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Inhibition of IFN-α/β signaling by two discrete peptides within measles virus V protein that specifically bind STAT1 and STAT2

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ABSTRACT

The V protein of measles virus (MV-V) is a potent inhibitor of IFN-α/β signaling pathway. We previously reported that when physically dissociated, the N-terminal and C-terminal regions of MV-V (PNT and VCT, respectively) could independently impair signal transduction. The PNT region inhibited IFN-α/β signaling by interacting with at least two components of this pathway: Jak1 and STAT1. Here we report a direct interaction between the VCT of MV-V and STAT2, a third component of IFN-α/β transduction machinery. This interaction with STAT2 is carried by the cysteine-constrained peptide of 49 amino acids localized in the VCT region, and is essential to the inhibition of IFN-α/β signaling. In parallel, we also mapped STAT1 binding site in the PNT region and identified a minimal peptide of only 11 amino acids. IFN-α/β signaling was impaired in human cells treated with this MV-V peptide fused to a cell-penetrating sequence. Finally, we show that signaling downstream of IFN-λ, a recently identified cytokine that also relies on STAT1, STAT2 and Jak1 to transduce, is blocked by MV-V. Altogether, our results illustrate how a single viral protein has evolved to achieve a robust inhibition of the antiviral response by interacting with several signaling molecules.

Introduction

In spite of the widespread use of an efficient live attenuated vaccine, measles remains a major cause of infant mortality in the world responsible for 345,000 deaths annually (Wolfson et al., 2007). Measles virus (MV) infection induces a transient but profound immunosuppression that accounts for the high frequency of opportunistic infections associated with this disease. MV is a member of *Paramyxoviridae* family and *Morbillivirus* genus (Fields et al., 2001). Its genome is a single-strand, negative-sense RNA that encodes for six structural proteins. While hemagglutinin (H) and fusion (F) are membrane glycoproteins associated with the envelope of MV particles, the nucleoprotein (N), the phosphoprotein (P) and the viral polymerase (L) are components of the ribonucleocapsid complex. The matrix protein (M) is located at the interface between glycoprotein tails and ribonucleocapsids, thereby contributing to the budding of viral particles.

In addition to phosphoprotein, the P gene of *Morbilliviruses* encodes two virulence factors, C and V (Devaux et al., 2008; Patterson et al., 2000). An opened reading frame embedded in the first half of the P gene encodes the C protein (Bellini et al., 1985). The C protein of MV is required for an optimal viral growth both *in vitro* and *in vivo* (Takeuchi et al., 2005), but its main function remains unclear. The V protein of measles virus (MV-V) is an essential virulence factor encoded by an editing strategy (Cattaneo et al., 1989; Devaux et al., 2008). A non-templated guanine nucleotide is inserted at a specific position of mRNAs transcribed from the P gene (Fig. 1A). As a consequence, MV-V is similar to the phosphoprotein P in its amino-terminal part (AA 1–231; PNT region) but exhibits a specific cysteine-rich C-terminus that folds in a zinc-finger domain (AA 232–299; VCT region). Although MV-V is probably multifunctional, its critical role in the inhibition of type I interferons (IFN-α/β) signaling has been well documented (Palosaari et al., 2003; Takeuchi et al., 2003; Yokota et al., 2003). IFN-α/β are major antiviral cytokines that transduce through well-characterized intermediates (Stark et al., 1998). Their binding to IFNAR1/IFNAR2c cell surface receptor activates Tyk2 and Jak1 kinases to phosphorylate STAT1 and STAT2 transcription factors. Activated STAT1 and STAT2, altogether with IRF9, form the Interferon-Stimulated Gene Factor 3 (ISGF3) that binds IFN-stimulated response element (ISRE) promoter sequences to stimulate the expression of antiviral genes, thus leading viruses to evolve inhibitory mechanisms. MV-V interferes with this pathway by interacting with components of the IFN-α/β signaling pathway in a multiprotein complex (Palosaari et al., 2003; Yokota et al., 2003). This interference mechanism protects the virus from the antiviral activity of IFN-α/β in infected cells, while these cytokines

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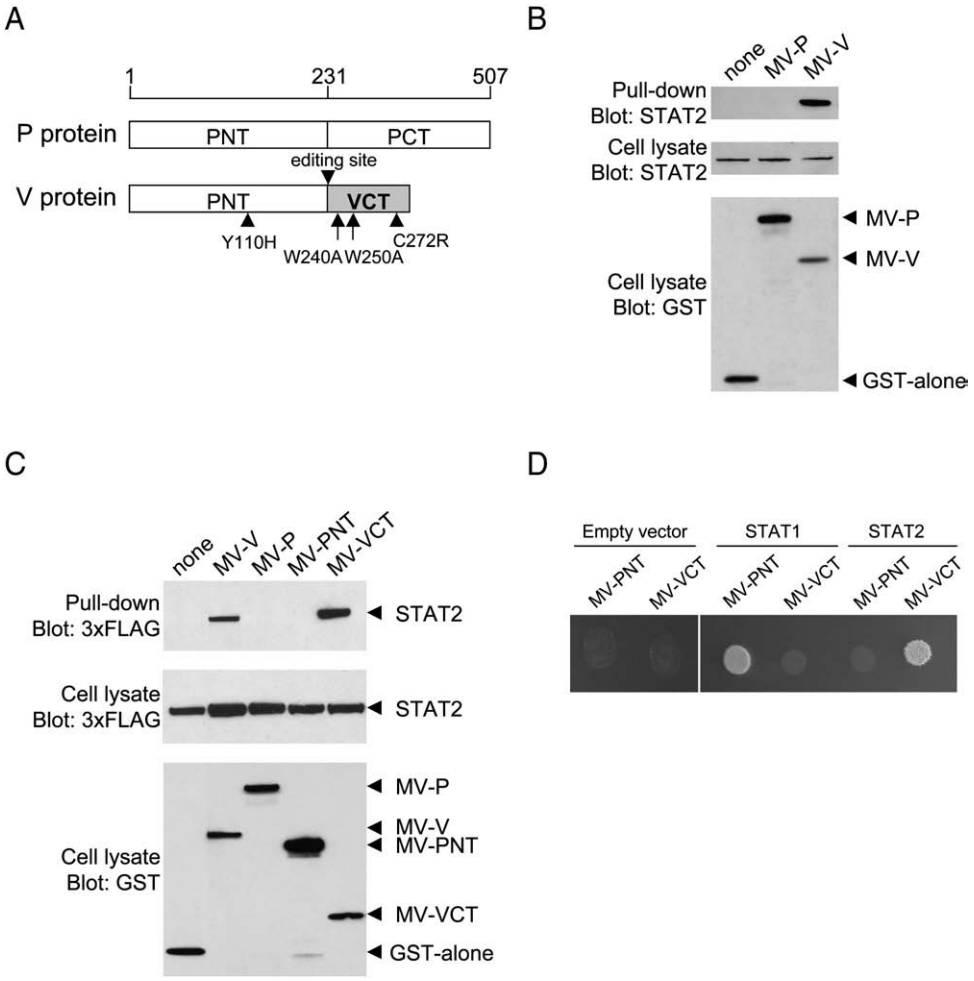

Fig. 1. The VCT region of MV-V interacts directly with STAT2. (A) Diagrams of MV-P and MV-V showing the position where RNA edition occurs. Deleterious mutations Y110H and C272R that are characteristic of Ed-Tag infectious clone (Ohno et al., 2004), and W240A/W250A mutations investigated in this report are indicated with arrows. (B) HEK-293T cells were transfected with expression vectors encoding GST alone or fused to MV-V or MV-P proteins, and tested for interaction with endogenous STAT2. Total cell lysates were prepared 48 h post-transfection (cell lysate; middle and lower panels), and co-purification of endogenous STAT2 was assayed by pull-down using glutathione-sepharose beads (GST pull-down; upper panel). GST-tagged MV-V and MV-P were detected by immunoblotting using anti-GST antibody, while endogenous STAT2 was detected with a specific monoclonal antibody. (C) Similar experiment, but HEK-293T cells were co-transfected with expression vectors encoding GST alone or fused to the PNT or VCT regions of MV-V, and 3xFLAG-tagged STAT2. Tagged proteins were detected by immunoblotting using anti-GST or anti-3xFLAG antibodies. (D) Interaction of STAT2 with the VCT region of MV-V was analyzed in the yeast two-hybrid system. Yeast cells expressing the PNT or VCT regions of MV-V fused to the Gal4 DNA binding domain (DB) were co-transformed with a plasmid encoding the Gal4 transactivation domain (AD) alone or fused to either STAT1 or STAT2. Yeast cells were plated on a selective medium lacking histidine and supplemented with 10 mM 3-amino-triazole (3-AT) to test the interaction-dependent transactivation of HIS3 reporter gene.

produced en masse during the infection exhibit deleterious effects on key components of the immune system such as dendritic cells (Hahm et al., 2005).

Recently, we identified STAT1 and Jak1 as direct interactors of the PNT region of MV-V, and documented how these two interactions inhibit STAT1 phosphorylation by Jak1 (Caignard et al., 2007). This provides molecular basis on how the PNT region alone can affect IFN-α/β signaling. However, inhibition by the PNT region is only partial, as compared to that observed with full-length MV-V, suggesting that the VCT region is also involved. Accordingly, a mutation in the PNT region (Y110H) that impairs STAT1 binding must be combined with a second mutation in the VCT region (C272R) to abrogate MV-V inhibitory activity (Caignard et al., 2007; Devaux et al., 2007; Fontana et al., 2008; Ohno et al., 2004) (Fig. 1A). We also demonstrated that the VCT region alone can impair IFN-α/β signaling (Caignard et al., 2007). Altogether, these observations suggested that the VCT region has a specific partner in the IFN-α/β receptor complex. In agreement with this hypothesis, we now demonstrate that the VCT region of MV-V from wild-type IC-B strain interacts directly with cellular STAT2. In addition, we precisely mapped the STAT1 and STAT2 binding sites embedded in the PNT and VCT regions of MV-V, respectively. When preparing this manuscript, Ramachandran et al. published a similar study on the MV-V protein of Edmonston ATCC strain (Ramachandran et al., 2008). Their data are compared to our results in the Discussion section of this manuscript.

Results

The VCT region of MV-V protein binds directly to STAT2

We previously reported that both the PNT and VCT regions contribute to the inhibition of IFN-α/β signaling by MV-V. This was demonstrated using a luciferase reporter gene controlled by an artificial promoter sequence containing multiple ISRE copies. We now report similar results with reporter constructs driven by promoter sequences from IFN-stimulated genes ISG54 and ISG56 (Table 1). STAT1 and Jak1 interactions account for the inhibitory activity of the PNT region (Caignard et al., 2007), but how IFN-α/β signaling is impaired by the VCT region remains unknown. Because co-affinity purification

Table 1
MV-V, MV-PNT and MV-VCT regions impair type I IFN signaling

IFN-β with	None	MV-V	MV-PNT	MV-VCT
pISRE-Luc	100	9.3±0.8	55.5±3.9	30.6±3.3
pISG54-Luc	100	1.7±0.0	3.5±0.3	14.4±0.8
pISG56-Luc	100	10.5±0.5	21.0±1.2	27.5±4.4

Transfected cells were stimulated with 1000 IU/ml of IFN-β and expression of luciferase reporter constructs controlled by ISRE repeats (pISRE-Luc) or promoter sequences of ISG54 or ISG56 (pISG54-Luc and pISG56-Luc, respectively) was quantified. Results were normalized so that reporter activity in cells transfected with a control vector equals 100. Experiments were performed in triplicates and data represent means±SD.

experiments previously demonstrated that MV-V induces the formation of a protein complex containing multiple cellular partners including STAT2 (Palosaari et al., 2003), we tested the possibility that the VCT region targets this essential component of IFN-α/β pathway. We first compared the ability of MV-V and MV-P viral proteins to interact with endogenous cellular STAT2 (Fig. 1B). HEK-293T cells were transfected with expression vectors encoding GST alone or fused to MV-V or MV-P proteins. GST-tagged viral proteins were purified with glutathione-sepharose beads and tested for interaction with endogenous STAT2. Cellular STAT2 co-purified with MV-V but not MV-P or GST alone (Fig. 1B). This confirms previous reports showing MV-V and STAT2 in the same protein complex (Palosaari et al., 2003), but also shows the critical role of the VCT region in this interaction. To demonstrate STAT2 binding to the VCT region alone, cells were transfected with either GST fused to PNT or VCT regions in the presence of 3xFLAG-tagged STAT2. The VCT region interacted with STAT2 identically to full-length MV-V, while the PNT region did not (Fig. 1C). To further validate this direct interaction in a heterologous system that does not express other IFN-α/β signaling components, VCT binding to STAT2 was tested in yeast (Fig. 1D). The VCT region fused to Gal4 DNA binding domain specifically interacted with STAT2 fused to Gal4 activation domain in the yeast two-hybrid system (Y2H; Fig. 1D), while the PNT region only interacted with STAT1 as previously reported (Caignard et al., 2007). Altogether, our data demonstrate the direct binding of STAT2 to the VCT region of MV-V, independently of the PNT region and the interaction with STAT1.

Mapping of MV-V binding site to STAT2

In order to characterize STAT2 binding interface on MV-V, we generated a full matrix of VCT overlapping fragments by PCR and tested their ability to interact with STAT2 in the Y2H system (Fig. 2). Both forward and reverse primers were designed every 36 nucleotides along VCT sequence and fused to appropriate sequences to allow gap-repair recombination with linearized Gal4-DB Y2H vector. All possible combinations of forward and reverse primers were used to amplify VCT fragments (Fig. 2A). Finally, corresponding PCR products were transformed in a yeast strain expressing AD-fused STAT2, and growth on selective medium was used to detect potential interactions. A 58 amino acid-long peptide encompassing position 231 to 288 of MV-V was sufficient to bind STAT2 (Fig. 2B). In an iterative process, we then generated a second and a third set of VCT fragments corresponding to one-by-one amino acid deletions (Figs. 2C and D), allowing to further reduce the STAT2 binding motif to a minimal peptide of 49 amino acids (MV-$V_{232-280}$). Because the VCT region alone was previously reported to impair IFN-α/β signaling when expressed in human cells, we tested the minimal MV-$V_{232-280}$ peptide for this activity. HEK293T cells were transfected with an ISRE-luciferase reporter gene in the presence of VCT, MV-V or MV-$V_{232-280}$ and stimulated 24 h later with IFN-β (Fig. 3). IFN-α/β signaling was strongly impaired by MV-V, whereas the inhibition by the VCT region alone was less pronounced as reported before (Caignard et al., 2007). Inhibition of IFN-α/β signaling by MV-$V_{232-280}$ was similar to the VCT region. As shown in Fig. 4A, this peptide extends from the beginning of the VCT region to the end of the

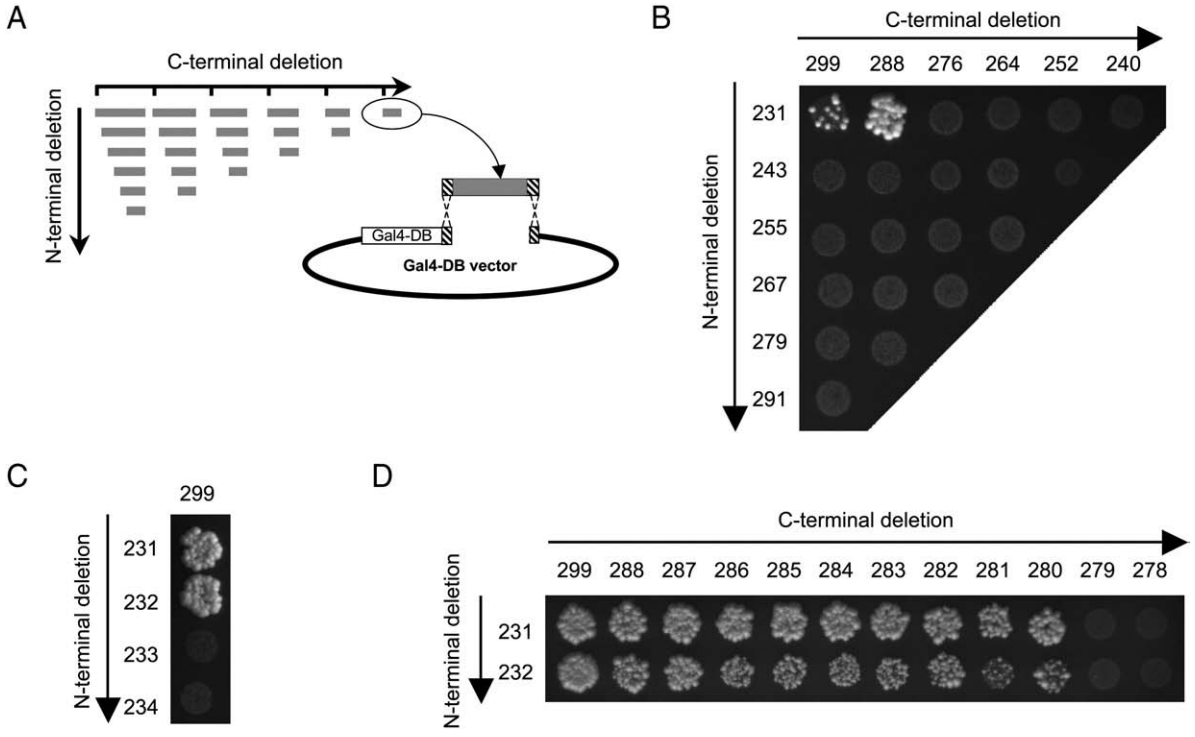

Fig. 2. Yeast two-hybrid mapping of MV-V binding region to STAT2. (A) Systemic deletion-based mapping procedure. Fragments of the VCT region were generated by PCR using a matrix combination of specific primers, and introduced into Gal4-DB vector by gap-repair in yeast cells expressing STAT2 fused to Gal4-AD. (B–D) Yeast cells were grown on selective medium lacking histidine and supplemented with 10 mM of 3-amino-triazole (3-AT) to test the interaction-dependent transactivation of HIS3 reporter gene. Vertical and horizontal axes indicate the first and the last amino acid residues of each fragment tested, respectively. (B–D) correspond to three iterations of this process. (D) The third iteration led to the identification of a 49 amino acid peptide encompassing position 232 to 280 of MV-V as the minimal STAT2 binding peptide.

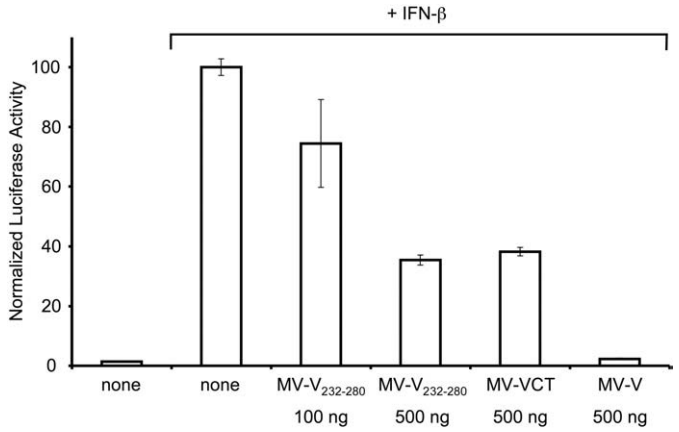

Fig. 3. Inhibition of IFN-α/β signaling by MV-$V_{232-280}$. HEK-293T cells were transfected with pISRE-Luc, pRL-CMV, and expression plasmids encoding either MV-V, the VCT region alone or MV-$V_{232-280}$. 24 h after transfection, 1000 IU/ml of recombinant IFN-β were added. After 24 h, relative luciferase activity was determined. Experiments were performed in duplicates, and data represent means ± SD.

cysteine-rich cluster that is highly conserved among all *Paramyxoviridae* expressing a V protein (Horvath, 2004). In contrast, the last 19 amino acids of MV-V (AA 281–299) were not critically required for the interaction with STAT2.

Using as a template the crystal structure of the V protein of Simian Virus 5 (SV5), another *Paramyxoviridae* (Li et al., 2006), we determined a 3D model of the C-terminal region of MV-V (AA 226–283) using SwissModel (Arnold et al., 2006; Guex and Peitsch, 1997) (Fig. 4B). On this structure prediction, the MV-$V_{232-280}$ peptide overlaps with the zinc-finger fold formed by the cysteine residues of the VCT region (Fig. 4B, left panel). The shorter peptides generated in Fig. 2D were probably unable to properly fold, thereby losing their capacity to bind STAT2. In addition to the well-characterized cysteine cluster, MV-$V_{232-280}$ contains two tryptophan residues separated by 9 amino acids just upstream of the cysteine cluster, respectively at positions 240 and 250 (Fig. 4A). This is reminiscent of a W-$(X)_3$-W-$(X)_9$-W tryptophan motif previously identified at the same position in the V protein of *Rubulaviruses* (Nishio et al., 2002, 2005). These viruses encode a V protein that interacts with STATs to induce their degradation, and the W-$(X)_3$-W-$(X)_9$-W tryptophan motif is required in this process (Nishio et al., 2002, 2005). Interestingly, tryptophan 240 from MV-V corresponds to tryptophan 179 from the V protein of SV5, a residue that is a part of bowl-shaped depression at the surface of the SV5-V protein involved in the interaction with STAT2 (Li et al., 2006). Tryptophan 240 and 250 are exposed at the surface of the VCT structure (Fig. 4B, right panel). We thus tested the consequences of substituting tryptophan by alanine at positions 240 and 250. As shown in Fig. 4C, W240A or W250A mutations strongly impaired VCT binding to STAT2. These mutants were also tested for their ability to affect IFN-α/β signaling using an ISRE-luciferase reporter assay. W240A and W250A mutants were severely affected for their capacity to block IFN-α/β signaling when compared to the wild-type VCT region (Fig. 4D). Thus, tryptophans 240 and 250 are required for the VCT region of MV-V to interact with STAT2 and to block IFN-α/β pathway. As expected, full-length MV-V carrying the same W250A mutation was only partially impaired for its ability to block IFN-α/β signaling (Fig. 4D). Indeed, interactions with STAT1 and Jak1 kinase domain (Jak1-TK) that bind to the PNT region of MV-V were not affected by this mutation and the loss of interaction with STAT2 (Fig. 4E).

Mapping of MV-V binding site to STAT1

The PNT region of MV-V interacts with another STAT protein, *i.e.* STAT1, but does not exhibit a zinc-finger domain like the VCT region. To precisely map STAT1 binding site, we applied the strategy described in Fig. 2A to the PNT region. A 31 amino acid peptide encompassing position 101 to 131 of the PNT region was sufficient to bind STAT1 (Fig. 5A). STAT1 binding motif was further characterized using a second and third set of PNT fragments (Figs. 5B and C) to finally map an 11 amino acid peptide that could not be reduced anymore (MV-$V_{110-120}$). The sequence of this peptide is $_{110}$YHVYDHSGEAV$_{120}$, and corresponds to the minimal portion of MV-V required to mediate an interaction with STAT1 in the yeast two-hybrid system. This peptide is highly conserved among all *Morbilliviruses* (Devaux et al., 2007). Its first residue corresponds to tyrosine 110 that is essential for the interaction between MV-V and STAT1 (Caignard et al. 2007, Fontana et al. 2008). When mutated to histidine in the MV-$V_{110-120}$ peptide, the interaction with STAT1 was lost as expected (Fig. 5C).

To demonstrate the critical role of this peptide in the interaction with STAT1 in human cells, different fragments of MV-V containing or not this peptide were expressed in HEK-293T cells (Fig. 6A). Co-affinity purification experiments showed that a fragment of PNT encompassing amino acid 110 to 231 (Fig. 6B, fragment 1) interacted with STAT1, whereas the same fragment depleted of the MV-$V_{110-120}$ peptide did not (Fig. 6B, fragment 2). Identical results were obtained using larger fragments containing the VCT region (Fig. 6B, fragments 5 and 6), again demonstrating the critical role of the MV-$V_{110-120}$ peptide in the interaction with STAT1. Surprisingly, fragment 3 encompassing amino acids 1 to 120 was inefficient to bind STAT1. This suggested that amino acids 121 to 231 (Fig. 6B, fragments 1 and 5) increase the affinity of the MV-$V_{110-120}$ peptide for STAT1, whereas amino acids 1 to 109 (Fig. 6B, fragment 3) are unable to perform this peculiar function. In agreement with this observation, we were also unable to co-purify STAT1 using the MV-$V_{110-120}$ peptide alone (data not shown). When the MV-$V_{110-120}$ peptide is taken out of its context, its affinity for STAT1 is probably too weak and the interaction cannot be detected by co-purification experiments. However, this interaction between the MV-$V_{110-120}$ peptide alone and STAT1 is detectable in a more sensitive assay like the two-hybrid system (Fig. 5C) (Yang, Wu, and Fields, 1995).

We tested the ability of the MV-$V_{110-120}$ peptide to block IFN-α/β signaling in human cells. First, HEK-293T cells were co-transfected with a plasmid expressing MV-$V_{110-120}$ and an ISRE-luciferase reported construct. Like the PNT region alone (Caignard et al., 2007), inhibition of the luciferase activity by MV-$V_{110-120}$ was significant, although relatively modest when compared to full-length MV-V (Fig. 7A). This demonstrated that in spite of a weak affinity for its cellular target, MV-$V_{110-120}$ could significantly alter IFN-α/β signaling. Then, we synthesized the MV-$V_{110-120}$ peptide in fusion with 8K or Tat_{47-57} cell-penetrating sequences (Tilstra et al., 2007). 8K or Tat_{47-57} cell-penetrating sequences belong to a family of short cationic peptides that are able to carry larger molecules across cellular membranes and were used to transduce MV-$V_{110-120}$. HEK-293T cells were transfected with the ISRE-luciferase construct and treated with synthetic peptides 6 h before the IFN-β stimulation. Treatment of the cells with Tat_{47-57}-MV-$V_{110-120}$ or 8K-MV-$V_{110-120}$ inhibited expression of the ISRE-luciferase gene when compared to cells treated with the MV-$V_{110-120}$ peptide without a cell-penetrating sequence (Fig. 7B). Tat_{47-57} alone was inactive, demonstrating that it was not responsible for the inhibition observed. Moreover, a mutant peptide carrying the Y110H mutation (Tat_{47-57}-MV-$V_{Y110H-120}$) and therefore impaired for its ability to interact with STAT1 was also inactive. Lastly, we analyzed the kinetics of 8K-MV-$V_{110-120}$ peptide activity. Cells transfected with the ISRE-luciferase construct were treated with 2 μg/ml of 8K-MV-$V_{110-120}$ at different time-points before or after IFN-β stimulation (Fig. 7C). The inhibition was maximal when treating the cells 6 h before stimulation. These results establish that an 11-amino acid peptide derived from MV-V protein is sufficient to alter the IFN-α/β signaling pathway when present in the cytoplasm of target cells.

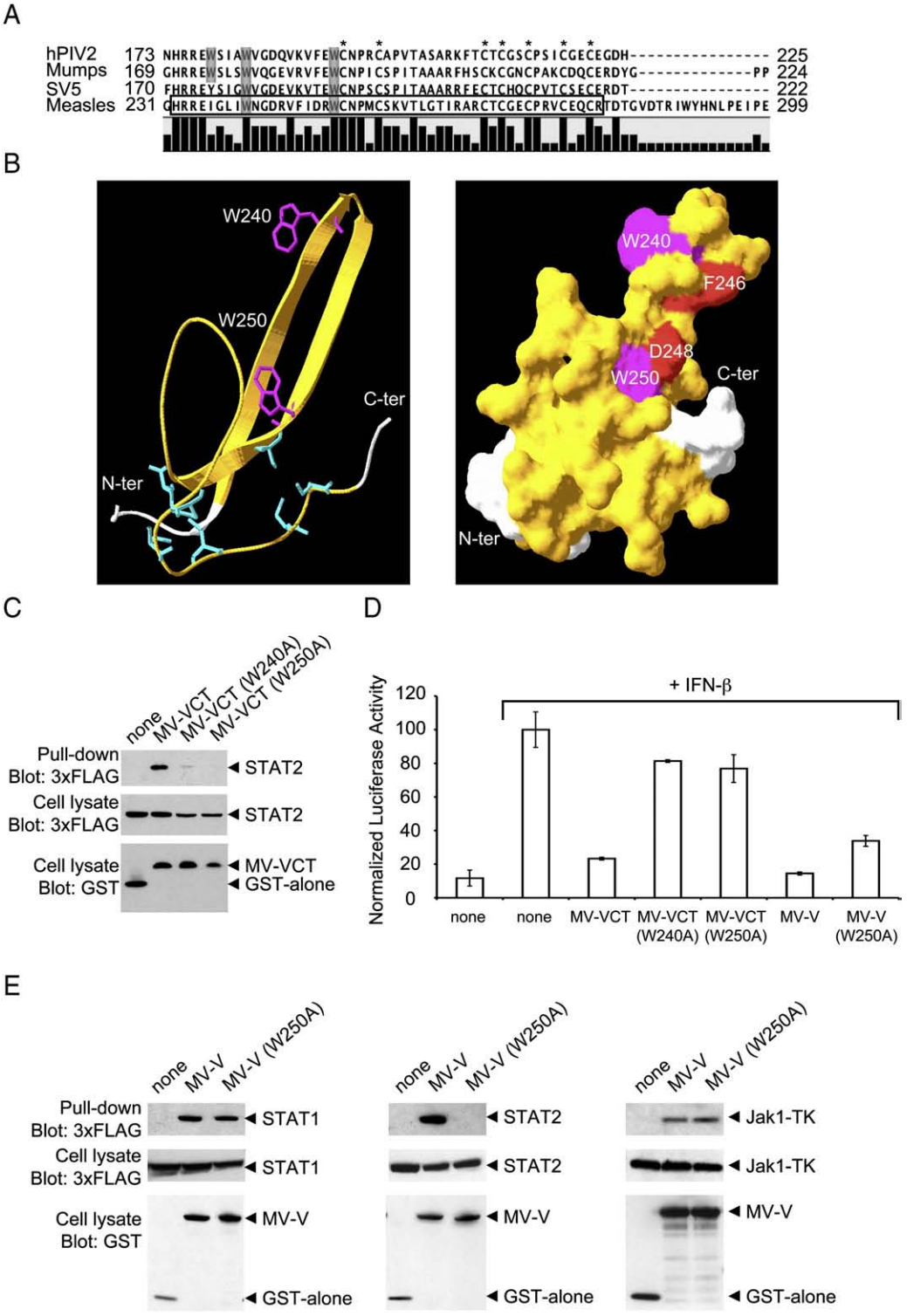

Fig. 4. Two tryptophan residues in the VCT region of MV-V are conserved among *Morbilliviruses* and *Rubulaviruses* and required for the interaction with STAT2. (A) Identification of tryptophan residues conserved upstream of the cysteine cluster in the VCT region of MV-V. Sequences from human parainfluenza virus 2 (hPIV2, NP_598402.1), Mumps virus (NP_054709.1), Simian Virus 5 (SV5, YP_138513.1), and Measles virus (NC_001498.1) were aligned with CLC Workbench 4.0.1. Amino acid conservation rate is indicated by a histogram below the alignment. Conserved tryptophan and cysteine residues are highlighted by shadow boxes and stars, respectively. STAT2 binding peptide MV-$V_{232-280}$ is delimited by a black box. (B) A 3D model of MV C-terminal region (AA 226–283) was determined by SwissModel (Arnold et al., 2006) using the crystallographic structure of V from SV5 as a template (Li et al., 2006). This structure is displayed as a ribbon model (left panel) or a molecular surface (right panel) using SPDB-Viewer (Guex and Peitsch, 1997). Cysteine residues that compose the zinc-finger are shown in turquoise, W240 and W250 in purple, and STAT2 binding peptide in yellow. F246 and D248, identified by others as essential for the interaction with STAT2, are shown in red (Ramachandran et al., 2008). (C) W240A and W250A mutants were deeply affected for the interaction with STAT2. HEK-293T cells were co-transfected with expression vectors encoding 3xFLAG-tagged STAT2 and GST alone or GST fused to wild-type or mutant VCT region. Tagged proteins were detected by immunoblotting using anti-GST or anti-3xFLAG antibodies. (D) W240A and W250A mutants were impaired for their ability to block IFN-α/β signaling when compared to the wild-type VCT region. HEK-293T cells were transfected with pISRE-Luc, pRL-CMV, and expression plasmids encoding wild-type or mutant MV-V or VCT. 24 h after transfection, 1000 IU/ml of recombinant IFN-β were added. After 24 h, relative luciferase activity was determined. Experiments were performed in triplicates, and data represent means ± SD. (E) MV-V mutant W250A interacts with STAT1 and Jak1 but not STAT2. HEK-293T cells were co-transfected with 3xFLAG-tagged STAT1, STAT2 or Jak1 kinase domain (Jak1-TK) and GST fused to wild-type MV-V or mutant with the W250A mutation. After purification of GST-fusion proteins, tagged proteins were detected by immunoblotting using anti-GST or anti-3xFLAG antibodies.

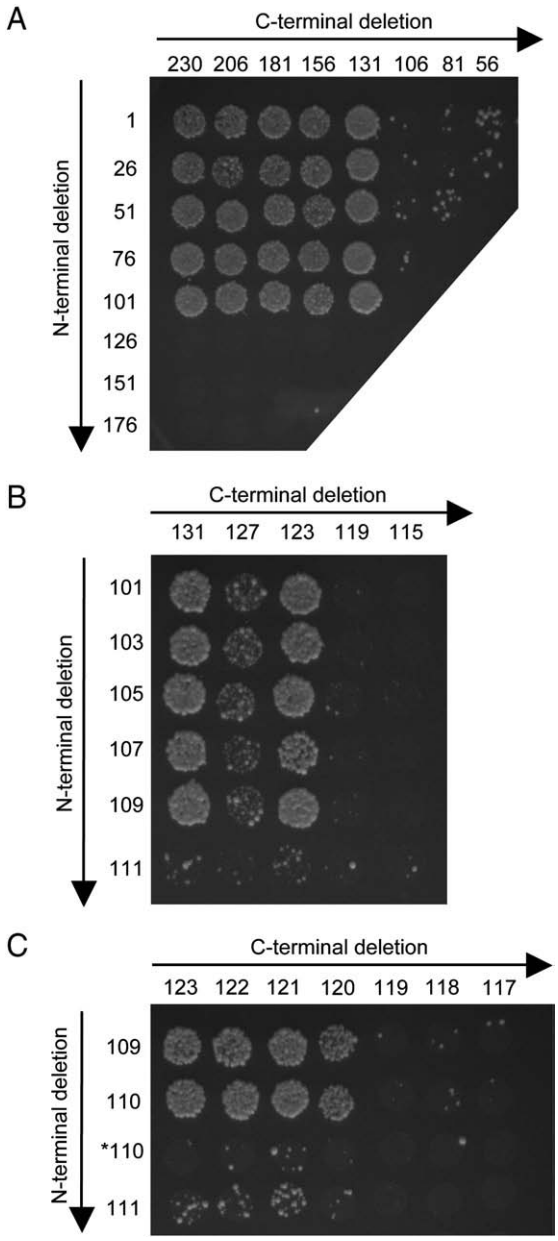

Fig. 5. An 11 amino acids peptide of MV-V is sufficient to bind STAT1. Fragments of PNT region were generated and tested for their ability to interact with STAT1 as described in Fig. 2A. (A) The first iteration identified a STAT1 binding peptide of 31 amino acids. After two (B) and three additional rounds (C), this peptide was finally reduced to 11 amino acids as a minimal STAT1 binding motif. *110 on the vertical axis refers to PNT fragments containing the deleterious Y110H mutation reported to disrupt the interaction with STAT1.

Inhibition of IFN-λ signaling by MV-V

Altogether, our data demonstrate that MV-V not only interacts directly with STAT1 and Jak1 by its PNT region, but also with STAT2 by its VCT region. Interestingly, these three signaling molecules are shared components of IFN-α/β and IFN-λ pathways. Recently identified type III interferons, that include IFN-λ1, IFN-λ2 and IFN-λ3 (also known as IL-29, IL-28A, and IL-28B), have been shown to bind a specific membrane receptor called IFNLR1/IL10R2 which is distinct from IFN-α/β receptor (IFNAR1/IFNAR2c). But IFN-λ requires Jak1, STAT1 and STAT2 to activate interferon-stimulated genes (Uze and Monneron, 2007). Upon IFN-λ stimulation, Tyk2 and Jak1 respectively associated with IL10R2 and IFNLR1 receptor subunits induce ISGF3 formation by phosphorylating STAT1 and STAT2. We thus tested the ability of MV-V to block signaling downstream of IFN-λ. Cells were transfected with an ISRE-luciferase reporter construct in the presence or not of MV-V, then stimulated with IFN-λ1. Signaling was deeply impaired by MV-V (Fig. 8). PNT and VCT regions were also tested for their ability to block IFN-λ signaling. In agreement with results obtained with IFN-α/β, both PNT and VCT regions impaired IFN-λ signaling although less efficiently than full-length MV-V protein. This demonstrates that signaling inhibition by MV-V is not restricted to IFN-α/β pathway as transduction downstream of IFN-λ receptor is efficiently blocked by this viral protein.

Discussion

MV-V is a potent inhibitor of IFN-α/β transduction pathway and critically contributes to the hijacking of cellular antiviral functions by MV. In this report, we have identified STAT2 as a direct target of the VCT region of MV-V (wild-type IC-B strain). Recently, the same observation has been reported by Ramachandran et al. for the Edmonston ATCC strain of MV (Ramachandran et al., 2008). Altogether with STAT1 and Jak1, STAT2 is the third component of the IFN-α/β signaling pathway reported to bind MV-V directly. These three interactions allow MV-V to form a multiprotein complex with IFN-α/β signaling components, and block signaling downstream of IFN-α/β receptor (Palosaari et al., 2003). We also showed that MV-V interferes with IFN-λ signaling, demonstrating that MV-V mediated inhibition is not restricted to IFN-α/β pathway and IFNAR1/IFNAR2c receptor complex. This supports a critical role of MV-V interactions with Jak1, STAT1 and STAT2 in the inhibition of both IFN-α/β and IFN-λ pathways.

MV-V interaction with STAT2 is carried by the zinc-finger domain of the VCT region (AA 232-280), but does not rely on the last 19 residues of MV-V (Fig. 4A) in agreement with (Ramachandran et al. 2008). This minimal STAT2 binding peptide contains the cysteine cluster that is a

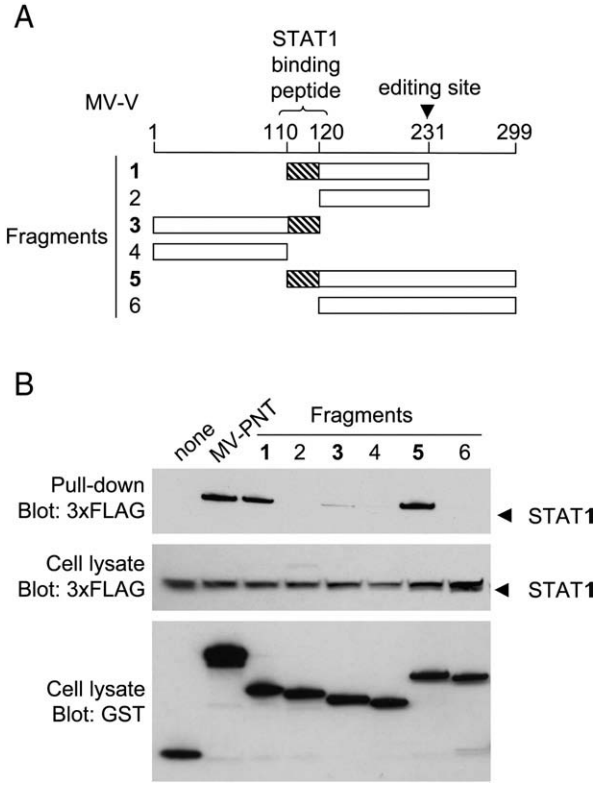

Fig. 6. MV-V requires the MV-$V_{110-120}$ peptide to interact with STAT1 in human cells. (A) Different fragments of MV-V were designed with or without STAT1 binding peptide. (B) HEK-293T cells were transfected to co-express GST fused to MV-V fragments and 3xFLAG-tagged STAT1. Tagged proteins were detected by immunoblotting using anti-GST or anti-3xFLAG antibodies.

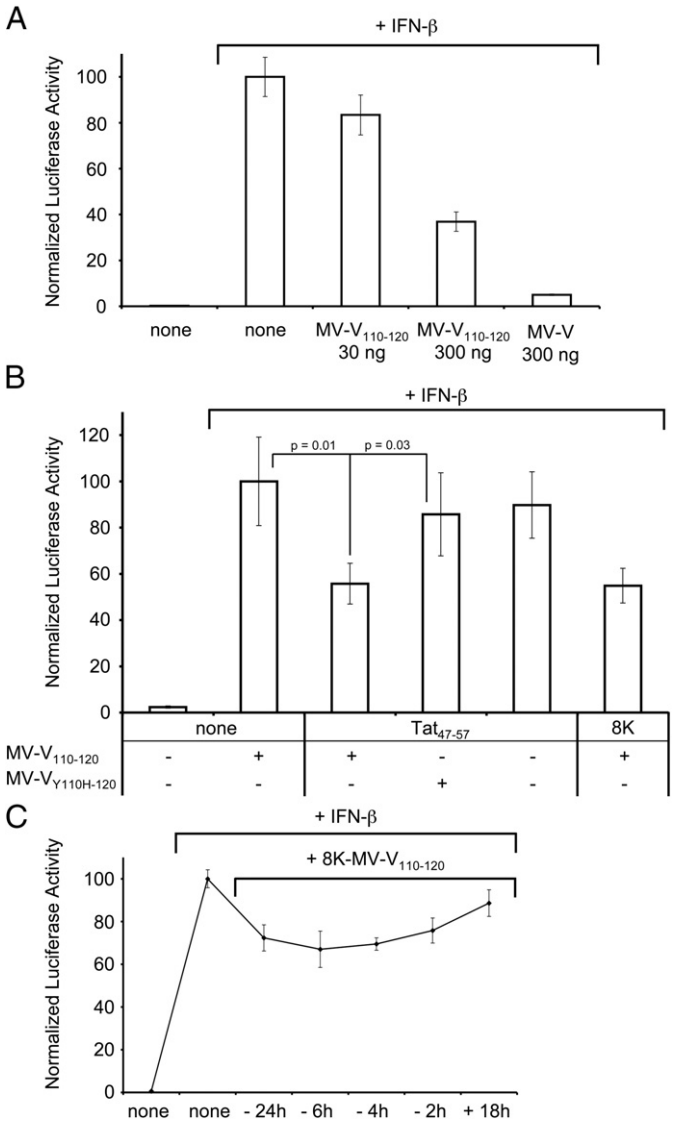

Fig. 7. Inhibition of IFN-α/β signaling by STAT1 binding peptide MV-$V_{110-120}$. (A) HEK-293T cells were transfected with pISRE-Luc, pRL-CMV, and expression plasmids encoding MV-V or MV-$V_{110-120}$. 24 h after transfection, 1000 IU/ml of recombinant IFN-β were added. After 24 h, relative luciferase activity was determined. Experiments were performed in triplicates, and data represent means ± SD. (B) HEK-293T cells were transfected with pISRE-Luc and pRL-CMV. 24 h later, cells were treated with 5 μg/ml of synthetic peptides corresponding to MV-$V_{110-120}$, the cell-penetrating sequence of Tat alone (Tat_{47-57}), MV-$V_{110-120}$ fused to Tat_{47-57} (Tat_{47-57}-MV-$V_{110-120}$), the same peptide carrying the Y110H mutation (Tat_{47-57}-MV-$V_{Y110H-120}$) or MV-$V_{110-120}$ fused to the cell-penetrating sequence KKKKKKKK (8K-MV-$V_{110-120}$). After 6 h, cells were stimulated with 1000 IU/ml of recombinant IFN-β. After 24 h, relative luciferase activity was determined. Experiments were performed in triplicates, and data represent means ± SD. *P*-values were calculated by the Student's *t*-test (C) The same experiment was performed with 2 μg/ml of 8K-MV-$V_{110-120}$ synthetic peptide. Cells were treated at 24 h, 6 h, 4 h, 2 h before the stimulation with IFN-β or 18 h later.

characteristic signature of the V proteins of *Paramyxoviridae*. In addition, we showed that tryptophans 240 and 250 are critical for the interaction with STAT2. Localized upstream of the cysteine cluster on the primary sequence, the position of these tryptophan residues is reminiscent of the W-$(X)_3$-W-$(X)_9$-W motif previously involved in the binding of the V protein of *Rubulaviruses* to STATs (Nishio et al., 2002, 2005). This motif may represent a conserved STAT-binding site for *Morbilliviruses* and *Rubulaviruses*. But in contrast to *Morbilliviruses*, STAT2 interaction with V from *Rubulaviruses* seems to require the PNT and VCT regions together (Li et al., 2006; Ramachandran et al., 2008; Ulane et al., 2005). According to the structure prediction currently available, tryptophan 240 and 250 are exposed at the surface of the protein fold defined by the VCT region. Although we cannot speculate on the exposition of these residues in the context of full-length MV-V, they are probably part of the binding interface to STAT2. Two other mutations (F246R and D248F) were also shown to disrupt the interaction with STAT2 (Ramachandran et al., 2008). Altogether with W240 and W250, these four residues exposed at the surface of the VCT region roughly define the STAT2 binding site (Fig. 4B, right panel). In addition to STAT2, the VCT region of MV-V was previously reported to interact with MDA5, thereby inhibiting the induction of IFN-β when stimulating cells with double-stranded RNA (Childs et al., 2007). An interaction with p53 family member p73 was also reported, allowing MV-V to inhibit apoptosis (Cruz et al., 2006). It will be interesting to precisely map the binding sites of MDA5 and p73 at the surface of the VCT region of MV-V, and understand how this relatively small domain has evolved multiple binding interfaces contributing to the fitness of measles virus *in vivo*.

Whereas STAT2 binds to the VCT region, the PNT region mediates MV-V interaction with STAT1 and Jak1. We previously reported that STAT1 and Jak1 binding sites are distinct (Caignard et al., 2007). Indeed, STAT1 does not compete with Jak1 for the interaction with MV-V when co-expressed. Moreover, MV-V carrying the Y110H mutation fails to bind STAT1 but still interacts with Jak1. In this report, we identified an 11 amino acid peptide, MV-$V_{110-120}$, as a minimal STAT1 binding sequence. This is in agreement with a recent report showing that AA 110–130 are required for MV-V interaction with STAT1 (Ramachandran et al., 2008). The MV-$V_{110-120}$ peptide alone was functional: human cells transfected with an expression vector encoding for MV-$V_{110-120}$ or treated with a synthetic peptide fused to a cell-penetrating sequence were significantly impaired for IFN-α/β signaling. Moreover, a mutant peptide carrying the Y110H mutation and therefore impaired for its ability to interact with STAT1 was unable to do so. Because the N-terminal region of MV-V is intrinsically disordered, we expected this kind of linear binding motif (Karlin et al., 2002). However, MV-$V_{110-120}$ affinity for STAT1 was relatively low, and the interaction was only detectable in a sensitive assay like the yeast two-hybrid system (Yang et al., 1995). Interestingly, this interaction was strengthened when MV-$V_{110-120}$ was fused to amino acids 121–231 of MV-V, and became detectable by co-affinity purification. These residues could stabilize the interaction of MV-$V_{110-120}$ with STAT1 by contributing to the interaction-induced folding of the PNT region when contacting STAT1. Another hypothesis is that amino acids 121–231 of MV-V bind Jak1. Because Jak1 and STAT1 interact with each other (Fujitani et al., 1997; Gupta et al., 1996), this could indirectly stabilize MV-$V_{110-120}$ binding to STAT1. Similarly to the STAT2 binding domain described above, the peptidic motif interacting with STAT1 is highly conserved among all

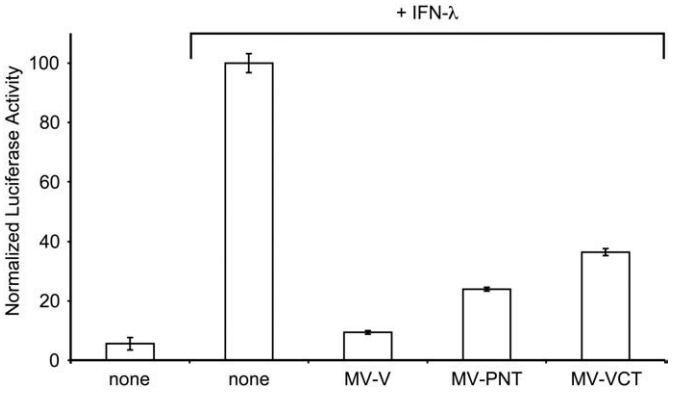

Fig. 8. Inhibition of IFN-λ signaling by MV-V. HEK-293T cells were transfected with pISRE-Luc, pRL-CMV, and expression plasmids encoding MV-V protein, PNT region, or VCT region. 24 h after transfection, 1000 IU/ml of recombinant IFN-λ1 were added. After 24 h, relative luciferase activity was determined. Experiments were performed in triplicates, and data represent means ± SD.

Morbilliviruses, attesting of the positive selection that applies on this short sequence.

In our STAT-binding assays, the PNT and VCT regions of MV-V behaved as autonomous modules. When dissociated, they were still able to interact with STAT1 and STAT2, respectively, and to alter IFN-α/β and IFN-λ signaling. In agreement with this observation, MV-V mutated in the VCT region to disrupt the interaction with STAT2 remained fully competent to recruit cellular STAT1 (Fig. 4E) (Ramachandran et al., 2008). Surprisingly, Ramachandran et al. reported that the PNT region expressed alone is significantly impaired for its ability to co-purify with endogenous STAT1. This could be explained if the VCT region, in addition to the interaction with STAT2, also contributes to the proper folding of the PNT region, and thereby stabilizes the PNT interaction with STAT1 when this cellular factor is expressed at low levels. Altogether, the identification of two STAT-binding sites in MV-V provides molecular basis to a previous report showing that two deleterious mutations, one in the PNT region (Y110H) and the other in the VCT region (C272R), are required to totally abrogate the inhibitory capacity of MV-V when tested against IFN-α/β (Ohno et al., 2004). Both STAT1 and STAT2 binding interfaces must be disrupted to abrogate the inhibitory activity of MV-V. It also explains why, in contrast to IFN-α/β, a single mutation in the PNT region at position 110 is sufficient to prevent MV-V from inhibiting the IFN-γ pathway (Fontana et al., 2008). Indeed, IFN-γ only relies on STAT1 homodimers to transduce, and the VCT interaction with STAT2 is needless in this context.

Finally, it was recently shown by others that in the absence of STAT2, MV-V interaction with STAT1 is greatly impaired, whereas MV-V mutants that fail to interact with STAT2 still efficiently bind STAT1 (Ramachandran et al., 2008). Because STAT1 and STAT2 are bound together in resting cells, this interaction is probably required for the proper folding of STAT1 and its interaction with the PNT region. Thus, STAT1–STAT2 interaction also contributes to the overall stability of the STAT1/STAT2/MV-V heterotrimer, freezing these signaling molecules in a complex where they are trapped. In the future, the precise structure of MV-V when interacting with STAT1, Jak1 and STAT2 will need to be determined. The identification of MV-V binding sites for STAT1 and STAT2 will be instrumental to reach this goal.

Materials and methods

ORF cloning, plasmid constructs and synthetic peptides

All viral constructs were amplified by standard PCR (ExTaq; Takara) method, and cloned into pDONR207 (Invitrogen) using an *in vitro* recombination-based cloning system (Gateway system; Invitrogen) as previously described (Caignard et al., 2007). ORFs encoding full-length MV-V, the PNT domain (AA 1–231) and the VCT domain (AA 232–299) were amplified from an infectious clone of IC-B strain kindly provided by Dr K. Takeuchi (University of Tsukuba, Japan; Genbank ID: NC001498)(Takeda et al., 2000). STAT2 coding sequence was amplified from a human spleen cDNA library (Invitrogen) before cloning into pDONR207 (Invitrogen). Plasmids containing STAT1 or Jak1 kinase domain (Jak1-TK) were previously described (Caignard et al., 2007). Tryptophan to alanine mutations at positions 240 and 250 of the VCT region of MV-V were introduced by site-directed mutagenesis (QuikChange® Lightning kit, Stratagene), then sequence verified. Subsequently, viral or cellular ORFs were transferred by *in vitro* recombination from pDONR207 in different Gateway-compatible destination vectors (see below) following manufacturer's recommendation (LR cloning reaction, Invitrogen). Synthetic peptides corresponding to the MV-$V_{110–120}$ sequence fused either to 8 lysine residues (8K-MV-$V_{110–120}$: KKKKKKKKGGYHVYDHSGEAV) or to the $Tat_{47–57}$ cell-penetrating sequence ($Tat_{47–57}$-MV-$V_{110–120}$: YGRKKRRQRRRGGYHVYDHSGEAV) were synthesized by Genecust (France). The cell-penetrating domain of Tat from Human Immunodeficiency Virus ($Tat_{47–57}$) was the first transduction peptide identified, whereas 8K is an optimal sequence selected by Mai et al. to transduce epithelial cell lines in culture. Both peptides labeled with Alexa Fluor 488 were able to transduce more than 99,7% of treated cells lines (Mai et al., 2002).

Co-affinity purification experiments

To perform co-affinity purification experiments, cloned ORFs were transferred from pDONR207 to pDEST27 expression vector (Invitrogen) to achieve GST fusion, and to pCI-neo-3xFLAG vector (Mendoza et al., 2006) for 3xFLAG-fusion. Tagged proteins were expressed in human HEK-293T cells maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen) containing 10% fetal bovine serum, penicillin, and streptomycin at 37 °C and 5% CO_2. Cell transfections were achieved using Lipofectamine 2000 (Invitrogen). Briefly, 5×10^5 HEK-293T cells were dispensed in each well of a 6-well plate, and transfected 24 h later with 600 ng of each plasmid DNA per well. Two days after transfection, HEK-293T cells were washed in phosphate-buffered saline (PBS), then resuspended in lysis buffer (0.5% Nonidet P-40, 20 mM Tris–HCl at pH=8, 120 mM NaCl and 1 mM EDTA) supplemented with Complete Protease Inhibitor Cocktail (Roche). Cell lysates were incubated on ice for 20 min, then clarified by centrifugation at 14,000 ×*g* for 10 min. For pull-down analysis, 400 µg of protein extracts were incubated for 1 h at 4 °C with 25 µl of glutathione-sepharose beads (Amersham Biosciences) to purify GST-tagged proteins. Beads were then washed 3 times in ice-cold lysis buffer and proteins were recovered by boiling in denaturing loading buffer (Invitrogen). Purified complexes and protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12% NuPAGE Bis–Tris gels with MOPS running buffer (Invitrogen), and transferred to a nitrocellulose membrane. 3xFLAG- and GST-tagged proteins were detected using standard immunoblotting techniques. Membranes were blotted with a mouse monoclonal HRP-conjugated anti-3xFLAG antibody (M2; Sigma-Aldrich) or a rabbit polyclonal anti-GST antibody (Sigma-Aldrich). Endogenous STAT2 was revealed using clone-22 (mouse monoclonal antibody; BD Biosciences). Secondary anti-mouse and anti-rabbit HRP-conjugated antibodies were from GE-Healthcare and Amersham, respectively.

Yeast two-hybrid procedure

Yeast culture mediums were prepared as previously described (Walhout and Vidal, 2001). STAT1 and STAT2 were transferred by *in vitro* recombination from pDONR207 in a Gateway-compatible pPC86 yeast expression vector (Invitrogen) to be fused with the activation domain of Gal4 (AD-Gal4). These constructs were transformed in AH109 yeast strains (Clontech) expressing either the PNT or VCT region of MV-V fused to the DNA binding domain of Gal4 (Gal4-DB; pDEST32 vector; Invitrogen). Yeast cells were plated on a selective medium lacking histidine and supplemented with 10 mM 3-amino-triazole (3-AT; Sigma-Aldrich) to test the interaction-dependent transactivation of HIS3 reporter gene.

To perform the refined mapping of PNT or VCT binding sites to STAT1 or STAT2, we used the gap-repair procedure (Walhout and Vidal, 2001). First, both forward and reverse PCR primers were designed every 75 nucleotides along the PNT sequence, or every 36 nucleotides along the VCT sequence. These 20 to 25 bp long primers were fused to specific tails allowing recombination in the Gal4-DB Y2H vector. Forward and reverse primers were fused to the following sequence tails, respectively: 5′-GAAGAGAGTAGTAACAAAGGTCAAAGACAGTTGACTGTATCGTCGAGGatg-3′ 5′-CCGCGGTGGCGGCCGTTACTTACTTAGAGCTCGACGTCTTACtta-3′.

Matrix combinations of forward and reverse primers were used to amplify PNT and VCT fragments by PCR. AH109 yeast cells expressing AD-fused STAT1 or AD-fused STAT2 were co-transformed with 5 µL of each PCR product in the presence 50 ng of linearized pDEST32 vector to achieve recombinatorial cloning by gap-repair. PNT or VCT fragments fused to Gal4-DB were then tested for interaction with AD-STAT1 or

AD-STAT2 by plating yeast cells on selective medium lacking histidine (−His medium) and supplemented with 10 mM of 3-AT.

Luciferase reporter gene assay

Luciferase reporter plasmid pISRE-Luc was from Stratagene. We thank Dr. Sandra Pellegrini (Institut Pasteur) for providing pISG54-Luc made by David E. Levy (Bluyssen et al., 1994). We thank Eliane Meurs (Institut Pasteur) for providing pISG56-Luc made by John Hiscott (Grandvaux et al., 2002). HEK-293T cells were plated in 24-well plates (2×10^5 per well). One day later, cells were transfected with pISRE-Luc, pISG54-Luc or pISG56-Luc reporter plasmid (0.3 μg/well), pRL-CMV reference plasmid that harbored a CMV promoter just upstream of the *Renilla* luciferase gene (0.03 μg/well; Promega), and specified ORFs cloned in pIRES2-DsRed2 vector (Clontech; 0.3 μg/well unless specified otherwise). Transfection efficiency was assessed under the microscope using dsRed protein fluorescence. 24 h after transfection, cells were stimulated with IFN-β (Biosource) or IFN-λ (R&D Systems) at 1000 IU/ml. After 24 h, cells were lysed, and both firefly and *Renilla* luciferase activities in the lysate were determined using the Dual-luciferase Reporter Assay System (Promega). Reporter activity was calculated as the ratio of firefly luciferase activity to reference *Renilla* luciferase activity, and normalized so that positive control activity equals 100.

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