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Measles virus V protein blocks Jak1-mediated phosphorylation of STAT1 to escape IFN-α/β signaling

Grégory Caignard [a], Mathilde Guerbois [a], Jean-Louis Labernardière [a], Yves Jacob [b], Louis M. Jones [c], The Infectious Mapping Project I-MAP [a,b,e], Fabian Wild [d], Frédéric Tangy [a,*], Pierre-Olivier Vidalain [a]

[a] *Laboratoire de Génomique Virale et Vaccination, CNRS URA 3015, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France*
[b] *Unité Postulante de Génétique, Papillomavirus et Cancer Humain, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France*
[c] *Groupe Logiciels et Banques de Données, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France*
[d] *INSERM U404-Immunity and Vaccination, IFR128-BioSciences, 21 Avenue Tony Garnier, Lyon-Gerland, 69365 Lyon Cedex 07, France*
[e] *INSERM U503-Immunobiologie Fondamentale et Clinique, IFR128-BioSciences, 21 Avenue Tony Garnier, Lyon-Gerland, 69365 Lyon Cedex 07, France*

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Abstract

Viruses have evolved various strategies to escape the antiviral activity of type I interferons (IFN-α/β). For measles virus, this function is carried by the polycistronic gene *P* that encodes, by an unusual editing strategy, for the phosphoprotein P and the virulence factor V (MV-V). MV-V prevents STAT1 nuclear translocation by either sequestration or phosphorylation inhibition, thereby blocking IFN-α/β pathway. We show that both the N- and C-terminal domains of MV-V (PNT and VCT) contribute to the inhibition of IFN-α/β signaling. Using the two-hybrid system and co-affinity purification experiments, we identified STAT1 and Jak1 as interactors of MV-V and demonstrate that MV-V can block the direct phosphorylation of STAT1 by Jak1. A deleterious mutation within the PNT domain of MV-V (Y110H) impaired its ability to interact and block STAT1 phosphorylation. Thus, MV-V interacts with at least two components of IFN-α/β receptor complex to block downstream signaling.

Keywords: Measles; Virus; V; IFN-α/β; Jak1; STAT1

Introduction

Interferons α and β (IFN-α/β) are essential mediators of the innate immune response against viruses (Stetson and Medzhitov, 2006; van Boxel-Dezaire et al., 2006). IFN-α/β signal through direct binding to a cell surface receptor composed of two transmembrane subunits, IFNAR1 and IFNAR2c (Darnell et al., 1994). To transduce signals, this receptor relies on two members of the Janus tyrosine kinase (JAK) family, Tyk2 and Jak1, which constitutively bind IFNAR1 (interferon-α receptor 1 subunit) and IFNAR2c, respectively (Muller et al., 1993; Velazquez et al., 1992). Jak1 and Tyk2 sequences exhibit seven regions, JH1 to JH7, that are conserved among members of the Jak family. Although their functions are not yet fully understood, JH1 is known to encode the kinase domain, JH2 represents a pseudokinase domain, and the amino-terminal domains JH3 to JH7 have been implicated in receptor association (Kisseleva et al., 2002). Two members of the signal transducer and activator of transcription (STAT) family, STAT1 and STAT2, are also essential components of this pathway (Stark et al., 1998). Six conserved domains have been identified through structural and functional analysis of STAT proteins, including a DNA-binding region, a SRC homology 2 (SH2) domain that ensures interaction with phosphorylated tyrosines, and a transcriptional activation domain (Kisseleva et al., 2002). Upon binding of IFN-α/β to its receptor, activated Jak1 and Tyk2 phosphorylate IFNAR1/2c cytoplasmic tails on tyrosine residues. These phos-

* Corresponding author. Fax: +33 1 40 61 31 67.
E-mail address: ftangy@pasteur.fr (F. Tangy).

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phorylation events allow the sequential recruitment of STAT2 to IFNAR1, its phosphorylation by Jak kinases, and the recruitment of STAT1 to phosphorylated STAT2 (Li et al., 1997). Phosphorylation of STAT1 finally triggers STAT1-STAT2 heterodimerization via SH2-phosphotyrosine interactions, and the release of this complex from the receptor. Together with the STAT-associated factor IRF9, STAT1 and STAT2 assemble into a heterotrimeric complex known as the IFN-stimulated gene factor 3, *ISGF3* (Levy et al., 1989). ISGF3 accumulates in the nucleus where it binds conserved IFN-stimulated response element (ISRE) sequences, and stimulates the expression of an antiviral gene cluster.

Paramyxoviruses have evolved the ability to block the induction of these genes through interference with IFN-α/β signaling pathway (Horvath, 2004). A virus-encoded protein called V is a major player of interferon evasion by Paramyxoviruses from *Rubulavirus*, *Henipavirus*, *Respirovirus* and *Morbillivirus* genera (Fields et al., 2001). All these viruses share common structural and genetic elements. Their genome is a single-strand RNA molecule that encodes six structural proteins. While hemagglutinin (H) and fusion (F) are transmembrane glycoproteins of the envelop, the nucleoprotein (N), the phosphoprotein (P), the viral polymerase (L) and the matrix protein (M) are internal to viral particles. The P locus also encodes nonstructural virulence factors, in particular V and C proteins (Bellini et al., 1985; Cattaneo et al., 1989). The C protein is encoded by an opened reading frame embedded in the first half of the P cistron. Moreover, an unusual editing strategy allows the insertion of non-templated guanine nucleotide at a specific position of P-encoded mRNAs (Fig. 1A). Thus, approximately half of P-encoded mRNAs are edited and encode for V, a protein similar to P in its amino-terminal part (PNT domain), but with a unique cysteine-rich C-terminus (VCT) (Cattaneo et al., 1989). Paramyxoviridae V proteins interfere with IFN-α/β signaling through interaction with STATs, but each genus within the family exhibits specific mechanisms of inhibition. *Rubulaviruses*, including mumps virus, SV5 and human parainfluenza virus type 2 (HPIV2), are able to recruit the cellular DDB1-Cul4A-Roc1 E3 complex to promote the rapid ubiquitination and degradation of the STAT proteins (Didcock et al., 1999; Parisien et al., 2001, 2002; Ulane et al., 2005; Yokosawa et al., 2002). *Henipavirus* V proteins sequester STAT1 and STAT2 in high-molecular-mass cytoplasmic complexes without inducing their degradation (Rodriguez and Horvath, 2004). Similarly, the V protein of measles virus (MV-V), the prototype of *Morbillivirus* genus, does not degrade STAT1 and 2, but effectively prevents their nuclear import (Palosaari et al., 2003). Whether MV-V only sequesters STAT1/2 in the cytoplasm, or also inhibits their phosphorylation after IFN-α/β stimulation remains controversial (Palosaari et al., 2003; Takeuchi et al., 2003; Yokota et al., 2003). Immunoprecipitation experiments performed on MV-infected cell using antibodies against IFNAR1 and RACK1, a scaffold protein interacting with IFNAR2c and latent STAT1, indicated that IFN-α/β receptor forms a tight complex containing the C and V proteins (Yokota et al., 2003). Other affinity purification experiments demonstrated that tagged MV-V associates with several cellular partners in a protein complex that includes at least STAT1, STAT2, STAT3, and IRF9 (Palosaari et al., 2003).

The Edmonston infectious clone (Ed-tag; Radecke et al., 1995) encodes an attenuated measles virus that fails to induce a protective immune response in monkeys (Combredet et al., 2003). The MV-V protein encoded by Ed-Tag virus does block IFN-α/β signaling, and two deleterious mutations, Y110H and C272R (Fig. 1A), were mapped within the PNT and the VCT domains, respectively (Ohno et al., 2004; Radecke et al., 1995).

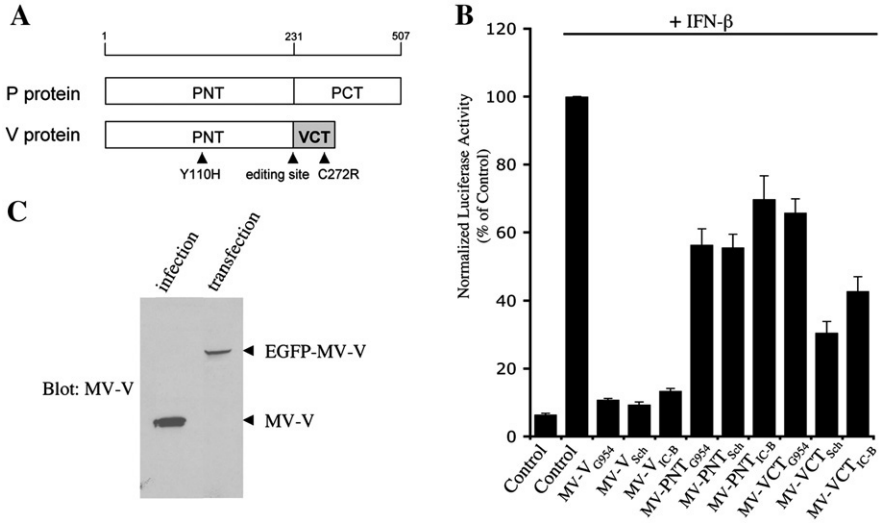

Fig. 1. (A) Diagrams of MV-P and MV-V. The position of the RNA editing site, and mutations Y110H and C272R that are characteristic of Ed-Tag infectious clone are indicated. (B) Inhibition of IFN-β signaling by MV-V proteins depends both on the PNT and the VCT domains. HEK-293T cells were transfected with pISRE-Luc, pRL-CMV, and pEGFP-C1 expression plasmids encoding indicated proteins. 24 h after transfection, 1000 IU/ml of recombinant IFN-β was added. After 24 h of incubation, the relative luciferase activities of IFN-treated cells were determined. Experiments were performed in triplicates, and data represent means ± SD. (C) Expression levels of MV-V protein in transfected or infected HEK-293T cells. Cells were transfected with 1 μg/well of pEGFP-C1 plasmid encoding MV-V_{Sch} or infected with 1 MOI of Schwarz virus then incubated for 24 h and 48 h, respectively. After cells lysis, MV-V expression level was analyzed by Western blot.

Both mutations are required to abrogate MV-V activity, suggesting that the two domains of MV-V can independently affect IFN-α/β signaling. So far, it has not been demonstrated that an isolated VCT domain inhibits IFN-α/β signaling, but the PNT domain was clearly shown to do so unless the Y110H mutation is introduced (Ohno et al., 2004). Interestingly, it was shown that MV-P, that shares the PNT domain with MV-V, can also prevent STAT phosphorylation and translocation to the nucleus, thanks to its amino-terminal domain (Devaux et al., 2006). In the present study, we investigated the molecular mechanisms of IFN-α/β signaling inhibition by MV-V protein and its domains. Identification of direct cellular targets by combined yeast two-hybrid and co-affinity purification was achieved, leading to the identification of STAT1 and Jak1 as interactors of MV-V. We further demonstrated that MV-V, or the PNT domain alone, can block the direct phosphorylation of STAT1 by Jak1. Interestingly, Y110H mutation was shown to impair MV-V inhibitory function, and specifically prevent interaction with STAT1, accounting for the loss-of-function phenotype of this mutant.

Results

Both the PNT and VCT domains of MV-V contribute to the inhibition IFN-α/β signaling

MV-V from Edmonston-ATCC or IC-B strains, but not Edtag, inhibits the transactivation of ISRE-luciferase reporter genes by IFN-α (Devaux et al., 2006; Ohno et al., 2004). Using a similar reporter assay, we show that MV-V from Schwarz vaccine and wild-type strains IC-B and G954 behaves accordingly, and strongly inhibits the transactivation of the ISRE-luciferase reporter gene by IFN-β (Fig. 1B). MV-V protein was expressed by transient transfection of a CMV-driven plasmid and its expression level was similar to that observed during MV infection. Therefore, it can be considered as physiological (Fig. 1C). A combination of two mutations specific of MV-V from Edtag was shown to impair its function: Y110H in the PNT domain and C272R in the VCT region (Ohno et al., 2004). Both mutations are required to abrogate MV-V activity, suggesting that the two domains independently contribute to the inhibition of IFN-α/β signaling. Thus, we tested separately the PNT and VCT domains of MV-V proteins in our reporter assay (Fig. 1B). Inhibition of IFN-α/β pathway was observed with the PNT domain alone as previously reported (Ohno et al., 2004), but was only partial when compared with full-length MV-V protein. Surprisingly, the VCT domains of Schwarz, IC-B and G954 strains also inhibited IFN-α/β signal transduction. This observation should be related to the reported interaction of the VCT domain of mumps virus V protein with RACK1, a key adaptor that recruits STAT1 to IFN-α/β receptor (Kubota et al., 2002). These results suggest that both the PNT and VCT domains of MV-V participate to the inhibition IFN-α/β signaling through interactions with cellular targets.

Yeast two-hybrid screens identified Jak1 and STAT1 as MV-V interacting proteins

To identify cellular partner(s) of MV-V protein, a human spleen cDNA library was screened by yeast two-hybrid using full-length MV-V proteins and corresponding PNT and VCT domains as baits. Proteins from the two wild-type (IC-B and G954), and one vaccine (Schwarz) strains were screened in parallel experiments. Full-length MV-V proteins from G954 and Schwarz and the PNT domain from IC-B identified STAT1 as an interactor in this system (11, 2 and 12 hits, respectively). In all cases, only cDNAs corresponding to full-length STAT1 were isolated. In addition, Jak1 kinase was isolated when screening with MV-V proteins from Schwarz and IC-B, and the PNT domain from IC-B (1, 1 and 3 hits, respectively). In all cases, cDNAs recovered corresponded to the tyrosine kinase domain of Jak1 (Jak1-TK, AAs 894-1154). To confirm these interactions, MV-V from all three strains and corresponding domains were retested in a pairwise setting against STAT1 or Jak1-TK (Fig. 2 and data not shown). MDA5, a known interactor of the VCT domain (Childs et al., 2007) that we also identified in our screens, was used as a positive control. A specific interactor of the phosphoprotein C-terminus, PIRH2, was used as a negative control (Chen et al., 2005). As shown in Fig. 2, MV-V and the PNT domain but not the VCT domain retested against STAT1 and Jak1-TK. In agreement with literature, MDA5 was found to bind MV-V or the VCT domain but not the PNT domain, while

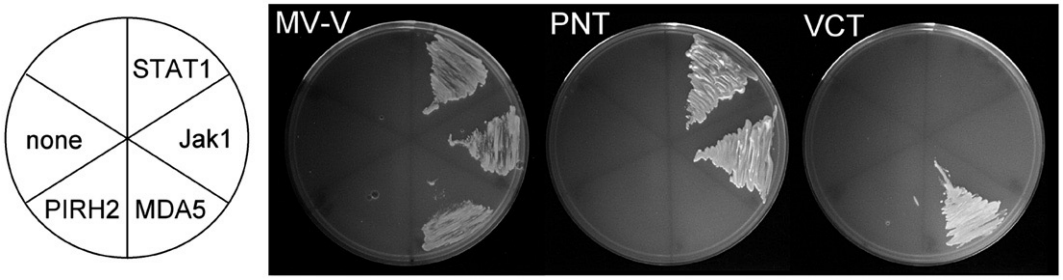

Fig. 2. MV-V interaction with STAT1 and Jak1 is mediated by the PNT domain of MV-V. Interaction of MV-V or its domains (PNT and VCT) with STAT1, Jak1, MDA5 or PIRH2 was analyzed in the yeast two-hybrid system. Yeast cells expressing MV-V from IC-B strain fused to the Gal4 DNA-binding domain (BD) were co-transformed by gap-repair (see Materials and methods) with a plasmid encoding the Gal4 transactivation domain (AD) fused to indicated cellular prey proteins (left panel). Because MV-V is a strong self-transactivator, interaction-dependent expression of *HIS3* reporter gene was tested on a growth medium supplemented with 100 mM 3-AT. Same experiments were performed with the PNT and the VCT domains of MV-V from IC-B strain. Because these two domains are not autonomous transactivators, yeast cells were plated on a growth medium supplemented with only 10 mM 3-AT to test interactions.

PIRH2 did not interact with MV-V or its domains. MV-V proteins of all three strains interacted similarly with STAT1 and Jak1-TK regardless of the viral strain tested (data not shown). The fact that STAT1 and Jak1-TK were not originally isolated with all MV-V proteins and PNT domains is inherent to the high false-negative rate of the two-hybrid system (Ito et al., 2001). These data suggest that MV-V interacts directly with STAT1 and Jak1, two major components of IFN-α/β signaling pathway, and that AAs 1–231 of MV-V that define the PNT domain are sufficient to mediate these interactions. Identification of the cellular proteins targeted by the VCT domain of MV-V to block IFN-α/β signaling will require additional investigations.

MV-V interacts with STAT1 and Jak1 in mammalian cells

To verify interactions identified by yeast two-hybrid in mammalian cells, we performed co-affinity purification experiments. First, HEK-293T cells were transfected with MV-V fused to glutathione-*S*-transferase (GST-MV-V) and 3xFLAG-tagged STAT1 (Fig. 3A). Interaction of MV-V with STAT1 was confirmed in this system (Fig. 3A), and binding efficiency to STAT1 was equivalent for all three strains, regardless of their wild-type (IC-B and G954) or vaccine (Schwarz) status. Similar experiments were performed to demonstrate that MV-V interacts with Jak1. However, non-specific binding of Jak1 to sepharose

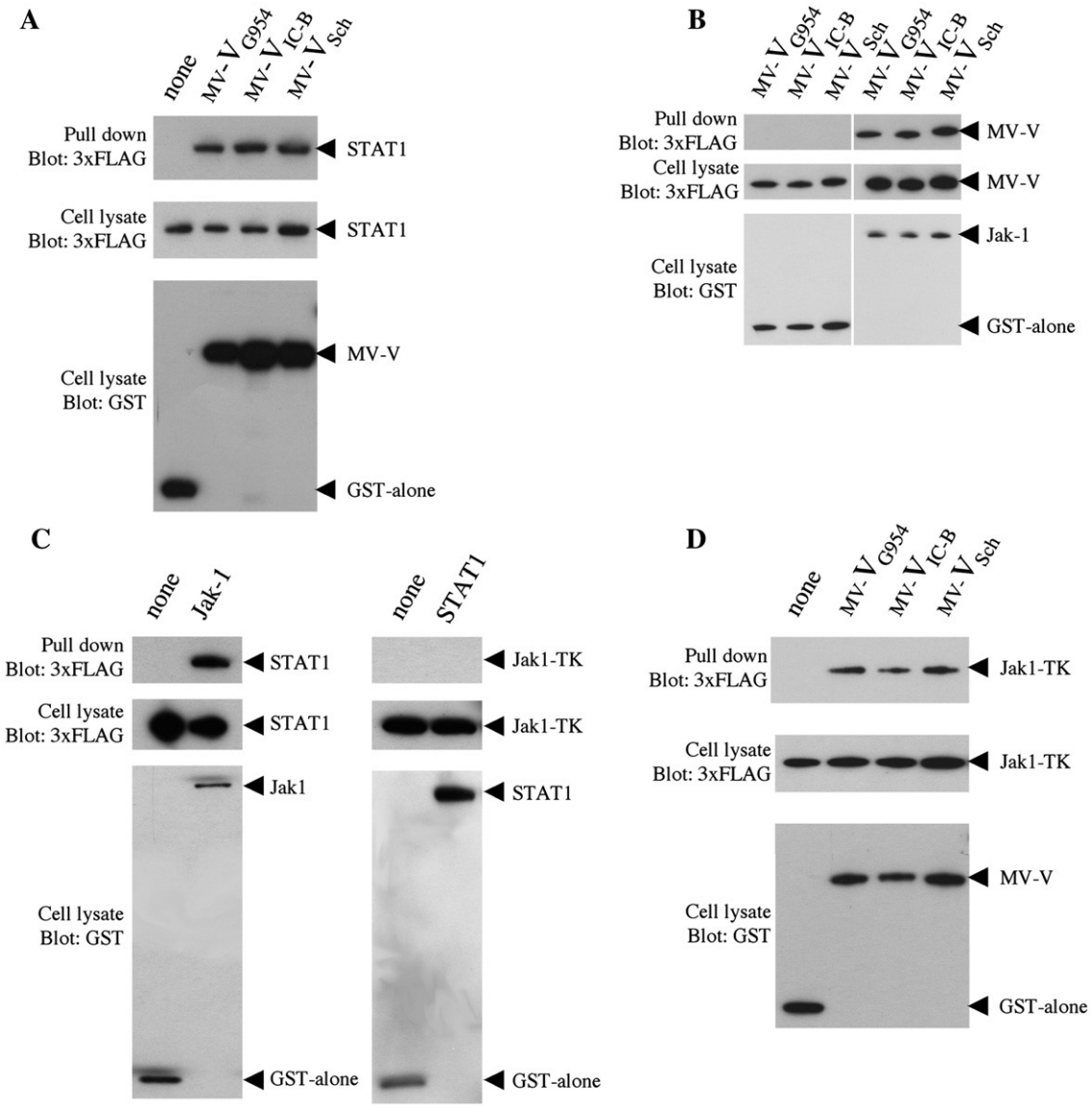

Fig. 3. MV-V proteins from wild-type and vaccinal MV strains interact with Jak1 and STAT1. (A) HEK-293T cells were co-transfected with expression vectors encoding GST alone or fused to MV-V proteins from G954 (MV-V_{G954}), IC-B (MV-$V_{IC\text{-}B}$) or Schwarz strain (MV-V_{Sch}), and either 3xFLAG-STAT1. Total cell lysates from transfected cells were prepared 48 h post-transfection (cell lysate; middle and lower panels), protein complexes were assayed by pull-down using glutathione-sepharose beads (GST pull-down; upper panel). 3xFLAG- and GST-tagged proteins were detected by immunoblotting. (B) Similar experiment, but HEK-293T cells were co-transfected with GST alone or GST-Jak1, and 3xFLAG-tagged MV-V proteins. (C) Same experiment but cells were co-transfected with GST alone, GST-Jak1 and 3xFLAG-tagged STAT1 (left panel), or GST alone, GST-STAT1 and 3xFLAG-tagged Jak1-TK (right panel). (D) Similar experiment, but HEK-293T cells were co-transfected with GST alone or fused to indicated MV-V proteins, and 3xFLAG-tagged Jak1-TK.

beads was observed. This technical issue forced us to change the two protein tags, and perform experiments with the GST and 3xFLAG tags fused to Jak1 and MV-V, respectively. Co-affinity purification demonstrated the specific interaction of full-length Jak1 with MV-V proteins of all three strains (Fig. 3B).

We identified Jak1 as a direct interactor of MV-V in the yeast two-hybrid system. Because STAT1 was previously reported to interact with Jak1 by its SH2 domain (Gupta et al., 1996), there is a possibility that Jak1 interaction with endogenous STAT1 contributes to MV-V co-purification with GST-Jak1. Interestingly, the kinase domain of Jak1 identified by yeast two-hybrid, and reported here as Jak1-TK, does not interact with STAT1 when co-expressed in HEK-293T cells (Fig. 3C). This suggests that Jak1-binding region to STAT1 is not localized within Jak1-TK domain, in agreement with previous reports that localized the STAT-binding site in the pseudokinase region (JH2) of Jak kinases (Fujitani et al., 1997). We thus tested if MV-V could co-purify with Jak1-TK independently of any interaction with STAT1. HEK-293T cells were transfected with GST-MV-V and 3xFLAG-tagged Jak1-TK (Fig. 3D). The interaction of MV-V with Jak1-TK was confirmed in this system, and the binding efficiency was the same for all three strains tested. These results confirmed our two-hybrid data, and verified MV-V direct interaction with both STAT1 and Jak1.

MV interaction with STAT1, but not Jak1, depends on tyrosine 110 in the PNT domain

The PNT domain of MV-V alone interacts with STAT1 or Jak1 in the yeast two-hybrid system. To validate this observation by co-affinity purification, HEK-293T cells were co-transfected with GST-tagged PNT or VCT domains of MV-V_{G954}, and 3xFLAG-tagged STAT1 or Jak1-TK. STAT1 was shown to interact with the PNT domain, but not the VCT domain of MV-V_{G954} (Fig. 4A). On the contrary, we could not co-purify Jak1-TK with the PNT domain alone (Fig. 4B). This interaction is

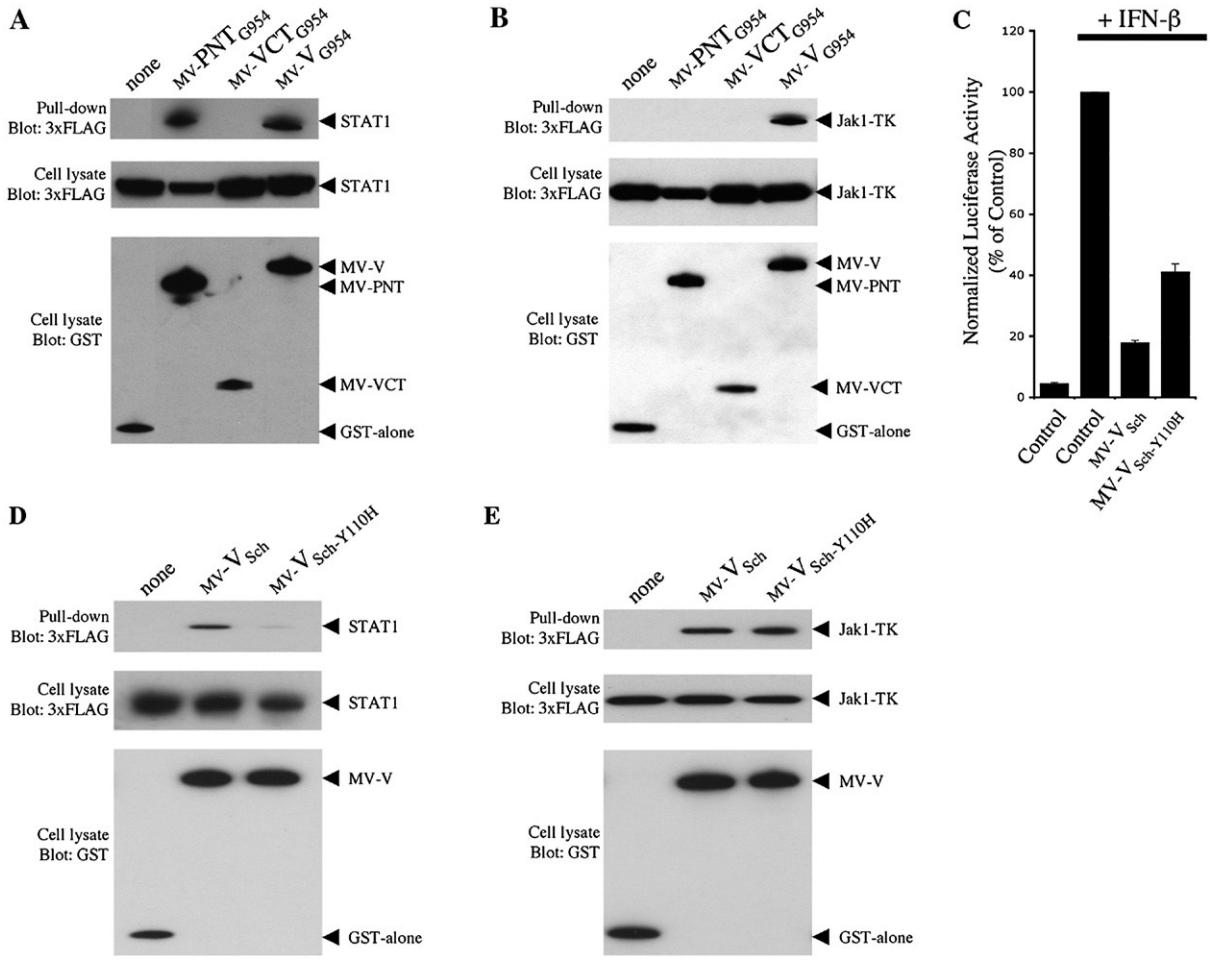

Fig. 4. Binding of the PNT domain of MV-V to STAT1 and Jak1, and consequences of Y110H mutation. (A and B) Cells were co-transfected with expression vectors encoding GST fused to the PNT or VCT domains of MV-V_{G954}, and either 3xFLAG-STAT1 (A) or 3xFLAG-Jak1-TK (B). 48 h post-transfection, protein complexes were assayed by pull-down using glutathione-sepharose beads. 3xFLAG- and GST-tagged proteins were detected by immunoblotting. (C) MV-$V_{Sch\text{-}Y110H}$ capacity to block IFN-α/β signaling. HEK-293T cells were transfected with pISRE-Luc, pRL-CMV, and pEGFP-C1 expression plasmids encoding MV-V_{Sch} or MV-$V_{Sch\text{-}Y110H}$. 24 h after transfection, 1000 IU/ml of recombinant IFN-β were added. After 24 h of incubation, the relative luciferase activities of IFN-treated cells were determined. Experiments were performed in triplicates, and data represent means ± SD. (D and E) Cells were co-transfected with expression vectors encoding GST alone or fused to MV-V_{Sch} or MV-$V_{Sch\text{-}Y110H}$, and either 3xFLAG-STAT1 (D) or 3xFLAG-Jak1-TK (E). After pull-down using glutathione-sepharose beads, 3xFLAG- and GST-tagged proteins were detected by immunoblotting.

probably too unstable to be characterized by co-affinity purification, while it could be detected in the yeast two-hybrid system which is known to be more sensitive. When expressed without the VCT region, the PNT domain is probably unable to properly fold as suggested by structural data (Li et al., 2006). Thus, one possibility to explain the discrepancy between yeast two-hybrid and co-affinity purification data is that the PNT domain cannot form a stable complex with Jak1-TK in mammalian cells unless it is structurally constrained through interactions with the VCT region of MV-V (see discussion).

Previous reports demonstrated that Y110H mutation, either introduced alone in MV-P or when combined to C272R in MV-V, impairs the PNT domain ability to block IFN-α/β signaling (Devaux et al., 2006; Ohno et al., 2004). In our reporter assay, we found that Y110H single mutation partially impaired the inhibition of IFN-α/β signaling by MV-V (Fig. 4C). We then tested the effect of this mutation on MV-V capacity to bind to STAT1 or Jak1-TK. Co-affinity purification experiments were performed using either MV-V_{Sch} or MV-$V_{Sch\text{-}Y110H}$ as a bait. As shown in Fig. 4D, MV-$V_{Sch\text{-}Y110H}$ interaction with STAT1 was greatly reduced. In contrast, MV-$V_{Sch\text{-}Y110H}$ and MV-V_{Sch} interacted with Jak1-TK similarly (Fig. 4E). These observations suggest that MV-$V_{Sch\text{-}Y110H}$ is impaired for its ability to block type I IFN signaling because it cannot bind STAT1, providing molecular basis for the loss-of-function phenotype of this mutant.

STAT1 and Jak1 do not compete for binding to the PNT domain of MV-V

Although Jak1 binding to MV-V relies on the PNT domain but is independent on tyrosine 110, it remained unclear whether STAT1 and Jak1 bind simultaneously to MV-V, or compete for the same region within the PNT domain. Thus, a competition experiment was designed where MV-V_{Sch} or MV-$V_{Sch\text{-}Y110H}$ was co-expressed with Jak1-TK in the presence of two different quantities of STAT1 (Fig. 5). In such setting, STAT1 expression did not affect Jak1-TK interaction with MV-V, thereby demonstrating that STAT1 and Jak1 do not compete for the same binding site within the PNT domain of MV-V. Moreover, MV-V_{Sch} and MV-$V_{Sch\text{-}Y110H}$ interacted similarly with Jak1-TK when co-expressed with STAT1 (Fig. 5). This observation also demonstrates that MV-V binding to Jak1 is not affected by its interaction with STAT1. These results validate a model where Jak1 and STAT1 bind to distinct regions in the PNT domain of MV-V protein.

MV-V binding to STAT1 prevents its direct phosphorylation by Jak1

Upon IFN-α/β stimulation, Jak1 phosphorylates STAT1 whereas MV-V was reported to block STAT1 phosphorylation (Takeuchi et al., 2003; Yokota et al., 2003). Our studies showed that MV-V interacts both with STAT1 and Jak1, and we therefore tested its ability to block the direct phosphorylation of STAT1 by Jak1. Co-expression of Jak1 and STAT1 in mammalian cells can trigger STAT1 phosphorylation and activation, bypassing the need for IFN-α/β stimulation (Quelle et al., 1995). Thus, GST-STAT1 and 3xFLAG-Jak1 were co-expressed in HEK-293T cells, in the presence or absence of 3xFLAG-tagged MV-V proteins. After 48 h, the protein extracts were tested for STAT1 phosphorylation status (Fig. 6A). When MV-V proteins from G954, IC-B or Schwarz were co-expressed, STAT1 phosphorylation was strongly inhibited. This demonstrates that MV-V can interfere with the direct phosphorylation of STAT1 by Jak1. Interestingly, MV-$V_{Sch\text{-}Y110H}$ was affected in its capacity to block STAT1 phosphorylation, probably because of the loss of interaction with STAT1, but still exhibited some inhibitory

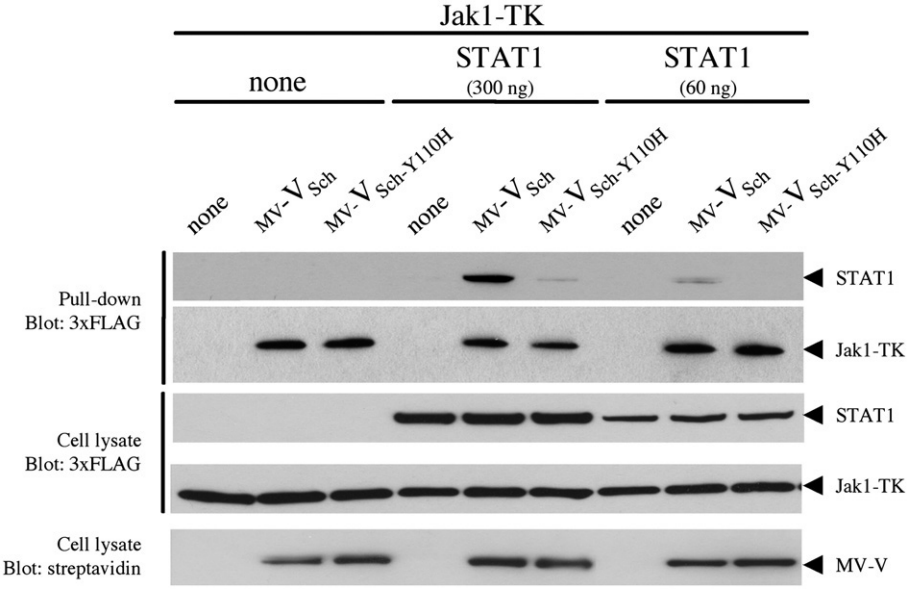

Fig. 5. STAT1 and Jak1 do not compete for binding to the PNT domain of MV-V. Cells were transfected with BioEase-tagged MV-V_{Sch} or MV-$V_{Sch\text{-}Y110H}$ (200 ng of plasmid per well of a 6-well plate), 3xFLAG-Jak1-TK (600 ng/well) and two doses of 3xFLAG-STAT1 (either 300 or 60 ng/well). 48 h post-transfection, protein complexes were assayed by pull-down using streptavidin–metacrylate polymer beads. 3xFLAG-tagged and BioEase-tagged proteins were detected by immunoblotting.

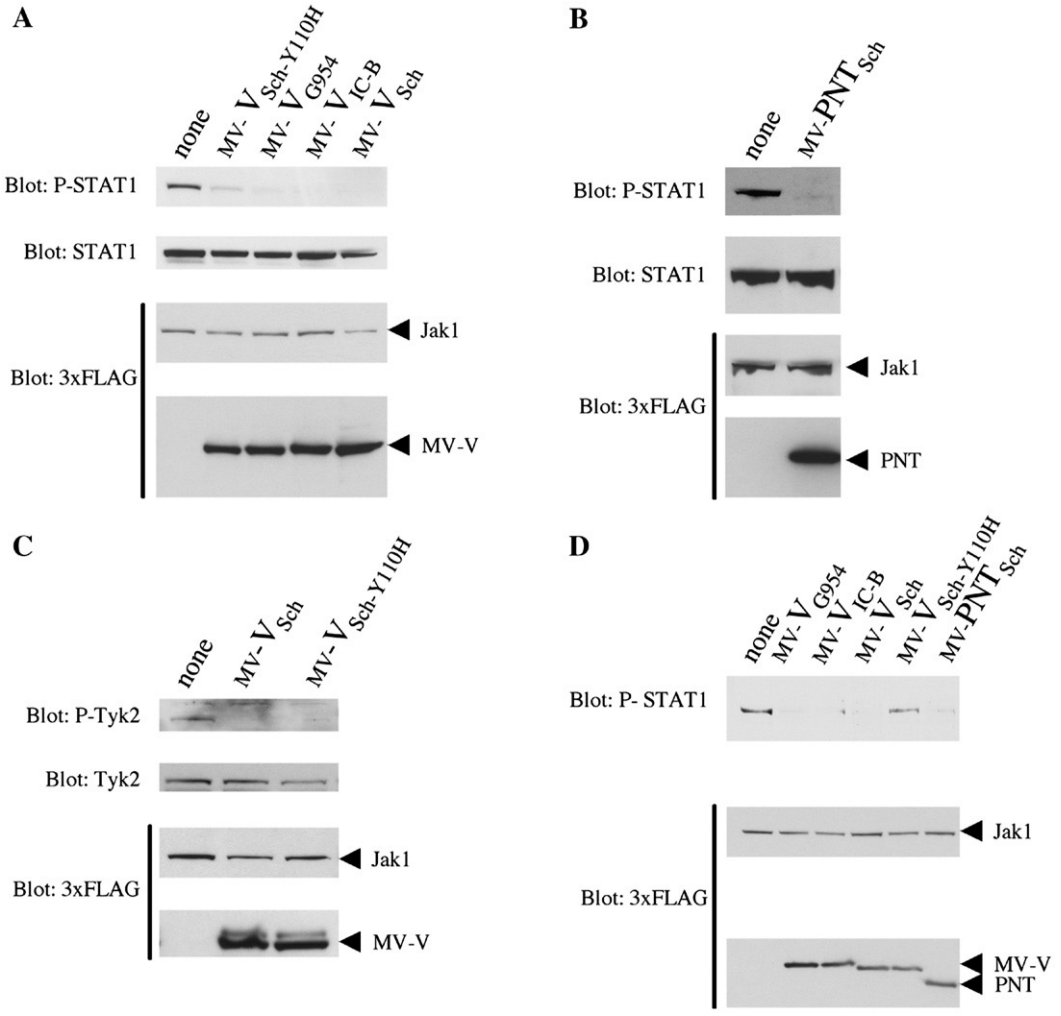

Fig. 6. MV-V inhibits STAT1 phosphorylation induced by Jak1 overexpression. (A) HEK-293T cells were co-transfected with expression vectors encoding GST-STAT1 (600 ng/well), 3xFLAG-Jak1 (10 ng/well), and 3xFLAG-tagged MV-V proteins (300 ng/well) or equal amounts of empty vector. Total cell lysates from transfected cells were prepared 48 h post-transfection. Phosphorylated STAT1, and 3xFLAG- or GST-tagged proteins were detected by immunoblotting. (B) The same experiment was performed with the 3xFLAG-PNT domain of MV-V_{Sch} (300 ng/well). (C) Cells were co-transfected with expression vectors encoding either Tyk2 kinase-defective mutant K930R (600 ng/well), 3xFLAG-Jak1 (10 ng/well), and 3xFLAG-tagged MV-V proteins (300 ng/well) or equal amounts of empty vector. Total cell lysates from transfected cells were prepared 48 h post-transfection. Phosphorylated Tyk2, total Tyk2, and 3xFLAG-tagged proteins were detected by immunoblotting. (D) Cells were co-transfected with 3xFLAG-Jak1 (600 ng/well) and 3xFLAG-tagged MV-V proteins (300 ng/well). Total cell lysates from transfected cells were prepared 48 h post-transfection. Endogenous phosphorylated STAT1, and 3xFLAG or GST-tagged proteins were detected by immunoblotting.

activity (Fig. 6A). In such a minimal system where only Jak1 and STAT1 are overexpressed, this observation suggests that MV-V interaction with Jak1, that is independent of Y110H mutation, is responsible for this partial inhibition. To substantiate this hypothesis, additional experiments were performed using Tyk2 instead of STAT1 as a target for Jak1. A kinase-defective mutant of Tyk2 (K930R) was used to discriminate Tyk2 self-phosphorylation from its phosphorylation by Jak1. As shown in Fig. 6C, MV-V_{Sch} and MV-$V_{Sch\text{-}Y110H}$ equally inhibited Tyk2 phosphorylation by Jak1. This observation supports a model where MV-V impairs the proper phosphorylation of various Jak1 targets.

It should be noted that transfections were performed with very low amounts of Jak1 encoding plasmid (10 ng/well of a 6-well plate). When high amounts of both Jak1 and STAT1 plasmids were used (600 ng/well), inhibition of STAT1 phosphorylation by MV-V was barely detectable, suggesting a competition between the three elements of the system. To test this hypothesis, cells were transfected with high amounts of Jak1 (600 ng/ml), and endogenous STAT1 phosphorylation was detected. In such setting, STAT1 instead of Jak1 is the limiting factor because endogenous STAT1 is expressed at very low levels. In the presence of MV-V, endogenous STAT1 phosphorylation was blocked (Fig. 6D). Moreover, MV-$V_{Sch\text{-}Y110H}$ was impaired in its ability to prevent STAT1 phosphorylation but this loss-of-function phenotype was only partial as reported above. These results demonstrate that MV-V can block endogenous STAT1 phosphorylation by Jak1, and that Y110H mutation impairs this function.

Finally, we tested the capacity of the PNT domain alone to inhibit overexpressed or endogenous STAT1 phosphorylation (Figs. 6B and D, respectively). Interestingly, this domain is sufficient to completely block STAT1 phosphorylation, although

interaction with Jak1 was unstable and could not be detected by co-affinity purification (Fig. 4B). This suggests that MV-V interaction with STAT1 alone is sufficient to block its phosphorylation and IFN-α/β signaling. However, reporter gene and kinase assays performed with the MV-$V_{Sch\text{-}Y110H}$ mutant (Fig. 4C, Figs. 6A, C and D) suggest that MV-V interaction with Jak1 by the PNT domain and yet unidentified IFN-α/β signaling components by the VCT region, can partially compensate for the loss of interaction with STAT1.

Discussion

MV-V is a potent inhibitor of IFN-α/β transduction pathway that contributes to the hijacking of cellular functions by MV. This viral protein was shown to associate with IFN-α/β receptor components in a tight complex that freeze signaling (Palosaari et al., 2003; Takeuchi et al., 2003; Yokota et al., 2003). We now demonstrate that both the N- and C-terminal domains of MV-V (PNT and VCT) contribute to the inhibition of IFN-α/β signaling. In addition, we identified by yeast two-hybrid and validated by co-affinity purification experiments the direct interaction of MV-V both with STAT1 and Jak1. Binding interfaces map to MV-V amino-terminal domain (PNT), and the deleterious mutation Y110H is shown to specifically disrupt MV-V interaction with STAT1. Finally, we report that MV-V inhibits the direct phosphorylation of STAT1 by Jak1, providing some molecular basis to the inhibition of IFN-α/β signaling by this virulence factor.

MV-V protein is composed of an intrinsically disordered amino-coterminal region shared with MV-P (PNT), and a highly conserved zinc-binding carboxyl-terminus domain (VCT). These two domains are involved in several processes through interactions with multiple viral and cellular partners. The VCT domain mediates MV-V oligomerization and interaction with MDA-5, thereby inhibiting the induction of IFN-α/β by double-stranded RNA (Childs et al., 2007; Ulane et al., 2005). In addition, this domain can bind RNA molecules, and such interactions repress MV genome replication (Parks et al., 2006). Functional assays also suggest that the VCT domain mediates MV-V interaction with p53 family member p73 (Cruz et al., 2006). We now report that the VCT domain alone can block IFN-α/β signaling. This would explain why a mutation that affects the PNT domain must be combined with a second mutation in the VCT region to abrogate MV-V inhibitory capacity (Ohno et al., 2004). Interestingly, the VCT domain of mumps virus was reported to interact directly with RACK1 (Kubota et al., 2002), an adaptor that bridges STAT1 to IFNAR2c, suggesting a mechanism for the inhibition of IFN-α/β signaling by the VCT domain of MV.

In contrast to the VCT domain which is self-constrained, folding of intrinsically disordered polypeptides like the PNT domain depends on interactions with specific binding partners (Bourhis et al., 2006). Interaction-induced folding of intrinsically disordered domains allow them to adopt various structures with specific binding interfaces. As a consequence, such regions favor interactions with multiple protein partners, and this is probably the case for the PNT domain of MV-V (Haynes et al., 2006). The structure of the V protein from simian virus 5 (SV5) suggests that the PNT domain of MV-V interacts with the VCT region to adopt a specific folding (Li et al., 2006). But this PNT domain must be differently folded when it interacts with components of the replication complex as part of the phosphoprotein P (Bourhis et al., 2006). Inhibition of IFN-α/β signaling by MV-V or MV-P relies on this domain, which is sufficient to block signal transduction downstream of IFNAR1/2c (Ohno et al., 2004). However, inhibition of IFN-α/β pathway by the PNT domain alone is only partial when compared with full-length MV-V protein (Fig. 1B). This supports a critical role for the VCT region that inhibits IFN-α/β pathway by itself, but could also contribute to the appropriate folding of the PNT domain.

We report here that STAT1 and Jak1 are direct interactors of the PNT domain when expressed in the context of MV-V protein. STAT1 was previously immunoprecipitated with FLAG-tagged MV-V as part of a protein complex containing STAT1, STAT2, STAT3, and IRF9 (Palosaari et al., 2003). Our yeast two-hybrid data supported by co-affinity experiments demonstrate that MV-V binds directly to STAT1. This observation should be related to the reported direct interaction of STAT1 with the V proteins of the *Morbillivirus* Rinderpest virus (RPV) and the *Henipaviruses* (Nanda and Baron, 2006; Rodriguez and Horvath, 2004). As reported here for MV, the amino-terminal domains of the V proteins from RPV, Nipah and Hendra viruses mediate the interaction with STAT1. Interaction with Jak1 is unexpected, and demonstrates that MV-V impairs IFN-α/β transduction by interacting with multiple components of this pathway. The PNT domain mediates MV-V interaction with Jak1 as shown by yeast two-hybrid, but the presence of VCT domain seems critically required to stabilize this interaction in mammalian cells. This led us to hypothesize that Jak1 binding to the PNT domain depends on its appropriate chaperoning by the VCT region, although this still requires a clear demonstration based on structural analysis of the complex. Our data also demonstrate that Jak1 and STAT1 have distinct non-overlapping binding sites. Thus, MV-V could bind simultaneously to Jak1 and STAT1 if these two cellular proteins are localized in the same vicinity.

We determined that MV-V binds to the kinase domain (JH1) of Jak1, a region critically involved in the phosphorylation of STAT proteins by Jak kinases. In an attempt to map the region of STAT1 that binds MV-V, STAT1 domains were cloned individually or in combinations, then tested for interactions with MV-V (data not shown). None of the constructs interacted with MV-V, suggesting that only full-length STAT1 is properly folded and displays the required binding site for MV-V. Complementary STAT1–STAT3 fusion proteins were previously used to demonstrate that the C-terminal region of STAT1 (AA 509–750) mediates the interaction with the V protein of Nipah virus (Rodriguez et al., 2004). Our data suggest that a similar strategy should be applied to MV-V. Interestingly, STAT1 is known to bind Jak1 by interaction of its SH2 domain with the JH2 pseudokinase domain of Jak1 (Fujitani et al., 1997; Gupta et al., 1996). This interaction is required for Jak1 to phosphorylate STAT1 in vitro, and likely represents an essential

step in the activation cascade downstream of IFNAR1/2c. Our results suggest that MV-V can form a stable heterotrimeric complex with Jak1 and STAT1, thereby freezing IFN-α/β receptor. It is tempting to speculate that MV-V has evolved to interact with STAT1-Jak1 heterodimeric complex at a binding interface localized at the junction between the two cellular proteins. With this model in mind, we tested whether MV-V can block the direct phosphorylation of STAT1 by Jak1 kinase. Our results demonstrate that MV-V inhibits Jak1-mediated phosphorylation of overexpressed or endogenous STAT1. This observation can provide a molecular basis for the inhibition of IFN-α/β signaling by this viral protein. MV-V was reported to sequester STAT1 in the cytosol (Palosaari et al., 2003), but our data sustain reports demonstrating that MV-V can also inhibit STAT1 phosphorylation in IFN-α/β stimulated cells (Takeuchi et al., 2003; Yokota et al., 2003).

MV-V from wild-type and vaccine strains have similar binding capacities for STAT1 and Jak1, and equally inhibit IFN-α/β signaling. This is expected, as MV vaccine strain must efficiently replicate in an interferon-competent environment when injected in vivo to trigger an adaptive immune response. In contrast, the infectious cDNA clone Ed-Tag encodes a virus defective for the inhibition of IFN-α/β signaling, and critically impaired for its capacity to trigger adaptive immunity (Combredet et al., 2003; Radecke et al., 1995). Mutations responsible for this deficiency were mapped to tyrosine 110 and cysteine 272 of MV-V (Ohno et al., 2004). Although mutation of cysteine 272 is clearly deleterious for the VCT domain zinc finger, we investigated how the Y110H mutation impairs MV-V function. As shown in the present study, the Y110H mutation affects MV-V capacity to block Jak1-mediated phosphorylation of STAT1. Failure to interact with STAT1 probably explains the phenotype of this mutant. Our observations raised the possibility that the tyrosine 110 of MV-V might be phosphorylated by Jak1 or other kinases. So far, we have not been able to detect the phosphorylation of MV-V on tyrosine residues, even if co-expressed with excess amounts of Jak1 (data not shown). In addition, interactions with STAT1 and Jak1 are likely independent on tyrosine phosphorylation events because (i) proteins are not phosphorylated on tyrosines in yeast where interactions have been identified by two-hybrid and, (ii) co-affinity purification experiments were performed in the absence of tyrosine phosphatase inhibitors. Thus, our observations suggest that MV-V is not phosphorylated on tyrosine 110.

Our data also demonstrate that the PNT domain alone binds STAT1 and blocks its phosphorylation by Jak1, although the interaction with Jak1 is probably weak or absent when this domain is separated from the VCT region. Thus, interaction with STAT1 is essential to explain the capacity of MV-V or MV-P to block STAT1 phosphorylation downstream of IFN-α/β receptor. However, the fact that MV-$V_{Sch\text{-}Y110H}$ mutant is only partially impaired for its ability to block STAT1 phosphorylation is surprising (Fig. 6A). This experiment performed in a minimal system where only Jak1 and STAT1 are overexpressed suggests that MV-V interaction with Jak1 is responsible for this inhibition. This model is supported by the observation that MV-V can block the phosphorylation of another well-characterized target of Jak1, the IFNAR1-associated kinase Tyk2. Although we cannot exclude that the VCT region targets Jak1-mediated phosphorylation of STAT1 through interactions with yet unidentified partners, our data suggest that MV-V interaction with Jak1 can partially compensate for the loss of interaction with STAT1. To verify this conjecture, we are planning to generate by reverse two-hybrid MV-V mutants that fail to interact with Jak1 but remain competent for binding to STAT1. Such mutants will be instrumental to demonstrate the relative contribution of MV-V interaction with Jak1 to the inhibition of IFN-α/β signaling.

In conclusion, our data support a model where MV-V blocks IFN-α/β signaling through interactions with multiple components of the pathway including STAT1, Jak1 and at least one yet unidentified cellular partner of the VCT domain that could be RACK1. Because of redundancy, this system probably ensures the complete inhibition of the pathway in MV infected cells.

Materials and methods

ORF cloning and plasmid constructs

All constructs were amplified by standard PCR (ExTaq; Takara) or RT-PCR (Titan One tube; Roche) methods, and cloned using a recombinational cloning system (Gateway system; Invitrogen) into pDONR207 (Invitrogen) or pDONR223 (kindly provided by D. Esposito and JL. Hartley; Rual et al., 2004). PCR primers used to amplify and clone viral or cellular ORFs displayed from 20 to 30 specific nucleotides matching ORF extremities so that their Tm is close to 60 °C. To achieve recombinational cloning of PCR products, 5′ ends of forward primers were fused to attB1.1 recombination sequence 5′-GGGGACAACTTTGTACAAAAAAGTTGGCATG-3′, while reverse primers were fused to attB2.1 recombination sequence 5′-GGGGACAACTTTGTACAAGAAAGTTGGTTA-3′. Primer pairs were designed using the ViralOrfeome interface (http://www.viralorfeome.com; manuscript in preparation) developed in collaboration with IFR-128 BioSciences (Lyon, France) and Modul-Bio (Marseille, France). Recombination of PCR products into pDONR207 or pDONR223 was performed following manufacturer's recommendation (BP cloning reaction, Invitrogen). All constructs were transformed and amplified in *Escherichia coli* DH5α strain. ORFs of full-length MV-V, PNT domain (AA 1-231) and VCT domain (AA 232-299) were amplified by PCR from previously described infectious clones of Schwarz strain (Genbank ID: AF266291; Combredet et al., 2003) or IC-B strain (Genbank ID: NC001498; Takeda et al., 2000), or by RT-PCR on total RNAs extracted from B95a cells infected with MV strain G954 (Genbank ID: EF472597; Kouomou and Wild, 2002). Since MV-V encoding ORF contains a non-templated extra guanine (G) residue, two step PCRs were carried out. Two PCR fragments overlapping at the editing site were amplified from IC-B and Schwarz infectious clones using either forward or reverse frameshift primers (5′-CATTAAAAA-GGGGCACAGACGCGAGAT-3′ and 5′-ATCTCGCGT-CTGTGCCCCTTTTTAATG-3′) that incorporate an extra G residue at the editing site in combination with attB-containing primers that hybridize at the extremities of MV-V encoding

sequence. Then the PCR fragments were mixed and a ligating PCR was carried out using only the attB-containing primers. To clone MV-V ORF from G954 strain, RT-PCR on total RNAs from infected cells was performed with specific primers. PCR products were cloned and sequenced to identify cDNAs with an extra non-templated G residue at the editing site. To clone MV-$V_{Sch\text{-}Y110H}$ encoding ORF, pDONR223 plasmids with MV-V_{Sch} or MV-$V_{Ed\text{-}tag}$ (Radecke et al., 1995) were cut in two with *Xho*I and *Hin*dIII. Then, fragments were swapped and religated to create MV-$V_{Sch\text{-}Y110H}$ with nucleotides 1–613 of MV-$V_{Ed\text{-}tag}$ fused to nucleotides 614–900 of MV-V_{Sch}. Resulting sequence is therefore identical to MV-V_{Sch}, but carries the Y110H mutation. ORFs encoding STAT1, Jak1 and Jak1-TK (AAs 894-1154) were amplified from cDNA clones kindly provided by Dr S. Pellegrini (Pasteur Institute, Paris), then cloned in pDONR207 (Invitrogen), and sequence verified. Subsequently, viral or cellular ORFs cloned into pDONR vectors were transferred by recombinatorial reaction into various Gateway-compatible destination vectors (see below) following manufacturer's recommendation (LR cloning reaction, Invitrogen). Previously described pRc-K930R vector was kindly provided by S. Pellegrini (Gauzzi et al., 1996).

Cell culture and transient expression of recombinant proteins

Human HEK-293T cells were 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. To achieve overexpression in mammalian cells, cloned ORFs were transferred by recombinational cloning (Gateway system; Invitrogen) from pDONR223 or pDONR207 to the following expression vectors to achieve GST-fusion (pDEST27; Invitrogen), 3xFLAG-fusion (pCI-neo-3xFLAG; Mendoza et al., 2006), biotinylation (pcDNA6/BioEase-DEST; Invitrogen) or EGFP-fusion (pEGFP-C1; Clontech). Unless specified, 5×10^5 cells per well were dispensed in 6-well plates and transfected 24 h later with 500 ng of each plasmid DNA per well. The total amount of transfected DNA per well was normalized using corresponding empty vectors whenever required. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendation.

Luciferase reporter gene assay

Expression of IFN-inducible genes was determined by transiently transfecting reporter plasmids pISRE-Luc containing the ISRE enhancer element upstream of the firefly luciferase gene (Stratagene). HEK-293T cells were plated in 24-well plates (2×10^5 per well). One day later, they were transfected with pISRE-Luc reporter plasmid (1 μg/well), pRL-CMV reference plasmid that harbored a CMV promoter just upstream of the *Renilla* luciferase gene (0.01 μg/well; Promega), and specified ORFs cloned in pEGFP-C1 vector (1 μg/well). After 24 h, IFN-β (Biosource) was added to the medium at 1000 IU/ml, and the cells were incubated for 24 h. The cells were lysed, and the firefly luciferase and *Renilla* luciferase activities in the lysate were measured using the Dual-luciferase Reporter Assay System (Promega) following manufacturer's recommendation. Reporter activity was calculated as the ratio of firefly luciferase activity to reference *Renilla* luciferase activity.

Yeast two-hybrid procedure

Yeast culture mediums were prepared as previously described (Walhout and Vidal, 2001). V, PNT and VCT coding sequences from IC-B, G954 and Schwarz MV strains were cloned into the Gal4-BD yeast two-hybrid vector pDEST32 (Invitrogen), and transformed into AH109 yeast cells (Clontech) according to standard procedures (Gietz et al., 1995). The GAL4-BD-PNT, -VCT and -V fusion proteins were tested for autonomous transactivation of *HIS3* reporter gene. The VCT domains of all three strains and PNT domains from IC-B and G954 MV strains did not induce autonomous transactivation, and the screens were performed on synthetic medium lacking histidine (-His medium) and supplemented with only 10 mM of 3-amino-1,2,4-triazole (3-AT; Sigma-Aldrich). As previously reported, the PNT domain from Schwarz strain was a weak transactivator and the selective medium for the screen had to be supplemented with 30 mM of 3-AT (Chen et al., 2003). Some of the few mutations that discriminate the PNT domain of Schwarz from G954 and IC-B, in particular proline substitutions, probably account for this difference. Full-length MV-V proteins were all strong transactivators in yeast. A synthetic medium supplemented with 100 mM of 3-AT was required to titrate this activity and prevent yeast growth in the absence of interactor. As previously described (Fromont-Racine et al., 1997), a mating strategy was used for screening the human spleen cDNA library cloned in the GAL4-AD pPC86 vector (Invitrogen) and previously transformed into the yeast strain Y187 (Clontech). After 6 days of culture on selective medium, [His+] colonies were picked, purified during 3 weeks by culturing on selective medium to eliminate false-positives (Vidalain et al., 2004). AD-cDNAs were amplified by PCR from zymolase-treated yeast colonies using primers (AD and Term; Walhout and Vidal, 2001) that hybridize within the pPC86 regions flanking cDNA inserts. PCR products were sequenced, and cellular interactors were identified by blast analysis (Louis M. Jones, Institut Pasteur). These PCR products were also used to retest interactions between viral baits and cellular preys following the gap-repair procedure (Walhout and Vidal, 2001). Briefly, 250 ng of linearized pPC86 empty vector was co-transformed with 5 μl of PCR product to allow recombinatorial repair in fresh yeast cells expressing BD-fused MV-V or its domains. AD-fused cDNAs encoding STAT1 (AAs 1-750), Jak1 (AAs 894-1154), MDA5 (AAs 577-1025) and PIRH2 (AAs 63-261) were retested in yeast cells using this procedure.

Cell lysis and co-affinity purifications

Two days after transfection, HEK-293T cells were washed in phosphate-buffered saline (PBS), then treated with 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) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich) when testing STAT1 or Tyk2 phosphorylation. 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 was incubated for 1 h at 4 °C with either 25 µl of glutathione-sepharose beads (Amersham Biosciences) to purify GST-tagged proteins, or 25 µl of streptavidin–metacrylate polymer beads (Boehringer Mannheim) to purify biotinylated BioEase-tagged proteins, respectively. Beads were then washed 3 times in ice-cold lysis buffer and proteins were recovered by boiling in denaturing loading buffer (Invitrogen).

Western blot analysis

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 with the iBlot Dry blotting system (Invitrogen). 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 STAT1 and STAT1 phosphorylated at position Y701 were revealed using clone-1 and clone-14, respectively (mouse monoclonal antibodies; BD Biosciences). MV-V_{Sch} was detected using a rabbit polyclonal antibody kindly provided by Dr. Kaoru Takeuchi (Takeuchi et al., 2003). Phospho-Tyk2 and Tyk2 were detected using a rabbit polyclonal antibody (Calbiochem) and the previously described mouse monoclonal antibody T10-2 (Gauzzi et al., 1996), respectively. Secondary anti-mouse and anti-rabbit HRP-conjugated antibodies were from GE-Healthcare and Amersham, respectively. Biotinylated BioEase-tagged proteins were detected using HRP-conjugated streptavidin (Invitrogen).

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