IFN treatment indicates that cytopathic assay (Fig. 1) is not due to the result of accelerated cell killing caused by VSV infection in V or Vsp expressing cells. Poor inductions of antiviral state and 2-5AS activity were strongly correlated with the expression of mumps virus V or Vsp protein (Table 2). Therefore, it is concluded that mumps virus V protein inhibits the establishment of antiviral state induced by IFNs.

TABLE 2

Mumps Virus V or Vsp Protein Mediated Inhibition of Antiviral State Induced by IFN

Expression plasmid or stable cell line	IFN-alpha (10^3 IU/ml)	VSV (pfu/ml)	Induction of 2-5AS[a]	Antiviral state
Transient system				
None	−	7.6×10^7	4.4	−
	+	2.4×10^2	186.6	+
pTM-P	−	6.4×10^6	5.2	−
	+	1.2×10^3	107.0	+
pTM-V	−	1.5×10^7	5.0	−
	+	7.0×10^6	12.3	−
pTM-Vsp	−	9.0×10^6	7.7	−
	+	1.6×10^5	38.5	−
Stable cloned cell				
FL/P	−	7.2×10^6	6.6	−
	+	2.0×10^2	112.4	+
FL/V	−	2.8×10^7	6.0	−
	+	1.0×10^7	8.4	−
FL/Vsp	−	5.4×10^7	6.0	−
	+	6.0×10^7	6.4	−

[a] 2-5AS activity (nmol/mg per h).

Poor Induction of STAT-1α, STAT-2, and IRF-9 in Cells Expressing V Protein

It is well known that STAT-1α is the essential component for IFN-α and IFN-γ signaling pathway to form transcription complex ISGF-3 and GAF. Didcock *et al.*, Young *et al.*, and we reported evidence showing a decrease of basal expression of STAT-1α and dysfunction of response to IFN-α and IFN-γ in SV5 or MV infected cells (2, 4, 7, 8, 9). After treatment with or without IFN, basal and IFN-induced levels of STAT-1α, STAT-2, and IRF-9 (p48) were investigated in FL/V and FL/Vsp clones by Western blot analysis using appropriate antibodies as previously described (3, 8). The basal/constitutive level of STAT-1α in MV-persistently infected cells (FLMP) was lower than that in uninfected FL cells, but not STAT-2 and IRF-9 (Fig. 2, lanes 1 and 4). Furthermore, little induction of the tree components for ISGF-3 transcription complex was found in the FLMP cells treated with 10^3 IU/ml of IFN-α (Fig. 2, lanes 2 and 5) or IFN-γ (Fig. 2, lanes 3 and 6). These results were consistent with those reported previously in our laboratory (3, 8). Response of FL/P clones to IFN-α and IFN-γ was identical to that of uninfected FL cells (Fig. 2, lanes 7, 8, and 9). On the contrary, in FL/V clones, STAT-1α, STAT-2, and IRF-9 were hardly induced by IFN treatment (Fig. 2, lanes 11, 12, 24, and 15). Drastic decrease in basal levels of STAT-1α was also found in these cells (Fig. 2, lanes 10 and 13). The absence of STAT-2 and IRF-9 induction after IFN treatment may be a result of a decrease in basal level of STAT-1α. The N-terminal region is common between the V and P proteins in MV, but the C terminal CYS-RICH region of V protein is different from that of P

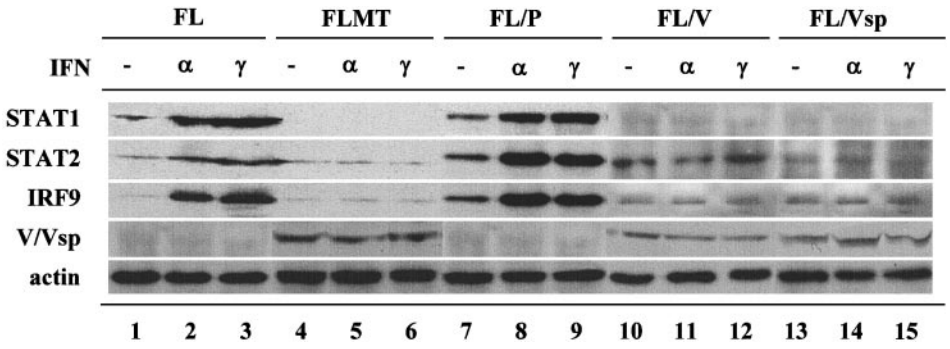

FIG. 2. Western blot analysis for STAT-1α, STAT-2, and IRF-9 (p48). FL, FLMP, FL/P, FL/V, and FL/Vsp cells were treated with 10^3 IU/ml of IFN-α (lanes 2, 5, 8, 11, and 14) or 10^3 IU/ml of IFN-γ (lanes 3, 6, 9, 12, and 15) for either 0 (lanes 1, 4, 7, 10, and 13) or 24 h (lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, and 15), and then STAT-1α, STAT-2, and IRF-9 (p48) in cell lysates were examined by Western blot analysis.

protein. No effect on a decrease in STAT-1α was demonstrated in FL/P clones (Fig. 2, lanes 7, 8, and 9). Therefore, it is suggested that the C terminal region (72 amino acid residues) of V protein participates in a decrease of STAT-1α. As expected, Fig. 2 shows that a decrease in STAT-1α in FL/Vsp clones was comparable to those of FLMT and FL/V. Furthermore, transiently expressing system also supported that poor induction of IFN-induced antiviral state (Table 2) is due to the failure of PKR induction through a decrease in basal levels of STAT-1α in Vsp-expressing cells (Fig. 3). Reduction of basal levels of STAT-1α was demonstrated in FL cells transfected with pTM-Vsp as compared with control cells (Fig. 3, lanes 1 and 4). As expected, IFN-induced STAT-1α, PKR, or IRF-1 induction was strongly suppressed in FL cells expressing Vsp protein (Fig. 3, lanes 5 and 6). The results for poor induction (mRNA and protein) of PKR and IRF-1 were also shown in stable clones of FL/V and FL/Vsp (data not shown). A small amount of STAT-1α was inducible by treatment with IFN-α or IFN-γ in transient expression of Vsp. The induction may be due to lower levels of Vsp expression in a transient expression system than those in a stable expression system and persistently infected cells.

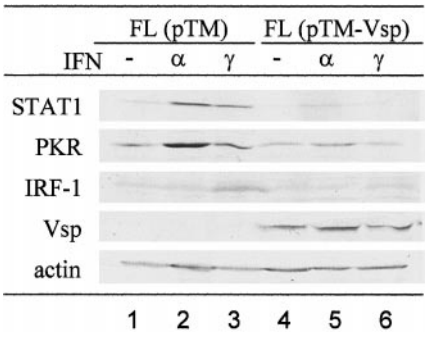

FIG. 3. Effect of Vsp expression on induction of IFN-inducible genes. After cultivation of FL cells transfected with control pTM vector (lanes 1–3) or expression plasmid pTM-Vsp (lanes 4–6) for 36 h, the cells were treated with IFN-α (lanes 2 and 5) or IFN-γ (lanes 3 and 6) for 24 h. The cell lysates were analyzed by Western blot technique.

Disappearance of STAT-1α in FLMP, FL/V, and FL/Vsp cells may be due to protein degradation because there was an almost similar level of STAT-1α transcripts (mRNA) between control FL cells and the others as described previously (3, 8). The decrease in basal and induced levels in STAT-1α is thought to be caused by its degradation through proteasome pathway. Recently, Didcock *et al.*, reported that the V protein of SV5 inhibits IFN signaling, and induces the degradation of STAT-1α, but not STAT-2 (2). The V protein of SV5 is almost identical to that of MV in the C terminal CYS-RICH region. The identity may indicate that STAT-1α disappeared through the function of MV-V protein. To address this question, the effect of the proteasome inhibitors, MG132 (Peptide Institute Inc., Mino, Osaka, Japan) on the levels of STAT-1α was examined in FLMT, FL/V, and FL/Vsp cells. MG132 partly inhibited the degradation of STAT-1α in these cells and basal levels of STAT-1α was detectable in the presence of MG132, though the recovered levels was lower than that of FL cells (unpublished data). On the contrary, E64d (lysosome inhibitor; Peptide Institute Inc., Mino, Osaka, Japan) had no effect on restoration of STAT-1α (data not shown).

C terminal CYS-RICH region of V protein could contribute to the failure of transcription complex formation (ISGF-3 and GAF) through decrease of STAT-1α. However, the restoration levels of STAT-1α was inadequate in spite of treatment of MG132. Therefore, it could not be excluded that there are some mechanisms to promote the degradation of STAT-1α other than proteasome system.

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