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Conservation of a Unique Mechanism of Immune Evasion across the *Lyssavirus* Genus

L. Wiltzer,[a,b] F. Larrous,[c,d] S. Oksayan,[a,b] N. Ito,[e] G. A. Marsh,[f] L. F. Wang,[f] D. Blondel,[g] H. Bourhy,[c] D. A. Jans,[b] and G. W. Moseley[a]

Viral Immune Evasion and Pathogenicity Laboratory[a] and Nuclear Signalling Laboratory,[b] Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Victoria, Australia; Institut Pasteur, Unit Lyssavirus Dynamics and Host Adaptation, Paris, France[c]; Université Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, Paris, France[d]; Laboratory of Zoonotic Diseases, Faculty of Applied Biological Sciences, Gifu University, Gifu, Japan[e]; Commonwealth Scientific and Industrial Research Organization Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, Australia[f]; and Laboratoire de Virologie Moléculaire et Structurale, CNRS, Gif sur Yvette, France[g]

The evasion of host innate immunity by *Rabies virus*, the prototype of the genus *Lyssavirus*, depends on a unique mechanism of selective targeting of interferon-activated STAT proteins by the viral phosphoprotein (P-protein). However, the immune evasion strategies of other lyssaviruses, including several lethal human pathogens, are unresolved. Here, we show that this mechanism is conserved between the most distantly related members of the genus, providing important insights into the pathogenesis and potential therapeutic targeting of lyssaviruses.

The principal host response to viral infection in humans is the expression of type I interferons (IFNs) (alpha IFN [IFN-α] and IFN-β), which activate intracellular signaling via phosphorylation of signal transducers and activators of transcription 1 (STAT1) and STAT2 at tyrosines Y701 and Y690, respectively. This results in the generation of STAT1/2 heterodimers, the principal mediators of IFN-α/-β signaling, as well as STAT1 homodimers, which translocate into the nucleus, where they induce gene transcription essential to the establishment of the antiviral state (10). Viruses have evolved powerful countermeasures to inhibit STAT signaling through the activity of virus-expressed IFN antagonist proteins (38), including the phosphoprotein (P-protein) of *Rabies virus* (RABV) (17, 44), the best-characterized member of the genus *Lyssavirus*. There are currently 14 viruses in this genus (8, 21, 26), with all characterized members documented to infect mammals (4, 29), including a broad range of livestock such as cattle, sheep, and horses (34). Human infection by RABV alone is estimated to cause >55,000 deaths/year, and six other lyssavirus species have been reported to cause lethal rabies disease in humans (4, 29), the incidence of which is almost certainly underreported (19, 22). Thus, emerging zoonotic lyssaviruses pose important threats to human health and agriculture.

Inhibition of IFN-dependent signaling in RABV infection is achieved through the interaction of the globular C-terminal domain (CTD; residues 173 to 297) of P-protein with STAT1 and STAT2 (3, 27, 45). This interaction is reported to require the C-terminal 10 amino acid residues of P-protein, as deletion of the 10 or 30 C-terminal residues abrogates both STAT1/2 binding and inhibition of IFN-dependent transactivation (3, 27, 45). The P-protein–STAT1/2 interaction inhibits IFN-activated nuclear translocation of STAT1/2 via the activity of a nuclear export sequence (NES) in the N-terminal region of P-protein (residues 49 to 58), which mediates active nuclear export of the P-protein–STAT1/2 complex, dependent on the activity of the cellular nuclear export protein CRM1 (37). The IFN antagonist proteins of several other viruses, including henipaviruses, Mapuera virus, measles virus, Ebola virus, and hepatitis C virus, also cause mislocalization of STAT1/2 (9, 13, 28, 40, 41), but RABV appears to be unique in that it selectively and directly targets the IFN-activated form of STAT1 (3, 27, 45). The evolution of this highly specific mechanism may relate to the limited coding capacity of the RABV genome (36), such that P-protein, which has several vital roles in genome replication in addition to IFN antagonism, is used to target STAT1/2 only when required. Importantly, defects in the NES of P-protein of the RABV strain Nishigahara-chicken embryo (Ni-CE) correlate with impaired nuclear export and IFN antagonist function of P-protein and with attenuated pathogenicity in mice (17). Thus, specific STAT1/2 interaction and active nuclear export by P-protein appear to be essential to the inhibition of STAT1/2 signaling, thus representing a critical mechanism in pathogenic RABV infection and a potential target for attenuated vaccine strain development and/or therapeutics. However, the immune evasion mechanisms of other highly pathogenic lyssaviruses and the role(s) therein of their P-proteins have not been investigated.

To examine the IFN antagonist functions of lyssaviruses, we selected several viral strains and species representative of lyssavirus diversity, including the RABV strains Challenge virus standard (CVS), silver-haired bat rabies virus (SHBRV), 8743THA (THA), 9001FRA (FRA), and 9704ARG (ARG), the closely related Australian bat lyssavirus (ABLV) and European bat lyssavirus 1 (EBLV-1), and the lyssaviruses most distantly related to RABV, Lagos bat virus (LBV) and Mokola virus (MOKV) (1, 5–7, 11, 12, 16, 24, 25, 43, 47). HeLa cells infected with these viruses were treated 24 h postinfection with IFN-α (1,000 U/ml, 0.5 h) or not treated, before fixation (31), immunostaining for Y701-phosphorylated STAT1 (pY701-STAT1), and fluorescence microscopic analysis (20). IFN treatment resulted in clear nuclear accumulation of pY701-STAT1 in mock-infected cells (Fig. 1A), but no nuclear accumulation of pY701-STAT1 was observed in cells infected with any of the lyssaviruses tested (Fig. 1B), indicating that the capacity

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Address correspondence to G. W. Moseley, greg.moseley@monash.edu.

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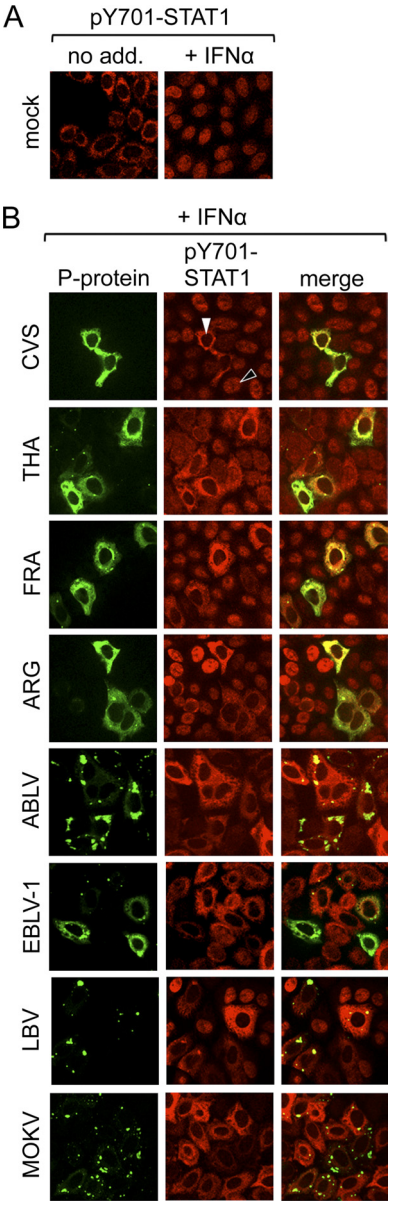

FIG 1 Inhibition of nuclear translocation of STAT1 in IFN-α-treated cells is conserved across the lyssavirus genus. (A) HeLa cells were treated with 1,000 U/ml IFN-α (PBL interferon source, catalog no. 11200-2; Pestka Biomedical Laboratories, Piscataway, NJ) for 0.5 h or not treated, before fixation and staining for pY701-STAT1. (B) HeLa cells infected with different RABV strains (CVS, THA, FRA, and ARG) and lyssaviruses (ABLV, EBLV-1, LBV, and MOKV) were treated 24 h postinfection with 1,000 U/ml IFN-α for 0.5 h or not treated, before fixation and immunostaining for pY701-STAT1 (Cell Signaling, Beverly, MA; catalog no. 9167) and P-protein (clone 25C2 [39]). Cells were analyzed using a Zeiss Axioplan (version 2.2) fluorescence microscope equipped with a Zeiss ApoTome system, as described previously (20). The white-filled and black-filled arrowheads indicate the nuclei of an infected cell and an uninfected cell, respectively.

to inhibit IFN-dependent STAT1 nuclear translocation is conserved in the genus.

To investigate the IFN antagonist functions of P-proteins from different lyssaviruses directly, we generated constructs using the pEGFP-C1 plasmid for the expression in mammalian cells of green fluorescent protein (GFP)-fused P-proteins, including those from the pathogenic laboratory RABV strains CVS and Nishigahara (Ni), which efficiently inhibit IFN-α-dependent STAT1/2 signaling, the highly pathogenic street strain SHBRV, the P-protein of which has not previously been assessed for IFN antagonist function, and the lyssavirus species ABLV and MOKV. We also included the P-protein of the attenuated Ni derivative strain Ni-CE, which is defective in inhibition of IFN-dependent STAT1 nuclear localization and signaling in infected and transfected cells due to mutations that impair the function of its NES (17). The P-proteins of Ni, Ni-CE, SHBRV, ABLV, and MOKV show 91.9%, 90.6%, 90.2%, 85.5%, and 64.0% sequence similarity to CVS P-protein, respectively, and the hydrophobic residues of the NESs are highly conserved throughout the genus (Fig. 2A; data not shown). However, the C termini differ markedly, particularly through their extensions in the P-proteins of several viruses, including MOKV (Fig. 2B; data not shown). Living Cos-7 cells transfected to express the GFP–P-proteins were imaged by confocal laser scanning microscopy (CLSM) to determine the ratio of nuclear to cytoplasmic fluorescence (Fn/c), a quantitative measure of nucleocytoplasmic protein localization (17, 30, 31, 33, 42). All P-proteins were excluded from the nucleus to similar extents, except for Ni-CE P-protein, in which the NES is mutated, resulting in a more nuclear phenotype (17) (Fig. 3A). Treatment of cells with leptomycin B (LMB; 5.2 nM, 3 h), a specific inhibitor of CRM1 (30, 31, 35), significantly increased the nuclear localization of all P-proteins, indicating that NES function is conserved in the genus (Fig. 2A) as the main determinant of P-protein subcellular localization, suggestive of an essential function in infection. Interestingly, the nuclear localization of ABLV and MOKV P-proteins in LMB-treated cells was significantly higher than that of P-proteins from the RABV strains, which showed equivalent nuclear localization (Fig. 3B). Thus, the activity of the nuclear localization signal, predicted to reside in the CTD of RABV P-protein (37), may differ across the genus.

To examine the capacity of lyssavirus P-proteins to inhibit IFN-activated nuclear translocation of STAT1, mock-transfected cells and cells transfected to express lyssavirus P-proteins or control proteins were treated with IFN-α (1,000 U/ml, 0.5 h) or not treated, before immunostaining for STAT1 and CLSM analysis (31). Truncated CVS P-protein lacking the C-terminal 30 residues (CVS PΔC30), which is known to be impaired for STAT1/2 interaction (2, 45), and CVS N-protein, which has no role in inhibiting STAT1/2 signaling (17), did not affect IFN-α-dependent STAT1 nuclear translocation compared with that in mock-transfected cells (Fig. 4). In contrast, STAT1 nuclear accumulation in IFN-α-treated cells expressing full-length P-proteins of different lyssaviruses was significantly inhibited (Fig. 4A). As previously reported, Ni-CE P-protein was impaired in this respect due to defective activity of its NES (17). Importantly, the Fn/c for STAT1 in IFN-treated cells expressing full-length P-proteins was significantly lower than that measured for STAT1 in nontreated cells, except for cells expressing the Ni-CE P-protein, in which the Fn/c of activated STAT1 was slightly increased, as previously reported (17) (Fig. 4B). This suggests that all P-proteins bind selectively to IFN-α-activated STAT1 and, with the exception of the NES-defective Ni-CE P-protein, cause STAT1 to be exported from the nucleus. Latent STAT1, however, is not bound by P-protein and thus can localize diffusely between the cytoplasm and nucleus. In LMB-treated cells, the P-protein–STAT1 complexes showed increased nuclear localization in all cases (data not shown), con-

A

Lyssavirus P	Amino acid sequence of the N-terminal NES									
	49	50	51	52	53	54	55	56	57	58
CVS	L	P	E	D	M	K	R	L	H	L
Ni	L	P	E	D	M	S	R	L	H	L
Ni-CE	L	P	E	D	M	S	R	P	H	P
THA	L	P	E	D	M	R	R	L	Q	L
FRA	L	P	E	D	M	R	R	L	Q	L
ARG	L	P	E	D	M	R	R	L	Q	L
SHBRV	L	P	E	D	M	R	R	L	Q	L
ABLV	L	P	E	D	I	K	K	L	D	I
EBLV-1	L	P	E	D	I	R	R	L	K	I
LBV	L	P	E	D	M	R	K	M	R	L
MOKV	L	P	E	D	M	S	R	L	R	I

B

Lyssavirus P	Amino acid sequence of the C-terminus																			
	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304			
CVS	Q	D	D	L	N	R	Y	T	S	C	-	-	-	-	-	-	-			
Ni	Q	D	D	L	N	R	Y	A	S	C	-	-	-	-	-	-	-			
Ni-CE	Q	D	D	L	N	R	Y	A	S	C	-	-	-	-	-	-	-			
THA	Q	D	D	L	N	R	Y	T	S	C	-	-	-	-	-	-	-			
FRA	Q	D	D	L	N	R	Y	A	S	L	-	-	-	-	-	-	-			
ARG	Q	D	D	L	N	R	Y	A	F	F	-	-	-	-	-	-	-			
SHBRV	Q	D	D	L	N	R	Y	A	F	P	-	-	-	-	-	-	-			
ABLV	Q	D	D	L	N	R	Y	M	S	C	-	-	-	-	-	-	-			
EBLV-1	Q	D	D	L	N	R	Y	L	A	Y	-	-	-	-	-	-	-			
LBV	Q	D	D	I	H	N	Y	M	T	R	I	E	E	I	D	H	N			
MOKV	Q	E	D	I	N	S	Y	M	A	R	L	E	E	A	E	-	-			

FIG 2 Comparison of the sequences of the N-terminal NESs and C termini of lyssavirus P-proteins. The sequences of the N-terminal NESs (residues 49 to 58) (A) and C termini (corresponding to the region from residue 288 to the C-terminal end of RABV P-protein, which is implicated in STAT1/2 binding) (B) are shown for the P-proteins of the RABV strains CVS, Ni, Ni-CE, THA, FRA, ARG, and SHBRV and for the lyssaviruses ABLV, EBLV-1, LBV, and MOKV. Conserved hydrophobic positions of the NES motif LXXXLXXLXL (where L can be replaced by V, I, M, or F) (23) and residues not conserved between the C termini of the different P-proteins are shaded. In the Ni-CE P-protein, the conserved leucines at position 56 and 58 replace the prolines.

firming that STAT1 nuclear exclusion by lyssavirus P-proteins is dependent on CRM1-mediated nuclear export, consistent with previous reports for RABV P-protein (46). Thus, the unique mechanism of selective targeting of IFN-α-activated STAT1 to cause its CRM1-dependent nuclear exclusion appears to be conserved in the lyssavirus genus.

We next examined the physical interaction of P-proteins with STAT1 and STAT2 by coimmunoprecipitation from transfected

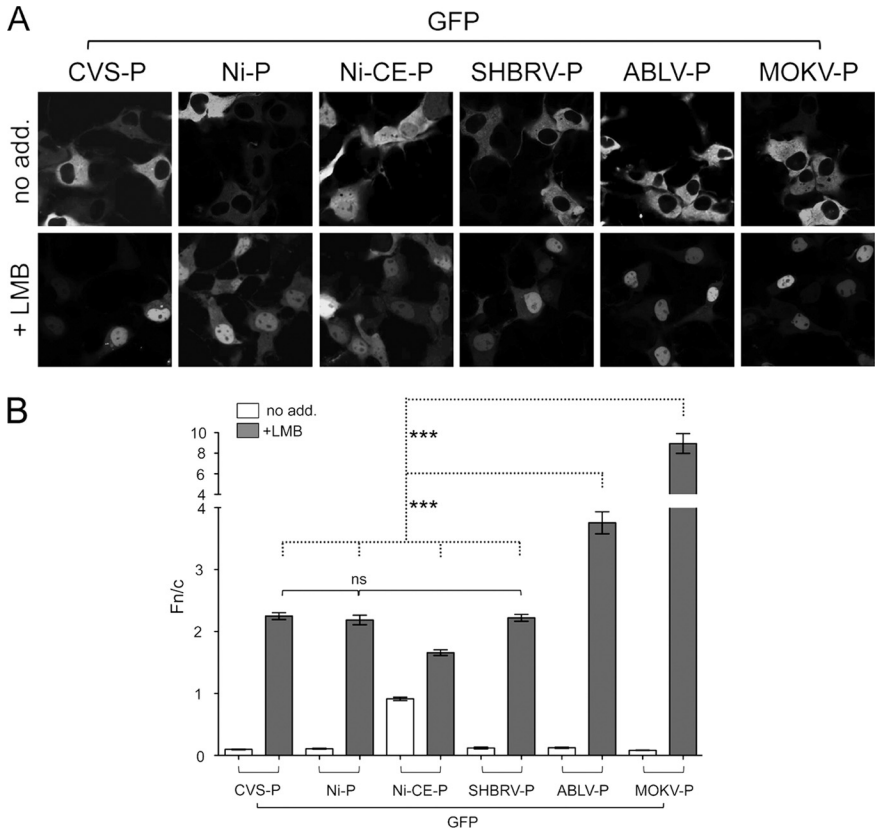

FIG 3 CRM1-dependent nuclear export of P-proteins is conserved in the lyssavirus genus. (A) Cos-7 cells expressing the indicated GFP-fused P-proteins were treated with 5 nM leptomycin B (a kind gift from M. Yoshida, RIKEN, Japan) for 3 h or not treated, before live imaging by CLSM using an Olympus FV1000 inverted confocal microscope with 60× water objective. (B) Images such as those shown in panel A were used to calculate the nuclear to cytoplasmic fluorescence ratios (Fn/c) for GFP-fused P-proteins (shown as means ± standard errors of the means [SEM]; $n > 30$ cells) corrected for background fluorescence by two-dimensional (2D) image analysis using Image J software as previously described (30–33, 42). The data shown are from a single assay, representative of 3 separate assays. Statistical analysis (Student's *t* test) was performed using GraphPad Prism software. ***, $P < 0.0001$; ns, not significant.

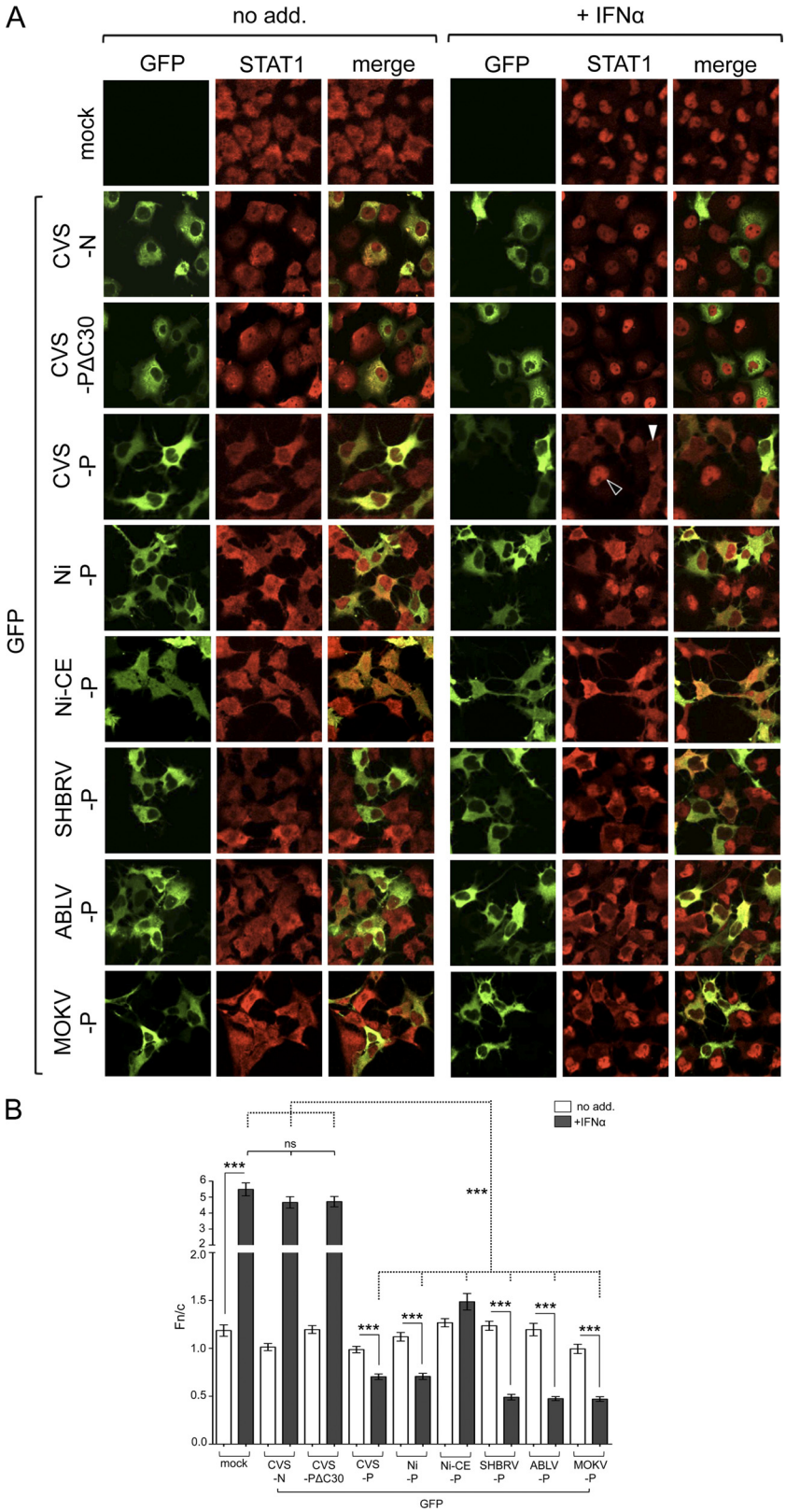

FIG 4 Inhibition of IFN-α-activated nuclear translocation of STAT1 by P-protein is conserved in the lyssavirus genus. (A) Mock-transfected Cos-7 cells or cells transfected to express the indicated GFP-fused P-proteins or N-protein were treated with 1,000 U/ml IFN-α for 0.5 h or not treated, before fixation and immunostaining for STAT1 and analysis by CLSM. Images are typical of >5 fields of view, with the white-filled and black-filled arrowheads indicating the nuclei of a transfected cell and a nontransfected cell, respectively. (B) Images such as those shown in panel A were analyzed to calculate the Fn/c (mean ± SEM; $n > 30$) for STAT1 as described in the legend to Fig. 3. Results are representative of 3 or more independent assays, with statistical analysis performed as for Fig. 3.

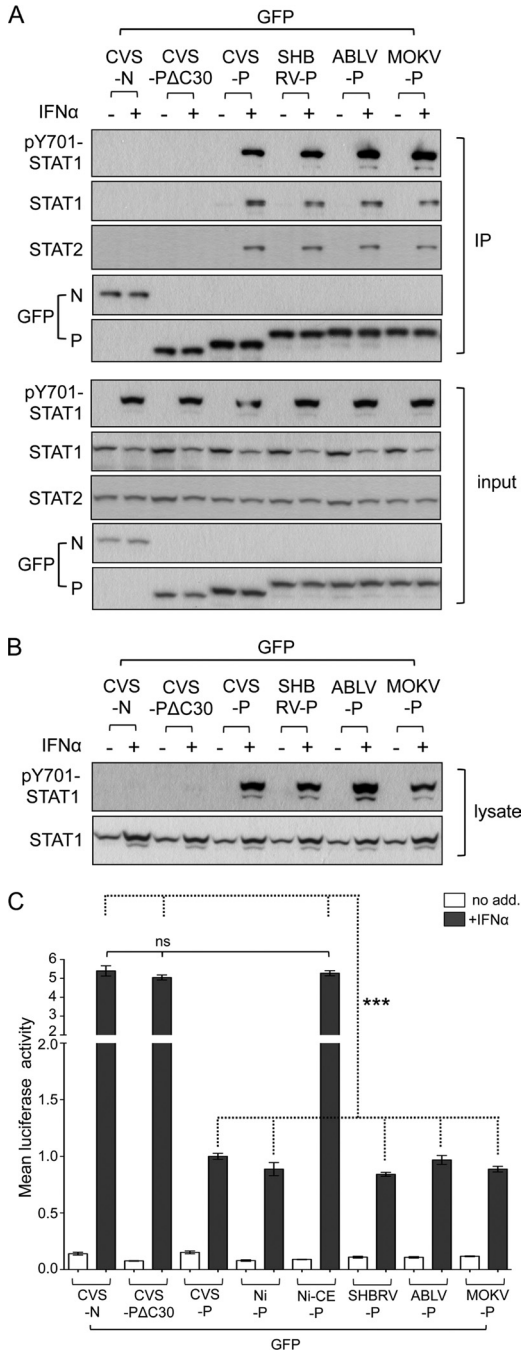

FIG 5 IFN-α-dependent interaction of P-protein with STAT1 and STAT2 and inhibition of IFN-α-dependent STAT1/2 signaling are conserved in the lyssavirus genus. (A and B) Cos-7 cells transfected to express the indicated GFP-fused P-proteins or GFP-fused RABV N-protein were treated with IFN-α (1,000 U/ml) for 0.5 h (A) or 16 h (B) or not treated, before lysis. Samples from cells treated for 0.5 h were subjected to immunoprecipitation of GFP-fused protein using GFP-trap (ChromoTek GmbH, Germany), followed by Western blot analysis of lysate (input) and immunoprecipitate (IP) using antibodies against pY701-STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-7988), STAT1 (BD Biosciences, San Jose, CA; catalog no. 610185), STAT2 (Santa Cruz Biotechnology; catalog no. sc-22816), or GFP (Roche Applied Science, Indianapolis, IN; catalog no. 11814460001). (B) Lysates of cells treated with 1,000 U/ml IFN-α for 16 h or not treated were analyzed by Western blotting for pY701-STAT1 and STAT1. (C) Cells cotransfected with plasmids for the expression of the indicated proteins and for the IFN-α/STAT1/2-specific dual luciferase reporter gene assay (14, 17) were treated with 1,000 U/ml IFN-α for 16 h or not treated, before analysis to measure IFN-α/STAT1/2-dependent transactivation of luciferase expression (14, 17). The graph shows firefly luciferase activity normalized to that of renilla luciferase, with values calculated relative to the value obtained for CVS P-protein (mean luciferase activity ± SEM, $n = 4$). The data were reproduced in 3 or more independent assays. Statistical analysis was performed as for Fig. 3.

Cos-7 cells (Fig. 5A). Consistent with the CLSM data, all P-proteins bound to STAT1 and STAT2 in an IFN-α-dependent manner, in contrast to the control proteins (Fig. 5A). RABV P-protein also inhibits dephosphorylation of pY701-STAT1, which is thought to be due to the inhibition of pY701-STAT1 nuclear translocation, preventing its interaction with nuclear phosphatases (3, 15). Using pY701-STAT1-specific antibody we could clearly detect pY701-STAT1 at 16 h after IFN-α treatment in lysates from cells expressing CVS, SHBRV, ABLV, and MOKV P-proteins but, as expected, no pY701-STAT1 could be detected in cells expressing control proteins (Fig. 5B). Thus, the capacity of P-protein to interact selectively with pY701-STAT1 and inhibit its dephosphorylation is maintained between distantly related lyssaviruses, indicating that the strategy for targeting STAT1 signaling is conserved across the genus.

To test the capacity of lyssavirus P-proteins to inhibit IFN-α-dependent STAT1/2 signaling, an IFN-α/STAT1/2-specific dual luciferase reporter-gene assay was used (14, 17). Cos-7 cells cotransfected with plasmids to express P-proteins or control proteins and the pISRE-luc/pRL-TK-luc vectors were treated with 1,000 U/ml IFN-α 6 h posttransfection or not treated and incubated for a further 16 h before analysis of IFN-α-specific luciferase activity. With the exception of the Ni-CE P-protein, which is defective for IFN antagonism due to its defective NES (17), all P-proteins suppressed IFN-α/STAT1/2-dependent luciferase transactivation activity to similar extents, compared with the control proteins CVS-PΔC30 and CVS-N protein (Fig. 5C). Thus, the capacity to antagonize IFN-α-dependent signaling is conserved in the lyssavirus genus, correlating with the conservation of IFN-α-dependent interaction with, and NES-dependent nuclear exclusion of, STAT1. Importantly, the conservation of this mechanism of IFN antagonism in distantly related viruses such as MOKV and LBV, the P-proteins of which differ significantly at their C termini compared with other lyssavirus P-proteins (Fig. 2B), suggests that this region may not directly contribute to the STAT1/2 binding site. Indeed, the C-terminal 10 residues of CVS P-protein include residues implicated in stabilization of the core CTD structure (18, 27), such that the impairment of STAT1/2 binding and IFN antagonist functions by deletion of this region (3, 45; this report) may be due to effects on the globular CTD structure that impact on a distinct CTD-localized STAT1/2 binding site.

In conclusion, we have shown that the unique mechanism of RABV P-protein inhibition of IFN-α-dependent STAT1/2 signaling is conserved in the lyssavirus genus. Because RABV P-protein-mediated inhibition of IFN-α-dependent STAT1/2 signaling correlates with the pathogenicity of RABV in infected animals (17), this step in viral infection is likely to represent a key pathogenicity factor and potential therapeutic target not only for RABV but also for emerging lethal human-pathogenic viruses, including ABLV and MOKV.

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Author/s:
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