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Brief Report

Phenotypic divergence of P proteins of Australian bat lyssavirus lineages circulating in microbats and flying foxes

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Abstract: Bats are reservoirs of many pathogenic viruses including the lyssaviruses rabies virus (RABV) and Australian bat lyssavirus (ABLV). Lyssavirus strains are closely associated with particular host reservoir species, with evidence of specific adaptation. Associated phenotypic changes remain poorly understood but are likely to involve phosphoprotein (P protein), a key mediator of the intracellular virus-host interface. Here, we examine the phenotype of P protein of ABLV, which circulates as two defined lineages associated with frugivorous and insectivorous bats, providing the opportunity compare proteins of viruses adapted to divergent bat species. We report that key functions of P protein in antagonism of interferon/signal transducers and activators of transcription 1 (STAT1) signaling and the capacity of P protein to undergo nuclear trafficking differ between lineages. Molecular mapping indicates that these differences are functionally distinct, and appear to involve modulatory effects on regulatory regions or structural impact, rather than changes to defined interaction sequences. This results in partial but significant phenotypic divergence, consistent with 'fine-tuning' to host biology, and with potentially distinct properties in the virus-host interface between bat families that represent key zoonotic reservoirs.

Keywords: Australian bat lyssavirus, lyssavirus, rabies virus, immune evasion, nuclear trafficking, interferon, STAT1, bats, virus reservoirs, adaptation

1. Introduction

Bats are reservoirs for viruses including henipaviruses, several coronaviruses, and most, if not all, members of the lyssavirus genus, and carry many viruses that are pathogenic in other animals without developing disease, indicative of a specialised relationship [1, 2]. Cross-species transmissions (CSTs) of viruses from bats pose significant threats to human health through emergence of novel diseases, particularly where spill over results in further transmission or sustained maintenance in new hosts. CSTs from zoonotic reservoirs without further transmission can also represent major burden; for example, humans are dead-end hosts for lyssaviruses that cause rabies, an invariably lethal acute meningoencephalitis, but rabies results in c. 59,000 human deaths each year [1, 3, 4]. This is largely due to transmission of RABV from dogs, although infections also include RABV and other lyssaviruses transmitted from bats and other mammals [1, 2].

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Lyssaviruses comprise at least 16 species and numerous lineages/strains, classified within three or more phylogroups that have distinct serologic reactivity [1, 3]. Cross-reactivity from RABV vaccines is evident within phylogroup 1 (that includes RABV and ABLV) but not more distantly related lyssaviruses [1, 3]. RABV/lyssviruses are thought to cause disease in most if not all mammals, but epidemiological and experimental data indicate that species or strains/lineages are adapted to particular host species, with CSTs typically resulting in dead-end infections [1, 3]. Notably, lyssaviruses can cause lethal disease in reservoir hosts, so adaptation does not appear to derive from major changes in the host-virus interface causing loss of pathogenesis, but rather from a balance of the virus-host dynamic enabling persistence in the population [3, 4]. Since lyssaviruses share a relatively broad tissue tropism and largely indistinguishable pathogenesis, adaptation probably involves subtle phenotypic changes including at molecular host-virus interface to 'fine-tune' strains to their host [3]. The nature of these changes remains poorly understood.

Among lyssaviruses, RABV uniquely exists in independent cycles in multiple volant and non-volant mammalian species, where the association of each host with a specific strain and absence of circulation of the same strain in multiple hosts is consistent with historical CSTs and adaptation [1, 3, 4]. Notably, RABV variants circulate in terrestrial mammals and, in the new world only, in diverse bat species [3]. In contrast, while most non-RABV lyssaviruses are maintained in bats, they have not been detected in new world bats and are maintained in single species or highly restricted species range [3]. RABV is considered the main cause of human rabies, but CSTs of other lyssaviruses occur, including in countries considered rabies-free such as Australia, where ABLV has infected humans and horses [1, 5-7]. Misdiagnosis, underreporting and a lack of discriminatory diagnostics suggest estimates of rabies deaths are conservative, and likely to include more non-RABV lyssavirus infections than currently assumed; this highlights risks to public health in the absence of pan-lyssavirus protection by current vaccines [3].

Lyssavirus P-protein is a multifunctional protein with critical roles in replication as an essential polymerase cofactor, and at the intracellular virus-host interface where its best-characterised roles are in evasion of antiviral signalling by interferon (IFN) cytokines [8]. Following infection, cells release type-I IFNs (IFNα/β), which induce phosphorylation of signal transducers and activators of transcription 1 (STAT1) at a conserved tyrosine (Y701) generating pY-STAT1. pY-STAT1 translocates to the nucleus and, in association with pY-STAT2 and IRF9, activates transcription of IFN-stimulated genes (ISGs) [9]. The globular C-terminal domain (CTD) of P-protein (Figure 1) binds pY-STAT1 to inhibit signalling [10-13]. P-protein also undergoes nuclear trafficking *via* several nuclear localization (NLS) and export (NES) sequences (see Figure 1; [14-19]); these were identified in RABV P-protein, but critical residues and trafficking are broadly conserved among lyssaviruses [20]. Since lyssavirus replication is cytoplasmic, nuclear trafficking is thought to enable host modulation, including STAT1 antagonism [8]. In full-length P protein (the most abundant isoform in infected cells [21]), a dominant N-terminal NES (N-NES) effects strongly cytoplasmic localization at steady state while a NLS (C-NLS) in the CTD enables nucleocytoplasmic shuttling [14]. A C-terminal NES (C-NES) and protein kinase C (PKC, S210) phosphorylation site, and a dynein light chain association sequence (DLC-AS) also regulate trafficking (Figure 1). Additional sequences important in smaller isoforms are not active in full-length P-protein [15, 22]. The N-NES enables IFN antagonism by effecting nuclear export of P-protein-associated pY-STAT1, such that the STAT1-binding site and N-NES of P-protein are critical to immune evasion, and are pathogenesis factors [12, 20, 23]. Nuclear import has also been implicated, as binding of P-protein to STAT1 inhibits DNA interaction, an intranuclear event [18]. However, the precise significance of P-protein nuclear import in STAT1 antagonism and pathogenesis remains unresolved.

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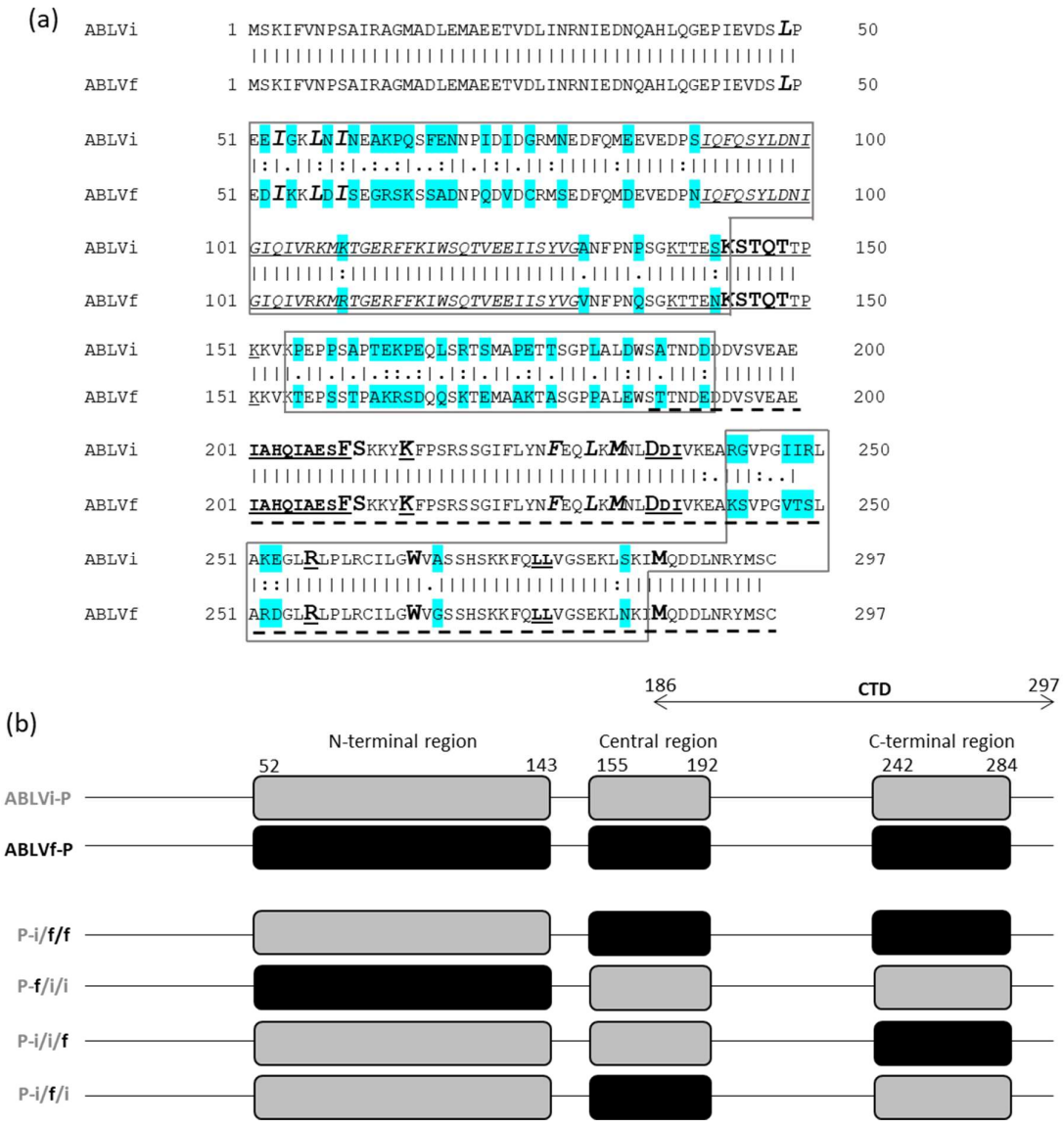

Figure 1. Sequence alignment of ABLV P-proteins and schematic of P-proteins and chimeras generated. (a) Alignment of sequences ABLVi and ABLVf P-proteins used; shading indicates substitutions between the proteins, with boxes indicating clusters of substitutions in the N-terminal (residues 52-143), Central (residues 155-192) and C-terminal (residues 242-284) regions. Key residues and sequences are indicated, including sequences important to nuclear trafficking: N-NES motif (residues 49-58, hydrophobic residues of motif in large bold font); DLC-AS motif (within residues 139-151, underlined; KSTQT motif is in large bold font); C-terminal NLS (C-NLS; key residues K214 and R260 in large bold font and underlined); S210 PKC site (large bold font); C-terminal NES (C-NES; residues F227, L230, M232 in large bold italicized font); sequences implicated in STAT1 binding: STAT1-binding surfaces (201-209, 235-237, 276-77, bold/underlined; residues F209 and D235 in large font); W-hole residues W265 and M297 (large bold font). (b) Schematic representation of ABLVf, ABLVi and chimeric P-proteins generated. The globular C-terminal domain (CTD) is indicated (dotted line in (a), CTD in (b)).

2. Materials and Methods

2.1. DNA constructs, cell culture and transfections

cDNA encoding the P-protein was amplified by PCR using isolates of ABLV held within the CSIRO Australian Centre for Disease Preparedness. The coding sequences were inserted into pEGFP-C1 in frame C-terminal to GFP to produce green fluorescent protein (GFP)-fused P proteins [20]. Plasmids to express chimeric proteins variously encoding regions containing clusters of sequence diversity between the ABLV strains (Fig. 1B;

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specifically: the N-terminal region (residues 52-143), Central region (residues 155-192), and C-terminal region (residues 242-284)) were generated using overlap PCR from the ABLVi-P and ABLVf-P plasmids. Plasmids to express GFP-fused negative control proteins from the RABV CVS strain (CVS-N protein [nucleoprotein] and CVS-P⊚30 protein [P protein deleted for the C-terminal 30 residues]), which lack antagonism/binding of STAT1 were described previously [20].

293T, Cos-7 and HeLa cells were maintained in DMEM with 10% fetal bovine serum (37°C, 5% CO_2). To express the P proteins, cells were transfected using Lipofectamine 2000 or 3000 (Invitrogen) as previously described [20].

2.2 Reporter gene assays

Reporter gene assays were performed as previously described [10, 20]. Briefly, constructs to express viral proteins of interest were co-transfected into 293T cells together with luciferase (luc) reporter plasmids for IFNα/STAT1/2-dependent signalling (pISRE-luc, in which firefly luc is under the control of the IFN-STAT1/2-sensitive ISRE element) and transfection control (pRL-TK, from which *Renilla* luc is constitutively expressed). Cells were treated 6 h post-transfection without or with 1000 U/ml IFNα (PBL Assay Science) for 16 h before measurement of luciferase activity as previously described [20]; the ratio of Firefly (FF)/Renilla (RL) luciferase activity was determined. Luciferase activity was then calculated relative to that for IFN-treated cells expressing CVS-N (negative control for antagonism of IFN signalling [20]).

2.3 Co-immunoprecipitation (IP)

Cos-7 cells were transfected for 6 h with plasmid to express viral proteins or controls before treatment with 1000 U/ml IFNα for the specified times, followed by IP using the GFP-trap system (Chromotek) according to the manufacturer's instructions; protease inhibitor and PhosSTOP (Roche) were included in lysis and wash buffers [10, 13, 20]. Proteins were detected by SDS-PAGE and immunoblotting (IB), using anti-pSTAT1 (Invitrogen #33-3400 or Santa Cruz #sc-7988), anti-GFP (Roche #11814460001 or Abcam #Ab6556), and anti-⊚-tubulin (Sigma #T8328) antibodies.

2.4 Confocal Laser Scanning Microscopy (CLSM)

Cells were transfected for 24 h before treatment without or with 5.2 nM Leptomycin B (LMB, a gift from Minoru Yoshida, RIKEN, Japan) for 3 h and imaging live in phenol-free DMEM with heated chamber using a Nikon C1 inverted confocal laser scanning microscope. Images were analysed using Fiji software (NIH) to calculate the nuclear to cytoplasmic fluorescence ratio (Fn/c) corrected for background fluorescence from > 40 cells/group, as described previously [13, 26].

2.5 Statistical Analysis

Unpaired Student's *t*-test was performed using GraphPad Prism 7.

3. Results and Discussion

3.1. Antagonism of IFN signalling differs between ABLVi and ABLVf P proteins

ABLVi and ABLVf P protein are 83.8% identical, with substitutions clustered broadly within three regions, corresponding to an N-terminal, Central and C-terminal region (Figure 1A). To assess whether the changes impact on IFN/STAT-antagonistic functions, we used a dual luciferase reporter assay for IFNα-STAT1/2-dependent IFN signalling [10, 12, 20]. 293-T cells were transfected to express the P-proteins, and with plasmids for the

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luciferase assay, before treatment with IFNα and measurement of luciferase activity. Our previous analysis of P-proteins of different lyssaviruses used ABLVf-P, which inhibited IFNα-STAT1/2 signalling to a similar extent as P proteins of several RABV strains and Mokola virus [20], and we confirmed that ABLVf-P significantly reduces IFN-activated luciferase expression compared to the control (Figure 2A). Consistent with conservation of STAT1 antagonism between lyssavirus species [10, 20], ABLVi-P also reduced luciferase activity [20] but to a significantly lesser extent than ABLVf-P, despite equivalent expression (Figure 2A,B); comparable results were obtained using Cos-7 cells (Supplementary Figure S1). Thus, ABLVi-P is a less potent antagonist than ABLVf-P.

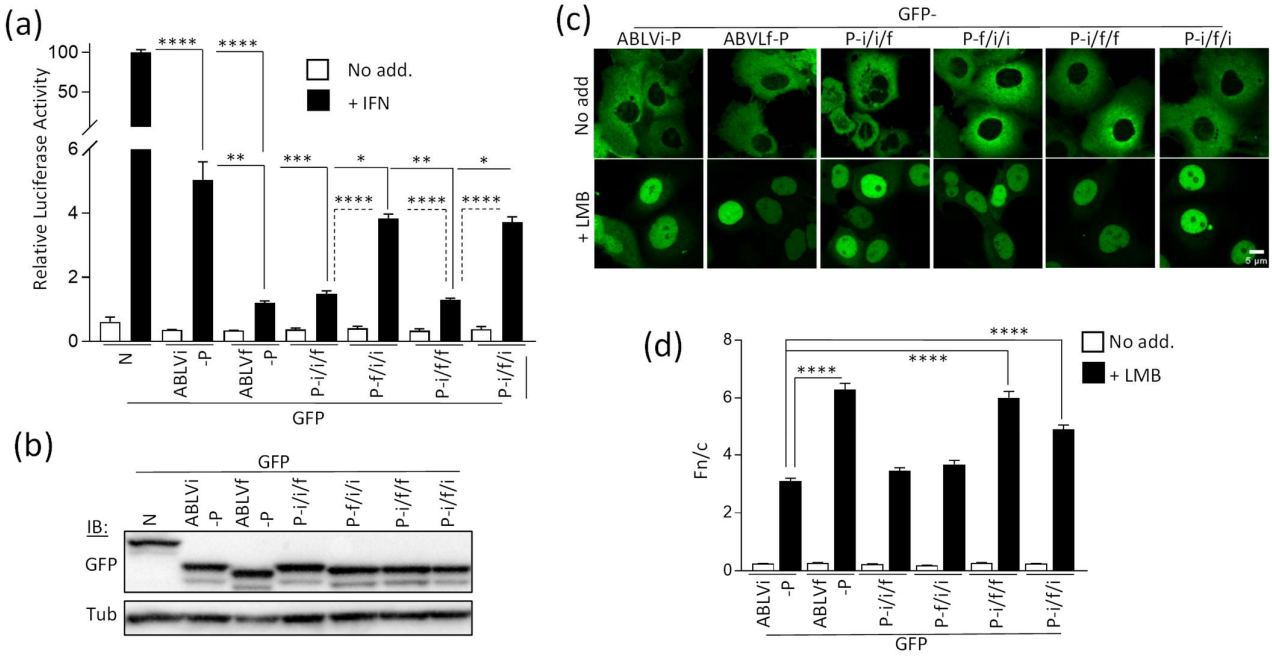

Figure 2. P-proteins of ABLVi and ABLVf differ in STAT1-antagonist function and nuclear trafficking. (a) 293T cells were transfected to express the indicated proteins and with plasmids for the IFNα/STAT1-dependent dual luciferase reporter assay before treatment with or without 1000 U/ml IFNα (16 h) and calculation of luciferase activity (ratio of firefly (FF)/renilla (RL) activity; mean ± SD). **** $P < 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ (Student's t-test); data representative of ≥ 3 assays. (b) Expression of GFP and tubulin (Tub., loading control) in lysates from reporter assays was determined by immunoblotting (IB) using the indicated antibody. (c) Cos-7 cells expressing the indicated proteins were treated with or without 5.2 nM Leptomycin B (LMB) before analysis by confocal laser scanning microscopy (CLSM). (d) Images such as those in (c) were analysed to calculate the ratio of nuclear to cytoplasmic fluorescence (Fn/c, mean ± SEM). **** $P < 0.0001$, $n > 40$ cells for each condition.

3.2 Nuclear trafficking differs between ABLVf and ABLVi P-protein

Antagonism of IFN/STAT1 signalling is associated with nucleocytoplasmic trafficking of P-protein [15, 20]. To determine whether differing antagonism correlates with altered trafficking, we analysed cells transfected to express GFP-fused P-proteins using confocal laser scanning microscopy (CLSM). In common with P-proteins of other lyssaviruses and consistent with conservation of the N-NES motif (Figure 1A; [20]) ABLVi and ABLVf P proteins were almost exclusively cytoplasmic at steady state (Figure 2C). Treatment of cells with leptomycin-B (LMB) to inhibit exportin-1 (which mediates N-NES-dependent nuclear export [19]) results in nuclear accumulation of P-protein in RABV-infected cells [16] and of transfected P-protein of RABV and lyssaviruses [19, 20], enabling analysis of nuclear import activity. Consistent with conservation of essential C-NLS residues (Figure 1A) [14, 17], LMB treatment resulted in nuclear accumulation of

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ABLVi-P and ABLVf-P proteins (Figure 2C,D). However, accumulation of ABLVf-P protein, determined by calculating nuclear to cytoplasmic fluorescence ratio (Fn/c) [13, 15, 20], significantly exceeded that of ABVLi-P protein (Figure 2C,D). Comparable results were observed in Hela cells (Figure S2). Thus, nuclear trafficking and IFN antagonism are maintained but differ in extent between ABLVi and ABLVf P proteins, such that differing STAT1 antagonism might relate to nuclear localization activity.

3.3 Differing nuclear trafficking and IFN antagonism depend on distinct regions of ABLV P-protein

Key sequences mediating nuclear trafficking by full-length RABV P protein are the N-NES, C-NLS/C-NES, S210, and the DLC-AS (Fig 1A) [14-17, 19, 27]. With respect to STAT1-binding, residues W265 and M287 of the CTD were initially implicated, as they form part of a hydrophobic pocket (referred to as the 'W-hole' [10, 12, 28]) in which naturally occurring substitutions between certain lyssaviruses (position 265, which is W in RABV P protein and G in DUVV P protein) or introduced mutations (W265G, M287V) inhibit STAT1 interaction [10]. These effects appear indirect, however, since the W hole is distant to the STAT1 interaction surface recently identified in the CTD using nuclear magnetic resonance spectroscopy (NMR) [12]. All of these key residues/motifs are conserved between ABLVi and ABLVf (Figure 1A), consistent with retention of major functions, and indicating that substitutions elsewhere impact as yet undefined regulatory sequences, or alter conformation.

To identify the regions responsible, we generated chimeric P-proteins (Figure 1B) variously containing clusters of substitutions (the N-terminal, Central and C-terminal regions) of ABLVi or ABLVf P (Figure 1). IFN/STAT1 reporter assays using these proteins (Figure 2A) indicated that the ABLVf-P C-terminal region is able to confer antagonist function comparable to that of wild-type ABLVf-P, indicating that changes in the CTD are the major determinant of the differing function in STAT1 inhibition. This is consistent with the CTD containing the STAT1 interface [11, 12]. However, the data also indicated minor effects of changes in the ABLVi-P protein N-terminal/Central regions, suggestive of accessory impact of other regions.

In contrast, CLSM analysis indicated that the C-terminal region of ABLVf-P protein is not sufficient to substantially enhance nuclear import of ABLVi-P protein (Figure 2 C,D). In fact, the ABLVf-P protein Central region was the only region sufficient to substantially increase nuclear import of ABLVi-P, with the N-terminal or C-terminal regions having little effect alone (Figure 2 C,D). Thus, the Central region is a principal determinant of differing nuclear import (Figure 2C,D), although recapitulation of nuclear import to levels comparable with wild-type ABLVf-P required both the Central and C-terminal regions of ABLVf-P protein. These data indicate that differences in nuclear localization of ABLVf-P and ABLVi-P are not due to discrete change in a specific interface with cellular trafficking machinery, but result from substitutions in several regions, with changes to the Central region of primary importance. Since the globular C-terminal domain contains the C-NLS, the data indicate that efficient activity of this sequence requires additional sequences in the Central region. Notably, the Central region lacks defined trafficking sequences for full-length P protein (Figure 1A), and key residues of the DLC-AS, C-NLS or the regulatory PKC site (S210) are not altered between ABLVf and ABLVi P protein (Figure 1A), so the effect of substitutions is likely to involve modulation of previously unrecognised regulatory sequences, or conformational effects impacting function/presentation of the NLS. Taken together, the distinct requirements for efficient IFN antagonism and nuclear trafficking, particularly with respect to the C-terminal region, indicate that differing antagonism does not result from altered nuclear import.

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3.4 Differing antagonistic functions of ABLV P protein are due to altered interaction with STAT1

Our data indicated that differences in STAT1 antagonism by ABLV P-proteins are dependent on the C-terminal region, which forms part of the CTD that binds to STAT1 [11, 12]. We thus hypothesised that ABLVf and ABLVi P proteins might differ in interaction with STAT1. Efficient P-protein-STAT1 interaction in cells requires IFN treatment, indicative of high affinity binding to pY-STAT1 such that P-protein selectively targets IFN-stimulated signalling processes [11, 20]. We thus examined interactions of P-proteins with pY-STAT1 by co-immunopreciptation (IP) of GFP-fused P-protein from cells following treatment with IFN for 0.5 h or 16 h (as previously [20]), and analysis of lysates and IPs by immunoblotting (IB) (Figure 3).

pY-STAT1 was not detected in untreated cells, but was clearly induced following 0.5 h treatment with IFN, with no inhibitory effect of P proteins of CVS RABV, ABLVi or ABLVf compared with CVS-P⊗30 (a standard control lacking STAT1-binding activity [11, 20]) (Figure 3A). This is consistent with previous reports that lyssavirus P-proteins do not impair phosphorylation of STAT1 at Y701 [20, 29].

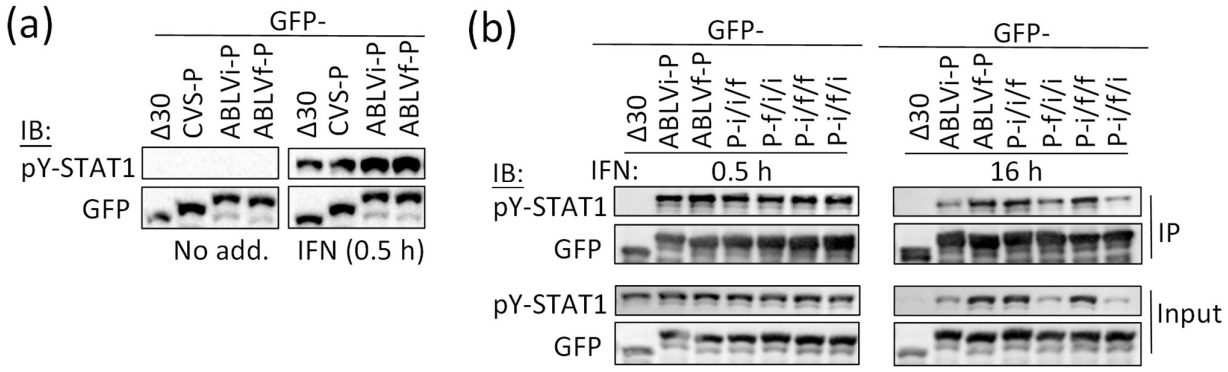

Figure 3. P-protein-STAT1 interaction differs between ABLV P-proteins and correlates with differing antagonist function. (A) Cos-7 cells expressing the indicated proteins were treated with or without 1000 U/ml IFNα for 0.5 h before lysis and analysis by IB for Y701-phosphorylated STAT1 (pY-STAT1) and GFP. (B) Cells expressing the indicated proteins were treated for 0.5 and 16 h with IFN before IP for GFP and analysis of lysate (input) and IP by IB. Results representative of 3 experiments.

Following an early peak in Y701 phosphorylation after IFN treatment (c. 0.5 h - 1 h), pY-STAT1 that has translocated to the nucleus to activate IFN-stimulated genes is inactivated by dephosphorylation by nuclear phosphatases [9, 12, 18, 20, 29]. This process is inhibited by P protein, likely due to inhibition of DNA interaction/cytoplasmic retention of pY-STAT1 to antagonise signalling [9, 12, 18, 20, 29]; this results in accumulation of pY-STAT1 in inhibitory complexes, presumably enabling sustained antagonism. We observed typical activation/dephosphorylation of STAT1 in cells expressing control protein, where pY-STAT1 accumulated at 0.5 h and was lost by 16 h IFN treatment (Fig 3B). At the 0.5 h time point, comparable levels of pY-STAT1 were detected in lysates of cells expressing wild-type or chimeric P-proteins (Figure 3B), and interaction detected by IP was also comparable. pY-STAT1 remained detectable in cells expressing all P-proteins after 16 h IFN treatment, indicative of interaction (Figure 3B). However, levels of pY-STAT1 were clearly higher in cells expressing ABLVf-P or chimeras containing the ABLVf-P C-terminal region (P-i/i/f, P-i/f/f) compared with cells expressing ABLVi-P or chimeras containing the ABLVi-P C-terminal region (P-f/i/i, P-i/f/i), indicating reduced interaction by the latter; this was supported by analysis of IPs (Figure 3B). Thus, differing IFN-antagonist function of ABLVf and ABLVi P-proteins

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appears to be due to altered interaction with STAT1 due to changes in the C-terminal region, proximal to the STAT-binding site in the CTD (Figure 1; [11, 12]). However, as interaction with pY-STAT1 was observed for all proteins, the substitutions do not disable the STAT1-binding site (consistent with conservation of critical STAT1 interaction residues (Figure 1A)) but rather impair interaction efficiency.

The finding that P proteins/chimeras bind well to pY-STAT1 after 0.5 h IFN treatment (at peak levels of pY-STAT1) with differences in proteins containing the ABLVi-P C-terminal region becoming apparent at 16 h indicates a reduced ability to retain pY-STAT1, probably relating to reduced affinity and an associated increase in dephosphorylation by cellular phosphatases. These observations are similar to recent findings using RABV P-protein mutated at the W-hole, which strongly inhibits antagonism of STAT1 signalling. Mutated protein retained significant STAT1-binding/antagonistic activity at early time points post-IFN treatment (albeit reduced compared with wild-type protein), and could not efficiently retain pY-STAT1 over extended incubation [12]. This is consistent the W-hole not forming part of the STAT1 binding site, such that mutations reduce binding efficacy without preventing interaction; in contrast, mutation of the STAT1 binding site at key residues F209/D235 (Figure 1A) ablated pY-STAT1 binding at all time points [12]. Our data using ABLV P-proteins support the idea that antagonistic function of P protein depends not only on initial binding to pY-STAT1, but also retention of pY-STAT1 in inactive complexes by inhibiting cycles of phosphorylation/dephosphorylation.

The W-hole was initially implicated in P protein-STAT1 interaction because differences between this region in DUVV and RABV P protein correlated with altered STAT1 antagonism. However, residues of the recently defined STAT1-binding interface (I201-F209, D235-I237, L276-V277) are highly conserved between lyssaviruses, including DUVV (having only a single conservative substitution compared with RABV, D236 to E236), and ABLV P proteins (residues are absolutely conserved (see Figure 1A; [12])), consistent with critical roles. Thus, functional variation between species and lineages appears to involve changes at regulatory/modulatory sites. Given the significance of STAT1 targeting in infection [10, 12], it is unlikely that DUVV/ABLVi have adapted primarily to attenuate STAT1-antagonism, and both viruses are pathogenic [1, 6]. Furthermore, STAT1 and IMPα2 (which binds to the P-protein C-NLS [14]) show >96% identity and >98% sequence similarity, respectively, between P. Alecto and human proteins, so changes are unlikely to reflect differing molecular interactions between bat species. Rather it seems likely that requirements for other functions, including rate of genome replication or the nature of the broader Chiropteran IFN system [2] might differ between bat hosts, resulting in subtle changes to fine tune the virus to the host, with the emergence of associated phenotypic changes in the multifunctional P protein.

Supplementary Materials: Supplementary Figure S1. Cos7 cells were transfected to express the indicated proteins and with plasmids for the IFNα/STAT1 reporter assay before treatment with or without IFNα and calculation of luciferase activity as described in the legend to Figure 2. Supplementary Figure S2. Hela cells expressing the indicated proteins were treated with or without LMB before analysis by CLSM and calculation of Fn/c (mean ± SEM), as described in the legend to Figure 2. **** $P < 0.0001$, $n \geq 40$ cells for each condition.

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