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Regulation of interferon signaling by the C and V proteins from attenuated and wild-type strains of measles virus ☆

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Abstract

The C and V proteins of the measles virus (MV) have been shown to block the signaling of type I and II interferon (IFN-α/β and IFN-γ). The relative contribution of the C and V proteins to the inhibition of IFN signaling and the extent to which this activity differs in attenuated or wild-type strains of MV remains undefined. This study presents a comparison of the IFN-antagonist activities of C and V proteins from four attenuated and two wild-type strains of MV. The V proteins were more potent inhibitors of IFN-inducible reporter gene expression than the C proteins, and this effect was unrelated to whether the protein originated from an attenuated or wild-type strain. The results also demonstrated the importance of the tyrosine at position 110 in the inhibition of IFN-α/β and IFN-γ signaling by the V protein, and identified a non-recombinant MV expressing a V protein that was impaired due to a mutation at this residue.
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Introduction

Measles virus (MV) infection is a major cause of childhood morbidity and mortality, accounting for approximately 345,000 deaths annually (Wolfson et al., 2007). Due to the widespread use of live attenuated vaccines for measles, this disease has been eliminated in the Western Hemisphere since 2000. Additionally, the European, Eastern Mediterranean and Western Pacific regions of the World Health Organization have set elimination goals for the near future (CDC, 2005). Despite these successes, measles remains endemic in many developing countries, especially in Sub-Saharan Africa and Asia, due to inadequate vaccine coverage.

MV is a member of the genus *Morbillivirus* of the family *Paramyxoviridae*. The genome of MV is 15,894 nucleotides in length and consists of six tandemly linked genes that encode, from 3′ to 5′, the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins (Griffin, 2001). In addition to coding for the P protein, an essential cofactor of the viral polymerase, the P gene of MV encodes two accessory proteins, C and V. The C protein is a small (186 amino acids), basic protein that is synthesized from a translation start signal on the P mRNA that is 22 nucleotides downstream from the P start site (Bellini et al., 1985). The C protein functions to downregulate viral polymerase activity (Bankamp et al., 2005; Reutter et al., 2001), enhance the production of infectious viral particles (Devaux and Cattaneo, 2004), and maintain long-term MV infection by preventing cell death (Takeuchi et al., 2005). In contrast, the V protein is translated from the same start codon as the P protein, but a co-transcriptionally inserted guanosine at the RNA editing site causes a frame shift. The resulting V protein shares its first 231 amino acids with the P protein, but has a unique 68 amino acid carboxy-terminal domain that includes seven conserved cysteine residues (Cattaneo et al., 1989; Liston and Briedis, 1994; Wardrop and Briedis, 1991). The V protein is known to downregulate viral polymerase activity (Tober et al., 1998) and inhibit apoptosis through interaction with the tumor suppressor p73 protein (Cruz

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et al., 2006). Both the C and V proteins of MV contribute to viral evasion of the host interferon (IFN) response (Andrejeva et al., 2004; Childs et al., 2007; Devaux et al., 2007; Nakatsu et al., 2006; Ohno et al., 2004; Palosaari et al., 2003; Shaffer et al., 2003; Takeuchi et al., 2003).

Type I IFN (IFN-α/β) and type II IFN (IFN-γ) are potent inflammatory cytokines that are induced in direct response to viral infection and have important roles in the inhibition of viral replication and the regulation of host immunity (Goodbourn et al., 2000; Stark et al., 1998). Both IFN-α/β and IFN-γ signal through similar, yet distinct Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal transduction pathways by binding to their cognate receptors on the surface of cells. This receptor-ligand interaction and signaling pathway results in the upregulation of IFN-stimulated gene (ISG) products that contribute to host immune regulation and the establishment of an antiviral state (Goodbourn et al., 2000; Stark et al., 1998).

The accessory proteins encoded by the P gene of many paramyxoviruses have the ability to interfere with the IFN signaling pathway. For example, the C proteins of Sendai virus (Garcin et al., 1999; Gotoh et al., 1999) human parainfluenza virus 3 (Malur et al., 2005), Nipah virus (Park et al., 2003), rinderpest virus (Nanda and Baron, 2006), and measles virus (Shaffer et al., 2003) block IFN signaling. Likewise, the V proteins of simian virus 5 (Didcock et al., 1999; Poole et al., 2002), simian virus 41 (Nishio et al., 2001), mumps virus (Kubota et al., 2001), human parainfluenza virus 2 (Nishio et al., 2001; Parisien et al., 2001), Nipah and Hendra viruses (Park et al., 2003; Rodriguez et al., 2002; Rodriguez et al., 2003), Newcastle disease virus (Huang et al., 2003), rinderpest virus (Nanda and Baron, 2006), and measles virus (Palosaari et al., 2003; Takeuchi et al., 2003) also block IFN signaling. Recently, a role in the inhibition of IFN signaling was demonstrated for the P proteins of Nipah virus (Shaw et al., 2004), rinderpest virus (Nanda and Baron, 2006), and measles virus (Devaux et al., 2007). Additionally, the accessory proteins of many paramyxoviruses inhibit the IFN induction pathway (Andrejeva et al., 2004; Childs et al., 2007; He et al., 2002; Kiyotani et al., 2007; Komatsu et al., 2007; Nakatsu et al., 2006; Poole et al., 2002; Strahle et al., 2007).

Although both the C and V proteins of MV have been shown to inhibit IFN signaling, there are inconsistencies in the results reported from different laboratories (Ohno et al., 2004; Palosaari et al., 2003; Shaffer et al., 2003; Takeuchi et al., 2003). Whereas Shaffer et al. (2003) demonstrated the inhibition of an IFN-α/β-responsive reporter gene by the C protein, other studies reported weak or no inhibition (Ohno et al., 2004; Takeuchi et al., 2003, 2005). There is agreement that the V protein inhibits IFN-α/β signaling (Devaux et al., 2007; Ohno et al., 2004; Palosaari et al., 2003; Takeuchi et al., 2003), but there are conflicting reports about the suppression of IFN-γ signaling by this protein (Palosaari et al., 2003; Takeuchi et al., 2003). Some of these discrepancies may be due to the use of different viral strains and cell types.

Since it is possible that the IFN-inhibitory functions of the C and V proteins of MV may depend on the strain from which they are derived, the relative abilities of the C and V proteins of four attenuated and two wild-type strains of MV to inhibit IFN-α/β and IFN-γ signaling in the absence of IFN induction were compared in this study. In all strains tested, the V protein of MV inhibited IFN signaling with greater efficiency than its corresponding C protein, an effect that was independent of whether the protein was derived from a wild-type or attenuated virus. Additionally, the V proteins from the EdTag and CAM-70 strains were impaired in their ability to inhibit both IFN-α/β and IFN-γ signaling due to a substitution at amino acid 110. Restoration of the tyrosine at this position recovered the IFN-antagonistic function of both proteins.

Results

Sensitivity of attenuated and wild-type MV titers to the presence of IFN

Six isolates of MV were chosen for analysis in this study, including three vaccine strains (CAM-70, Moraten, and Edmonston–Zagreb (EZ)), a recombinant virus derived from a laboratory strain of Edmonston (EdTag), and two wild-type strains (Indiana and D87-V/S). To investigate the sensitivity of these strains to IFN, we infected cells, treated them with IFN or left them untreated, and measured the resulting viral titers. Vero/hSLAM cells, which cannot produce IFN (Emeny and Morgan, 1979), were used to rule out the effects of IFN induction by MV infection. All MV strains grew to comparable titers in the absence of IFN (Table 1). EdTag was extremely sensitive to the presence of IFN-β. Moraten, EZ, and Indiana were intermediately sensitive to IFN-β, with Moraten exhibiting approximately 60-fold higher sensitivity than EZ or Indiana. CAM-70 and D87-V/S were minimally sensitive to IFN-β (Table 1). Interestingly, the two wild-type strains exhibited statistically different levels of sensitivity to IFN-β ($p<0.05$). Regarding sensitivity to the presence of IFN-γ, Moraten and EdTag were both intermediately sensitive, while the other strains were only minimally sensitive (Table 1). These data do not reveal any distinction between wild-type and attenuated strains of MV based on their ability to replicate in the presence of an IFN-induced antiviral state. Encephalomyocarditis (EMC) virus was used as a control in this study since its replication is extremely sensitive to the presence of IFN (Green et al., 1980). As

Table 1
Differences in sensitivity of attenuated and wild-type MV titers to the presence of IFN[a]

Virus	Titer (p.f.u./mL)	Fold decrease of viral titer in presence of IFN	
	No IFN	IFN-β	IFN-γ
EdTag	5.38×10^6	3093.94	53.14
Moraten	2.98×10^5	89.11	96.46
CAM-70	1.36×10^5	6.20	4.84
EZ	1.89×10^6	25.78	5.29
Indiana	1.98×10^5	23.42	2.65
D87-V/S	2.63×10^5	1.89	1.94
EMC	6.50×10^4	3300.00	800.00

[a] This table was derived from an average of three experiments done in triplicate.

expected, the viral titer of EMC decreased dramatically upon exposure to both IFN-β and IFN-γ (Table 1).

Transient expression of C and V proteins

The C or V open reading frame (ORF) of each MV strain was cloned into the mammalian expression vector, pCAGGS, which permits high levels of protein expression from a ubiquitously strong promoter (Niwa et al., 1991). The C ORF was silenced in the V constructs without altering the V amino acid sequence. The nucleotide sequences of the P genes for EdTag, Moraten, CAM-70, EZ, Indiana, and D87-V/S were confirmed by comparison to the published sequences (Table 2).

All six C plasmids expressed proteins of approximately 20 kDa that reacted with antiserum to the C protein of MV (Fig. 1). All six V plasmids expressed proteins that reacted with antiserum to the shared region of the P and V proteins of MV (Fig. 1). The proteins

Table 2
Amino acid variability among V proteins used in this study

Amino acid	MV V protein						
	Ed[a]	ET	Mor	CAM	EZ	Ind	V/S
29	I[b]					V	V
33	M					L	
46	E						D
51	R						K
54	K						E
63	P					S	
83	P						S
84	G					R	
93	L					V	
97	P						S
98	R			G			
99	N			D			
110[c]	Y	H		C			
111	Y					H	H
121	K			E			
130	M			V			
138	D			G			
146	N					D	D
165	I			V			
171	A					T	
195	R						K
199	R					K	
217	D					Q	N
219	G						S
220	R					K	
221	A						G
225	E	G	G	G	G		
237	S						G
266	R			S			
272	C	R					
285	V			A			
291	Y	H					

[a]Strain names are abbreviated as Edwt (Edmonston wild-type), ET (EdTag), Mor (Moraten), CAM (CAM-70), EZ (Edmonston–Zagreb), Ind (Indiana), V/S (D87-V/S). The GenBank accession numbers for the P gene sequences were: Edwt (AF266288), ET (Z66517), Mor (UO1984), CAM (DQ435723), EZ (AF266290), Ind (EU026136).
[b]Single-letter amino acid code.
[c]The amino acids found by Ohno et al. (2004) to control IFN-α/β-antagonism in the V protein are indicated by shading.

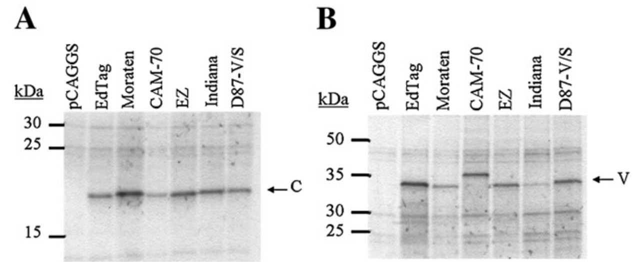

Fig. 1. Expression of the C proteins (panel A) and V proteins (panel B) of MV. Vero cells were transfected with the indicated C or V protein-expressing plasmid or an empty vector (pCAGGS). Proteins were ^{35}S-methionine labeled, immunoprecipitated with peptide-specific antiserum, and separated by SDS-polyacrylamide gel electrophoresis. The positions of the C and V proteins are indicated on the right (arrows), and the molecular weight marker is indicated on the left.

expressed by five of these plasmids were approximately 34 kDa, and the protein expressed by the CAM-70 V plasmid was approximately 36 kDa. The C and V proteins expressed from plasmid DNA co-migrated with the C and V proteins immunoprecipitated from Vero/hSLAM cells infected with the same strain (data not shown). The predicted molecular weights of the V proteins are similar despite amino acid differences. Therefore, the slower migration of the CAM-70 V protein could be caused by differences in post-translational modifications, such as phosphorylation. The P and V proteins of MV can be phosphorylated on tyrosine, serine, and threonine residues, but an effect on protein function has not yet been demonstrated (Das et al., 1995; Liu et al., 1997; Ofir et al., 1996).

Effect of C and V proteins from wild-type and attenuated strains of MV on IFN-α/β signaling

The relative abilities of the C and V proteins of MV to inhibit IFN-α/β signaling were determined by measuring the effect of different doses of each protein on the expression of an IFN-α/β-responsive reporter gene. Transfection of increasing amounts of plasmids expressing the C proteins resulted in marginal inhibition of firefly luciferase production. The level of inhibition of reporter gene expression with 0.5 μg of plasmid expressing the C proteins of CAM-70, Moraten, and Indiana was 16.4%, 15.5%, and 13.0%, respectively. The inhibition increased slightly to 28.6%, 24.9%, and 25.5%, respectively, with the addition of 1 μg of C protein-expressing plasmid, but it remained approximately the same with plasmid concentrations up to 2 μg (Fig. 2A). In contrast, the V protein of MV inhibited IFN-α/β-responsive reporter gene expression in a dose-dependent manner, reaching levels of 82–90% with the addition of 1 μg of V protein-expressing plasmid (Fig. 2B). These data suggest that the V protein of MV is a more potent inhibitor of IFN-α/β signaling than the C protein.

To determine whether the ability of the C and V proteins of MV to inhibit IFN-α/β signaling correlated with the derivation of that protein from an attenuated or wild-type virus, C and V proteins of different strains were compared in their ability to inhibit IFN-α/β-responsive reporter gene expression. The EZ and D87-V/S C proteins inhibited reporter gene expression by 10.1% and 29.9%, respectively ($p<0.05$), while the EdTag, Moraten, CAM-70, and Indiana C proteins did not inhibit (Fig. 2C). In

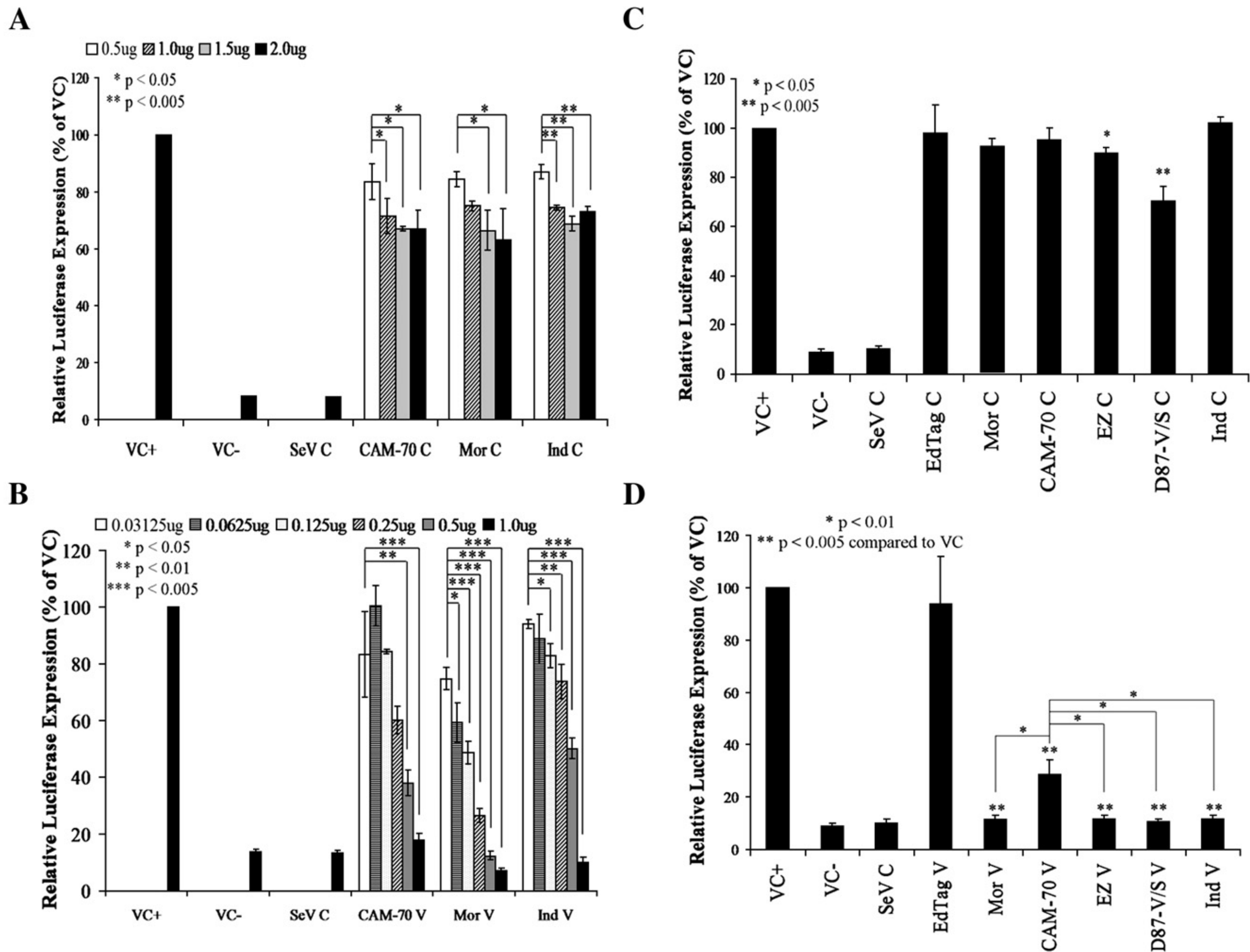

Fig. 2. The C and V proteins of MV inhibit IFN-α/β-responsive reporter gene expression. Vero cells were transfected with a plasmid constitutively expressing renilla luciferase and a plasmid expressing firefly luciferase under the control of an IFN-α/β-responsive promoter. The cells were also transfected with plasmids expressing (A) C proteins or (B) V proteins from CAM-70, Moraten, or Indiana strains of MV at varying doses, or with 1.0 μg of plasmid expressing (C) C proteins or (D) V proteins from each of six strains of MV. 48 h post-transfection, the cells were stimulated with 100 U IFN-β for 6 h, lysed, and tested for luciferase activity. Stimulated pCAGGS constituted a negative control for inhibition of IFN-responsive gene expression (VC+), and unstimulated pCAGGS was used to show background levels of firefly luciferase luminescence (VC−). A plasmid expressing the Sendai virus C proteins was used as a positive control for inhibition of IFN-responsive gene expression. Results are expressed as a ratio of firefly to renilla luciferase luminescence taken as a percentage of the luminescence obtained using IFN-stimulated, empty pCAGGS vector. The data shown are an average of six experiments done in triplicate. Statistics were performed using Student's *t*-test.

contrast, all of the V proteins tested, with the exception of the EdTag and CAM-70 V proteins, inhibited reporter gene expression at levels between 88.5% and 89.4%. The EdTag V protein poorly inhibited reporter gene expression (6.3%) and this result was consistent with a previous report (Ohno et al., 2004). The V protein from CAM-70 was also not as potent an inhibitor as the other V proteins (71.5%) (Fig. 2D). These results provide further evidence that the V proteins of MV are more potent inhibitors of IFN-α/β signaling than the C proteins. There was no indication that C or V proteins that displayed greater inhibition of IFN-α/β signaling were from either attenuated or wild-type strains of MV. Additionally, the inability of the EdTag V protein to inhibit IFN-α/β signaling was confirmed in these experiments, and an additional V protein was found to be impaired in this function.

Since it has been suggested that the C and V proteins of MV might exist in a complex with components of the IFN-α/β receptor (Yokota et al., 2003), we investigated the effect of co-expressing the C and V proteins. The V protein was expressed at a level that inhibited reporter gene expression by approximately 40–80%, and an increasing amount of plasmids expressing C proteins from the corresponding strain of MV was added. The CAM-70 and Moraten C proteins did not increase the level of inhibition observed with the V protein of these strains alone (Fig. 3A). However, the EZ and D87-V/S C proteins increased the level of inhibition when co-expressed with the V protein of the same strain (Fig. 3B). Taken together, these data suggest that while the C protein of MV has a weak inhibitory effect on IFN-α/β signaling, the V protein of MV is a stronger inhibitor of IFN-α/β signaling. Furthermore, the C and V proteins of some strains of MV may act in an additive manner to inhibit IFN-α/β signaling.

Effect of C and V proteins from wild-type and attenuated strains of MV on IFN-γ signaling

While there is some evidence to suggest that the C protein of MV can inhibit IFN-γ signaling (Shaffer et al., 2003), the

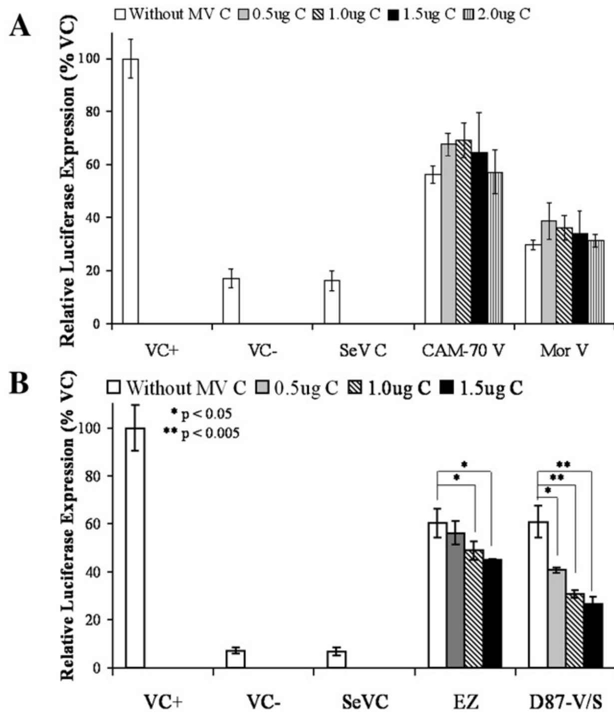

Fig. 3. The C and V proteins of some strains of MV may act in an additive manner to inhibit IFN-α/β-responsive reporter gene expression. Vero cells were transfected with a plasmid constitutively expressing renilla luciferase and a plasmid expressing firefly luciferase under the control of an IFN-α/β-responsive promoter. The cells were also transfected with plasmids expressing the V protein from (A) CAM-70 or Moraten or (B) EZ or D87-V/S strains of MV at doses which inhibited IFN-α/β signaling by 40–80% and varying doses of plasmids expressing the C protein from the same virus. 48 h post-transfection, the cells were stimulated with 100 U IFN-β for 6 h, lysed, and tested for luciferase activity. Results are expressed as a ratio of firefly to renilla luciferase luminescence taken as a percentage of the luminescence obtained using IFN-stimulated, empty pCAGGS vector. As described in the legend to Fig. 2, stimulated (VC+) and unstimulated (VC−) pCAGGS were used as negative controls, and a plasmid expressing the Sendai virus C proteins was used as a positive control. The data shown are an average of three experiments done in triplicate. Statistics were performed using Student's *t*-test.

inhibitory effect of the V protein is under debate (Palosaari et al., 2003; Takeuchi et al., 2003). The relative abilities of the C and V proteins of MV to inhibit IFN-γ signaling were determined by measuring the effect of different doses of each protein on the expression of an IFN-γ-responsive reporter gene. Addition of increasing concentrations of plasmids expressing the C protein to this system did not inhibit IFN-γ-responsive reporter gene expression (data not shown). In contrast, the V proteins of CAM-70, Moraten, and Indiana inhibited IFN-γ-responsive reporter gene expression in a dose-dependent manner, reaching maximum inhibition levels of 36.0%, 83.0%, and 81.6%, respectively (Fig. 4A). These data indicate that the V protein is a potent inhibitor of IFN-γ signaling, while the C protein does not inhibit at all.

To test whether the ability of the C and V proteins to inhibit IFN-γ signaling was dependent on the strain of MV from which it was derived, C and V proteins from attenuated and wild-type strains of MV were tested for inhibition of IFN-γ-responsive reporter gene expression. None of the C proteins were able to inhibit IFN-γ-responsive reporter gene expression (data not shown). In contrast, the V proteins exhibited marked inhibition of IFN-γ-responsive reporter gene expression, with the exception of the V proteins from EdTag and CAM-70, which did not inhibit to the same extent as the other V proteins (11.0% and 24.5%, respectively) (Fig. 4B). These results show that the V proteins of MV are more potent inhibitors of IFN-γ signaling than the C proteins, which do not inhibit. As shown above for IFN-α/β, there was no correlation between the potency of inhibition of IFN-γ signaling by a C or V protein and its derivation from an attenuated or wild-type strain. Furthermore, both the EdTag and CAM-70 V proteins were impaired in their ability to inhibit IFN-γ signaling.

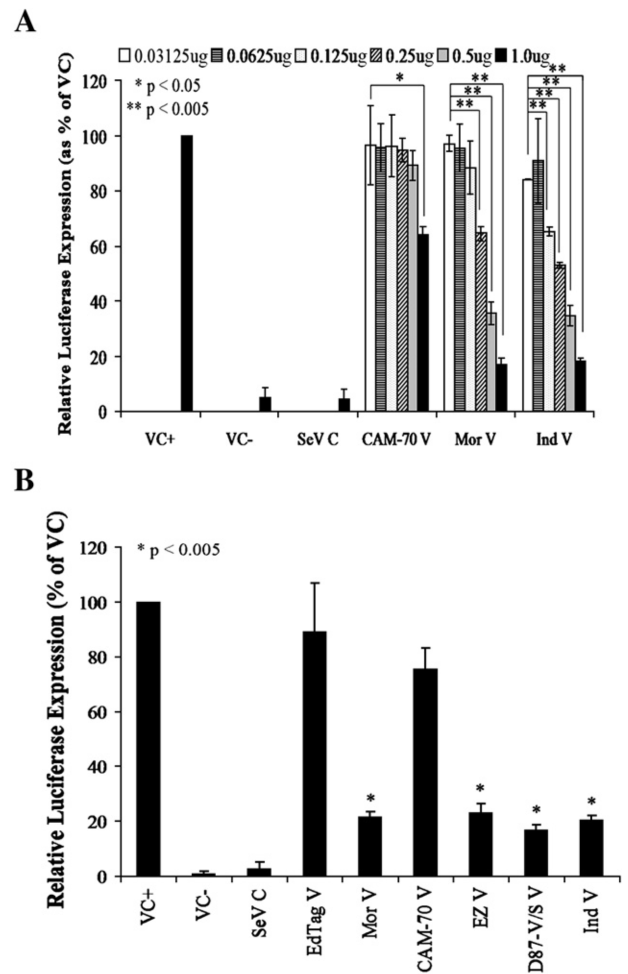

Fig. 4. The V proteins of MV inhibit IFN-γ-responsive reporter gene expression. Vero cells were transfected with a plasmid constitutively expressing renilla luciferase and a plasmid expressing firefly luciferase under the control of an IFN-γ-responsive promoter. The cells were also transfected (A) with plasmids expressing V proteins from CAM-70, Moraten, or Indiana strains of MV at varying doses, or (B) with 1.0 μg of plasmid expressing V proteins from each of six strains of MV. 48 h post-transfection, the cells were stimulated with 1000 U IFN-γ for 6 h, lysed, and tested for luciferase activity. Results are expressed as a ratio of firefly to renilla luciferase luminescence taken as a percentage of the luminescence obtained using IFN-stimulated, empty pCAGGS vector. As described in the legend to Fig. 2, stimulated (VC+) and unstimulated (VC−) pCAGGS were used as negative controls, and a plasmid expressing the Sendai virus C proteins was used as a positive control. The data shown are an average of six experiments done in triplicate. Statistics were performed using Student's *t*-test.

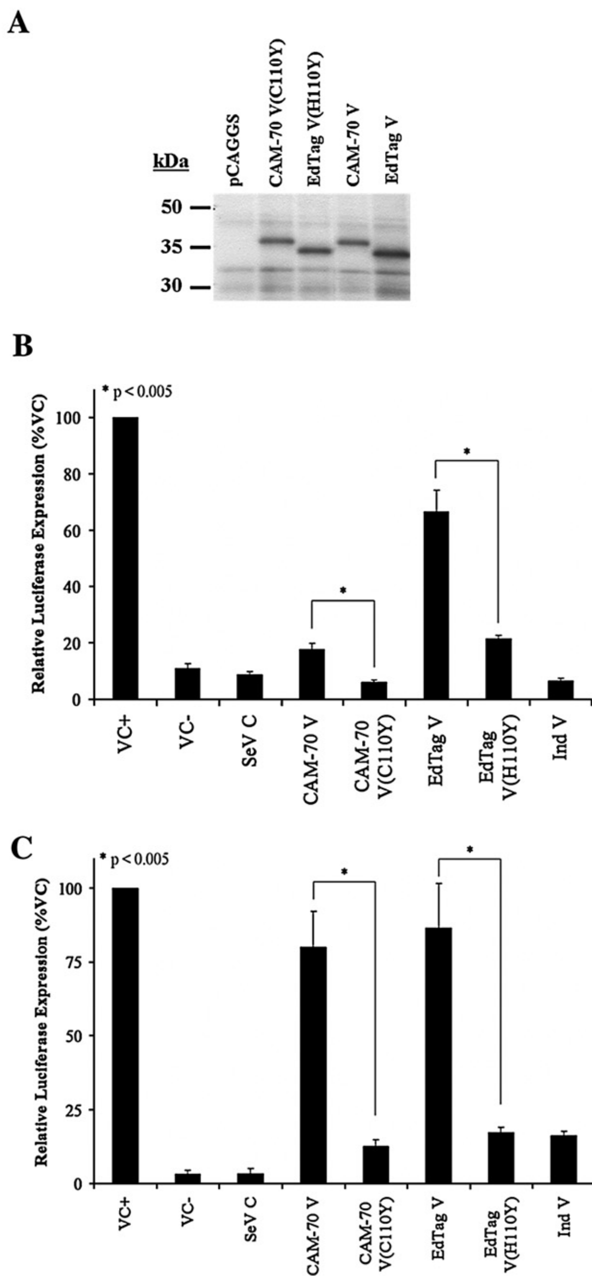

Fig. 5. Tyrosine 110 is important for the IFN−antagonistic activity of the V protein. (A) The CAM−70 and EdTag V proteins and their mutants were ^{35}S−methionine labeled and detected by immunoprecipitation with rabbit antiserum specific to the shared amino−terminus of the P and V proteins and SDS−PAGE analysis, as described in Figure 1. As described in Figures 2 and 4, luciferase activity was determined in Vero cells that were transfected with plasmids encoding renilla luciferase, firefly luciferase under the control of (B) an IFN−α/β−responsive promoter or (C) an IFN−γ−responsive promoter, and the indicated protein. Results are expressed as a ratio of firefly to renilla luciferase luminescence taken as a percentage of the luminescence obtained using IFN−stimulated, empty pCAGGS vector. As described in the legend to Figure 2, stimulated (VC+) and unstimulated (VC−) pCAGGS were used as negative controls, and a plasmid expressing the Sendai virus C proteins was used as a positive control. The data shown are an average of three experiments done in triplicate. Statistics were performed using Student's *t*-test.

Tyrosine 110 controls the IFN-Inhibitory activity of the V protein from the CAM-70 strain of MV

As previously reported, the EdTag V protein differs from the Edmonston V protein at amino acid positions 110 (tyrosine to histidine), 272 (cysteine to arginine), and 291 (tyrosine to histidine), with the substitutions at positions 110 and 272 shown to be important for the inhibition of IFN-α/β-responsive reporter gene expression (Ohno et al., 2004). The EdTag V protein used in the present study contained the three mutations described above and an additional amino acid substitution at position 225 (glutamic acid to glycine) (Table 2), which was reported to be conserved in vaccine strains of MV (Parks et al., 2001). The CAM-70 V protein contained 10 amino acid differences compared to the Edmonston V protein, including a tyrosine to cysteine change at amino acid position 110 (Table 2).

To explore the role that amino acid 110 in the V protein of MV plays in inhibition of IFN signaling, the plasmids expressing the V protein of EdTag or CAM-70 were modified to change the histidine or cysteine at position 110 to tyrosine. Expression of these mutant proteins, referred to as CAM-70 V(C110Y) and EdTag V(H110Y), was confirmed by immunoprecipitation with antiserum to the shared region of the P and V proteins of MV and SDS-PAGE analysis (Fig. 5A). The modified V proteins were compared with the unmodified V proteins in their ability to inhibit the expression of an IFN-α/β- or IFN-γ-responsive reporter gene. Both CAM-70 V(C110Y) and EdTag V(H110Y) displayed significantly increased inhibition of both IFN-α/β and IFN-γ signaling. As expected, the mutation in the EdTag V protein did not fully restore its ability to inhibit IFN-α/β-responsive reporter gene expression (Ohno et al., 2004) (Fig. 5B). However, the mutation in the CAM-70 V protein was sufficient to bring its IFN-α/β-antagonistic activity to the level observed for the other V proteins tested (Fig. 5B). The introduction of a tyrosine at amino acid residue 110 was sufficient to restore the ability of both proteins to inhibit IFN-γ-responsive reporter gene expression (Fig. 5C). These data indicate that amino acid 110 plays a role in the ability of the V protein of MV to inhibit both IFN-α/β and IFN-γ signaling.

Discussion

Although the C and V proteins of MV have been previously implicated as inhibitors of IFN signaling, the relative contribution of each protein to this activity was unclear. This report demonstrated that the C protein marginally blocked the expression of an IFN-α/β-responsive reporter gene, but was unable to block IFN-γ-responsive reporter gene expression. In contrast, the V protein blocked the expression of IFN-α/β- and IFN-γ-responsive reporter genes. The level of IFN-inhibition was unrelated to whether the protein was derived from an attenuated or wild-type strain of MV. We confirmed the importance of a tyrosine at amino acid 110 for the IFN-α/β-antagonist function of the V protein, and demonstrated a role for this residue in the inhibition of IFN-γ signaling. Notably, this report is the first to identify a non-recombinant MV that produced a V protein that was impaired due to a mutation at this residue.

Our laboratory previously showed that the C protein from the Edmonston wild-type strain of MV inhibited the expression of an IFN-α/β-responsive reporter gene and, to a lesser extent, an IFN-γ-responsive reporter gene in Vero cells (Shaffer et al., 2003). The data presented here correspond with subsequent studies performed in other cell types suggesting that C has a minor role in the inhibition of IFN signaling (Nakatsu et al., 2006; Ohno et al., 2004; Takeuchi et al., 2005). Discrepancies between the data in this report and our previous results were probably due to improvements in the IFN-responsive reporter gene assay, including the use of a more sensitive detection method, an expression vector which produced lower background, and the inclusion of more biologically relevant strains of MV. Additionally, since the MV that lacked the C protein in the study by Shaffer et al. (2003) also expressed impaired V and P proteins, the contribution of the C protein to the inhibition of IFN signaling may have been overestimated. A recent study suggested that the C protein of MV blocks IFN induction (Nakatsu et al., 2006), and there is evidence that the C proteins of other paramyxoviruses may also have this ability (Strahle et al., 2007; Van Cleve et al., 2006). It is therefore possible that, while the C protein may have a minor role in the inhibition of IFN-α/β signaling, it is more important in limiting IFN induction.

Although it is widely recognized that the V protein of MV inhibits IFN-α/β signaling, the impact of this protein on IFN-γ signaling is a subject of debate in the literature. This study and a study by Palosaari et al. (2003) both support the conclusion that the V protein inhibits IFN-α/β and IFN-γ signaling. In contrast, Takeuchi et al. (2003) reported that the V protein could not inhibit IFN-γ signaling. A major difference between these studies was that Takeuchi et al. (2003) used a HeLa cell line that stably expressed the V protein of MV, while the other studies relied on transient expression of the V protein, a method that may more accurately reflect the *de novo* synthesis and accumulation of the V protein that occurs during MV infection.

All V proteins tested in this study were more potent inhibitors of IFN-α/β and IFN-γ signaling than were the corresponding C proteins from each strain. This result suggests that the V protein might be the major inhibitor of IFN signaling during MV infection. Devaux et al. (2007) recently found that while the P protein of MV inhibited the IFN-α/β response, the level of inhibition was 10-15 fold less than that observed for the V protein. In rinderpest virus, a closely related paramyxovirus in the genus *Morbillivirus*, the V protein was the dominant inhibitor of IFN compared to the P and C proteins of that virus (Nanda and Baron, 2006). Additionally, the P protein of Nipah virus, a member of the genus *Henipavirus*, exhibited weaker IFN-antagonistic activity than the V and W proteins of that virus (Shaw et al., 2004). Further studies that directly compare the C, V, and P proteins of MV will be needed to strengthen the argument that the V protein is the primary inhibitor of IFN signaling during infection with MV.

This is the first report to investigate the effect of co-expressing the C and V proteins of MV on IFN-α/β signaling. The two C proteins (EZ and D87-V/S) that improved the inhibition of an IFN-α/β-responsive reporter gene when co-expressed with their corresponding V protein were also the two C proteins that inhibited IFN-α/β signaling in the absence of V. Of course, the inability to detect significant inhibition with the C proteins from other strains of MV could be a function of the sensitivity of the reporter gene assay. Future studies will be needed to determine the mechanism of the additive effect of the C and V proteins on the inhibition of IFN signaling.

In this study, the IFN-antagonist activity of the V protein was unrelated to whether the protein was derived from an attenuated or wild-type strain of MV, but it correlated with the presence of a tyrosine at amino acid 110. Three previous studies have noted the importance of this residue in the shared region of the P and V proteins of MV (Caignard et al., 2007; Devaux et al., 2007; Ohno et al., 2004). This is the first report of a cysteine substitution at amino acid 110 in the V protein of a non-recombinant strain of MV that resulted in impaired IFN-antagonism. Additionally, this is the first report that demonstrated the importance of this residue for the inhibition of IFN-γ signaling by the V protein. Interestingly, changing amino acid 110 to a tyrosine in the EdTag V protein improved, but did not fully restore its ability to inhibit IFN-α/β signaling. This observation was also reported by Ohno et al. (2004), who pointed out that a cysteine to arginine change at position 272 in the unique carboxy-terminal region was also important. Caignard et al. (2007) demonstrated that the amino- and carboxy-terminal domains of the V protein could individually inhibit IFN-α/β signaling. This indicates that both regions of the V protein may be needed to inhibit IFN-α/β signaling. Further studies are needed to fully map the regions involved in the inhibition of IFN signaling by the V protein.

The involvement of amino acid 110 in the inhibition of both IFN-α/β and IFN-γ signaling indicates that it may be important for the interplay of the V protein with common components of these pathways, such as STAT1 or Jak1. It was previously observed that the V protein of MV co-purified with STAT1 and that MV infection suppressed the phosphorylation of Jak1 and STAT1 (Palosaari et al., 2003; Yokota et al., 2003). A direct interaction was recently demonstrated between the V protein of MV and both Jak1 and STAT1, and the tyrosine at amino acid 110 was necessary for interaction with STAT1, but not Jak1 (Caignard et al., 2007). Devaux et al. (2007) reported that a tyrosine at amino acid 110 was required for the P protein of MV to inhibit STAT1 phosphorylation and nuclear translocation, and described a putative hexapeptide, Y(Y/H)VYDH, beginning at position 110 that is conserved among six morbilliviruses. For Nipah virus, amino acids 100 to 150 in the common amino-terminal domain of the P, V, and W proteins were sufficient for their STAT-1-binding activity (Rodriguez and Horvath, 2004; Shaw et al., 2004). Preliminary observations identified a short sequence, DVVYHD, in this region that resembles the hexapeptide observed in genus *Morbillivirus*, and is conserved between Nipah and Hendra viruses, both members of the genus *Henipavirus*. These data suggest a common mechanism for V protein antagonism of the IFN-α/β and IFN-γ signaling pathways, as well as continuity in this function across genera.

Previous studies have investigated the sensitivity of attenuated and wild-type strains of MV to IFN treatment. Naniche et al. (2000) showed that wild-type strains of MV were more

sensitive to IFN-α/β treatment than attenuated strains. Two additional studies showed that wild-type strains were less sensitive than attenuated strains to IFN-α treatment (Obojes et al., 2005; Ohno et al., 2004). Wild-type and vaccine-like strains of MV were equally sensitive to the presence of IFN-γ (Obojes et al., 2005). In our study, there was no association between the IFN-sensitivity of MV and its designation as a wild-type or attenuated strain. The discrepancies between our result and the results of previous studies could be due to the use of different cell types, the addition of IFN at different times relative to infection, or the number and choice of strains studied. Taken together, these studies indicate that wild-type and attenuated strains of MV cannot be distinguished based on their sensitivity to IFN treatment.

A surprising finding was that the vaccine strains were highly variable in their sensitivity to IFN treatment. Although all MV vaccine strains in use have been proven safe and efficacious, some biologic differences between strains have been reported. Immunization with EZ induced higher rates of seroconversion compared to the Schwarz vaccine strain at equivalent titers (Cutts et al., 1995), and it has been suggested that EZ is more immunogenic than other Edmonston-derived MV vaccine strains (Sabin et al., 1984, 1983). It is possible that the EZ strain more effectively inhibits innate defense mechanisms than some of the other MV vaccines. Our study supports this conclusion because EZ was less sensitive to IFN treatment than Moraten. Another interesting finding was that CAM-70 was less sensitive to IFN-β than all other attenuated strains. CAM-70 was the only non-Edmonston-derived attenuated strain in this study and it has been shown to be the most genetically divergent, especially with regards to the P gene, of all genotype A vaccine viruses (Rota et al., 1994; Santos et al., 2003).

Although no association between attenuation status and IFN-sensitivity was detected, the level of sensitivity of a majority of the strains (EdTag, EZ, Indiana, and D87-V/S) correlated to the ability of its V protein to inhibit IFN signaling. Most notably, EdTag was extremely sensitive to the presence of IFN-α/β and IFN-γ and its V protein was impaired in its ability to inhibit signaling. No such correlation was seen for the Moraten and CAM-70 strains. This inconsistency could be attributed to the involvement of other viral proteins in the inhibition of IFN, such as the P protein (Devaux et al., 2007). Since the IFN-antagonist activity of the V protein can be regulated by a substitution at amino acid 110 in its shared domain with the P protein, it is difficult to separate the functions of these two proteins in the context of a viral infection. Additionally, the attenuation of these strains could have resulted in other changes leading to altered viral replication in the presence of IFN. For example, the predicted amino acid sequences of the V proteins of Moraten and EZ are identical. Therefore, other viral factors may attribute to the different IFN-sensitivity of these viruses. Another explanation could be that the time course of P, V and C protein production varies among different strains of MV.

In the context of viral infection, evasion of the IFN response is complicated and may involve interplay between multiple viral and host proteins as well as the need to block IFN induction in addition to signaling. There is increasing evidence that the C and V proteins of paramyxoviruses may also inhibit IFN induction (Andrejeva et al., 2004; Childs et al., 2007; He et al., 2002; Kiyotani et al., 2007; Komatsu et al., 2007; Nakatsu et al., 2006; Poole et al., 2002; Strahle et al., 2007). This report furthers our understanding of how the C and V proteins of MV function to inhibit IFN signaling. Studies regarding the other functions of the C and V proteins will be necessary to fully understand the contribution of these proteins to viral pathogenesis.

Materials and methods

Cells and viruses

Vero cells were maintained in Eagle's Minimum Essential Medium (EMEM; Mediatech) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals). Vero/hSLAM cells (Ono et al., 2001) which stably express the human signaling lymphocyte activation molecule (SLAM), a receptor of MV infection (Tatsuo et al., 2000), were maintained in EMEM supplemented with 10% FBS and 400 μg/mL G418 sulfate (Mediatech).

The Kitasato submaster seed of the CAM-70 strain (Isomura et al., 1986; Okuno et al., 1971; Sangkawibha et al., 1971; Ueda et al., 1970) was provided by I. Yoshida, Kanonji Institute, Osaka, Japan. The Moraten strain (Enders and Peebles, 1954) was obtained from Merck, Sharp and Dohme. The Edmonston–Zagreb strain (EZ) (Bennett et al., 1999; Ikic et al., 1972) was provided by D. Ikic, Institute of Immunology, Zagreb, Croatia. The EdTag virus, which was recovered using reverse genetics from cloned cDNA based on the sequence of the Edmonston B strain of MV (Radecke and Billeter, 1996; Schneider et al., 1997), was provided by M. Billeter, University of Zürich, Zürich, Switzerland. All attenuated virus strains were plaque purified and propagated in Vero cells. The Indiana strain (MVi/Indiana.USA/22.05) is a wild-type virus of genotype D4 (World Health Organization, 2006) that was isolated in 2000 from a human clinical isolate and has been passaged four times on Vero/hSLAM cells. The Davis87 strain is a wild-type virus of genotype D3 that causes disease in rhesus macaques and was isolated on rhesus PBMCs after an outbreak at a primate facility (McChesney et al., 1997). The original Davis87 stock was provided by M. McChesney, University of California, Davis, California and has been passaged nine times in Vero/hSLAM cells, resulting in a stock that is referred to as D87-V/S. The D87-V/S virus was sequenced to confirm the absence of nucleotide changes compared to the parental strain (Bankamp et al., in press).

IFN sensitivity assay

IFN sensitivity tests were performed as previously described (Ohno et al., 2004). Briefly, Vero/hSLAM cells were infected with various strains of MV at a multiplicity of infection (m.o.i.) of 0.001. 1 h post-infection, inoculum was removed and cells were treated with either 1000 U/mL rhIFN-β (Biosource International), 1000 U/mL rhIFN-γ (R&D Systems), or left untreated in 5 mL EMEM/G418 and 2% FBS. After 48 h, virus was harvested and titrated on Vero/hSLAM cells.

Levels of virus sensitivity to IFN were defined by a 10-fold decrease in viral titer. A decrease in viral titer of 1- to 10-fold was defined as "minimally sensitive," a decrease of 10- to 100-fold was defined as "intermediately sensitive," and decrease of 100-fold and beyond was defined as "extremely sensitive."

Derivation of plasmids

Total RNA was extracted from MV-infected Vero cells using Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR with gene-specific primers was performed using SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) to obtain cDNAs encoding the C and V genes. The primers used to amplify the C gene were as previously described (Shaffer et al., 2003), and were designed to exclude the first 12 nucleotides of the P ORF. V cDNAs were identified by screening cDNA obtained using previously described primers (Bankamp et al., 2002) that amplified the entire P ORF and silenced the C ORF for the presence of a non-templated guanosine insertion at the RNA editing site. The C and V amplicons were digested with EcoRI and XhoI and ligated into the plasmid vector, pCAGGS, to which a linker containing EcoRI and XhoI recognition sites was added. The mammalian expression vector, pCAGGS, has been previously described (Niwa et al., 1991), and was provided by C. Basler, Mount Sinai School of Medicine, New York, New York.

Site-directed mutagenesis was performed by amplifying the coding region from the plasmid containing MV V by using a three-step PCR reaction with the primers described above and primers containing the desired mutation (underlined), CAMF (5′-GCA CTG GGT TAC AGT GTT ATT ATG TTT ATG ATC ACA GCG G-3′), CAMR (5′-CCG CTG TGA TCA TAA ACA TAA TAA CAC TGT AGC CCA GTG C-3′), EdTagF (5′-GCA CTG GGC TAC AGT GTT ATT ATG TTT ATG ATC ACA GCG G-3′), and EdTagR (5′-CCG CTG TGA TCA TAA ACA TAA TAA CAC TGT AGC CCA GTG C-3′). The mutated amplicons were digested with EcoRI and XhoI and ligated back into the pCAGGS vector. All pCAGGS-C and pCAGGS-V clones were sequenced using ABI PRISM Dye Terminator Reaction Kit and the ABI 3100 and 3130xL Genetic Analyzer machines (Perkin Elmer-Applied Biosystems) according to the manufacturer's instructions. Sequence data were analyzed with the Sequencher™ DNA sequencing program (Gene Codes Corporation), and confirmed by comparison to the published sequence for each strain.

A pGEM-4 expression vector encoding the four C proteins of Sendai virus was provided by S. Moyer, University of Florida College of Medicine, Gainesville, Florida. The C gene of Sendai virus was excised from this vector using MluI and XhoI and cloned into the pCAGGS vector.

The pISRE and pGAS plasmids were gifts from R.E. Randall, North Haugh University of St. Andrews, Fife, Scotland. pISRE encodes the firefly luciferase gene following four tandem repeats of the IFN-stimulated response element (ISRE) from the IFN inducible gene 9-27 (Didcock et al., 1999; Garcin et al., 1999; King and Goodbourn, 1998). pGAS contains the minimal thymidine kinase promoter and two tandem repeats of the IFN-γ activating sequence (GAS) from the IRF-1 gene (King and Goodbourn, 1998). The pRL-TK plasmid (Promega) encodes the renilla luciferase gene constitutively expressed from a thymidine kinase promoter and was used as a transfection control.

Expression of C and V proteins

Vero cells were cultured to 70% confluency in 6-well plates and transfected with plasmids expressing C, V, or V mutants in Opti-MEM medium (Life Technologies) and TransIT®-LT1 (Mirus Bio) following the manufacturer's recommendation. pCAGGS without a coding sequence was used as a negative control for protein expression. 48 h post-transfection, cells were starved for 1 h in methionine-free medium (ICN), labeled for 2 h with ^{35}S-methionine, and then lysed in RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 0.2% SDS, 150 mM NaCl, and 50 mM Tris–HCl at pH 7.5). Aliquots of cell extracts were precleared with normal rabbit serum and then incubated with C-specific or P/V-specific rabbit antiserum overnight on a rotator at 4 °C. Complexes were precipitated with GammaBind G-Sepharose (Amersham Pharmacia Biotech) and washed with RIPA buffer. Samples were boiled for 5 min in SDS-PAGE sample buffer and separated on an 8-16% gradient SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, bands were visualized by autoradiography.

IFN-responsive reporter gene assay

Vero cells were cultured to 70% confluency in 24-well plates and transfected with Opti-MEM medium and TransIT®-LT1 (Mirus Bio) following the manufacturer's recommendation. pISRE or pGAS and pRL-tk were co-transfected with various doses of pCAGGS-C or pCAGGS-V plasmid DNA. pCAGGS without a coding sequence was used to make up the difference in total plasmid concentration, and also constituted a negative control for inhibition of IFN-responsive gene expression. A plasmid encoding the C proteins of Sendai virus, which are known to inhibit both IFN-α/β and IFN-γ signaling (Garcin et al., 2002; Kato et al., 2001; Saito et al., 2002), was used as a positive control for inhibition of IFN-responsive gene expression. 48 h post-transfection, cells were stimulated with 100 U rhIFN-β or 1000 U rhIFN-γ for 6 h. pCAGGS that was unstimulated constituted a negative control for IFN signaling, and was used to demonstrate background levels of firefly luciferase luminescence. Cell lysates were harvested for detection of luciferase activity using the Dual Reporter Luciferase System (Promega) according to the manufacturer's protocol. Chemiluminescence was quantified on a Luminoskan Ascent FL microplate luminometer (Thermo Electron). To control for transfection efficiency, data were expressed as a ratio of firefly to renilla luciferase luminescence and taken as a percentage of luminescence obtained using IFN-stimulated, empty pCAGGS vector.

Statistical analysis

For two group comparisons, a two-tailed Student's *t*-test was used, and a value of $p < 0.05$ was considered statistically significant.

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