Interaction of Rabies Virus P-Protein With STAT Proteins is Critical to Lethal Rabies Disease

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Background. Rabies virus (RABV) causes rabies disease resulting in >55,000 human deaths/year. The multifunctional RABV P-protein has essential roles in genome replication, and forms interactions with cellular STAT proteins that are thought to underlie viral antagonism of interferon-dependent immunity. However, the molecular details of P-protein-STAT interaction, and its importance to disease are unresolved.

Methods. Studies were performed using sequence/structure analysis, mutagenesis, immunoprecipitation, luciferase and qRT-PCR-based signaling assays, confocal microscopy and reverse genetics/in vivo infection.

Results. We identified a hydrophobic pocket of the P-protein C-terminal domain as critical to STAT-binding/antagonism. This interface was found to be functionally and spatially independent of the region responsible for N-protein interaction, which is critical to genome replication. Based on these findings, we generated the first mutant RABV lacking STAT-association. Growth of the virus in vitro was unimpaired, but it lacked STAT-antagonist function and was highly sensitive to interferon. Importantly, growth of the virus was strongly attenuated in brains of infected mice, producing no major neurological symptoms, compared with the invariably lethal wild-type virus.

Conclusions. These data represent direct evidence that P-protein-STAT interaction is critical to rabies, and provide novel insights into the mechanism by which RABV coordinates distinct functions in interferon antagonism and replication.

Keywords. Viral disease; interferon; signal transducers and activators of transcription; immune evasion; interferon antagonist; lyssavirus; rabies virus; Duvenhage virus; pathogenicity; replication.
heterodimerize and translocate to the nucleus where they activate IFN-stimulated genes (ISGs) including ISG15 and MxA, which are important to the establishment of an antiviral state. The STATs are then dephosphorylated by nuclear phosphatases, and exported to the cytoplasm [3, 4].

Viruses counter these responses by expressing IFN antagonist proteins [4]. Although the specific mode of action of these factors can vary between different viruses, STATs are major targets of many IFN antagonists, including those of RABV/lyssavirus, dengue virus, influenza virus and paramyxoviruses [5], presumably due to the critical role of STATs in antiviral responses. The STAT-targeting activity of IFN antagonists is thus considered a determining factor in pathogenicity [3, 4, 6]. However, for many viruses, including lyssavirus and paramyxoviruses, the genuine importance of STAT-targeting in infection and disease is unknown, primarily due to the fact that IFN antagonists are often multifunctional proteins with roles both in inhibiting immune signaling and in genome replication [3]. Thus, deletion or mutation of these proteins can impair viral growth/replication independently of effects on IFN antagonism [7].

The P-proteins are considered the major IFN antagonists of lyssavirus due to their capacity to bind STATs via their C-terminal domain (CTD, residues 186–297 of RABV P-protein [8–10]), and cause nuclear exclusion of P-protein–STAT complexes via a strong export sequence within P-protein [11]; P-proteins can thereby inhibit activation of IFN-dependent reporter genes in protein expression studies [12]. Importantly, P-protein is multifunctional, having essential roles in genome replication as the polymerase cofactor through interaction of the CTD with viral N-protein [13, 14]. This suggests that the CTD has dual functions critical to infection, but the molecular details of P-protein–STAT interaction, including the location and constituent residues of the STAT-binding interface, and the structural mechanisms by which P-protein coordinates interactions with N- and STAT proteins, are unresolved. Importantly, no mutations have been identified that can inhibit STAT-binding without also inhibiting replication such that no viable mutant lyssavirus lacking STAT-binding function has been generated, preventing specific examination of the role of STAT antagonism in infection.

Here, we demonstrate that P-protein interactions with and antagonism of STATs is dependent on residues within a unique hydrophobic pocket (the “W-hole”) of the CTD. Structural and mutagenic analysis indicated that P-protein thus utilizes functionally and spatially distinct interfaces in the CTD to coordinate interactions with N- and STAT proteins. Based on these findings, we introduced mutations into RABV to specifically inhibit STAT interaction, generating viable virus with growth kinetics indistinguishable from the parental strain in vitro, but which lacked IFN/STAT antagonist activity. This virus was highly sensitive to IFN and severely attenuated in vivo causing no lethality in mice, in contrast to the invariably lethal parental strain.

### METHODS

**Constructs, Cells, Transfections and Infections**

Constructs were generated by standard techniques (see Supplementary Information) or are described elsewhere [12, 15, 16].

Cells used were Cos-7, HEK293T, NA, Vero, SK-N-SH and BHK/T7-9 (see Supplementary Information for culture conditions). Transfections using Lipofectamine2000 (Invitrogen) and infections were performed as previously [12, 17].

**Luciferase Assays**

For IFNα-dependent signaling assays, Cos-7 cells cotransfected with pISRE-luc, pRL-TK, and pEGFP-C1 encoding P-proteins (6 hours) were treated without or with 1000 U/mL recombinant human IFNα (PBL Interferon Source) for 16 hours before analysis in a dual luciferase assay, and calculation of relative luciferase activity as previously [12].

For minigenome assays, HEK293T cells transfected with pRVD1-luc, pC-RN, pC-RL, and pEGFP-C1 encoding P-proteins (48 hours) were analyzed for firefly luciferase activity as above (see Supplementary Information).

**qRT-PCR**

HEK293T cells mock-transfected or transfected to express GFP-P-protein were treated 24 hours later without or with 1000 U/mL IFNα (8 hours) to activate STAT-dependent signaling. To activate IFNβ, cells were cotransfected with Flag-RIG-I (24 hours). Following activation, cells were lysed for total RNA extraction (QIAGEN) and analysis by qRT-PCR using the SensiMix SYBR Hi-ROX kit (Bioline) (Supplementary Information).

**Immunoprecipitation (IP)**

IP used the GFP-Trap system (Chromotek GmbH) with wash buffer supplemented with 1 × PhosSTOP and 1 × protease inhibitor, followed by immunoblotting (IB) analysis as previously [12] (see Supplementary Information).

**Yeast-2-hybrid (Y2H)**

Two-hybrid analysis was performed using L40 yeast strain as previously [16] (see Supplementary Information).

**Reverse Genetics**

Mutations were introduced to the CE-NiP-WT genome plasmid by overlap PCR as previously [17], and recombinant virus rescued in BHK/T7-9 cells. Viral stocks were prepared in NA cells, which are commonly used to prepare IFN-sensitive strains [18–20] and titers were determined by focus formation assay to calculate focus forming units (ffu)/mL as previously [17] (see Supplementary Information).

**IFN Sensitivity Assays**

Growth of virus in Vero cells inoculated at multiplicity of infection (MOI) 0.001 was analyzed daily by focus formation assay.
In some assays, infected cells were cultured after 1 day post inoculation (dpi) with 500 U/mL IFNα (see Supplementary Information).

**Mouse Infection**
12 6-week-old female ddY mice (Japan SLC Inc.) per group were inoculated intracerebrally (i.c.) with 0.03 mL of diluent (mock) or diluent containing 10⁴ ffu of virus. Mice were inspected for symptoms over 21 days (see Supplementary Information). To measure viral titer in brains, mice were euthanized at 5 dpi and brains homogenized for analysis by focus formation assays. Experiments were approved by the Committee for Animal Research and Welfare of Gifu University (Approval No. 10086).

**Confocal Laser Scanning Microscopy**
SK-N-SH cells infected at MOI of 0.01 (18 hours) were treated without or with IFNα (4000 U/mL, 0.5 hours) before fixation and immunostaining for STAT1 and N-protein or Y701-phosphorylated STAT1 (pY-STAT1) and P-protein, followed by confocal microscopy analysis to calculate the nucleocytoplasmic fluorescence ratio (Fn/c) (mean Fn/c, n > 30 cells) [12] (see Supplementary Information).

**Statistical Analysis**
Unpaired two-tailed Student’s t-test was performed using GraphPad Prism (5.0c).

**RESULTS**

**STAT-binding and IFN Antagonist Activity of DUVV P-protein is Reduced Compared With That of RABV P-protein**

We previously found that the P-proteins of several RABV strains, ABLV, and the distantly related MOKV interact with STATs and inhibit IFN-signaling to similar extents, indicating that this function is conserved across the genus [12]. DUVV causes lethal human disease, but is less pathogenic in mice than a WT RABV street strain [21]. To compare STAT antagonism by DUVV and RABV P-proteins, we expressed GFP-fused P-proteins in Cos-7 cells, and assessed effects on IFNα-signaling using a luciferase reporter gene assay [12]. IFNα-signaling, indicated by induction of luciferase activity, was significantly (P < .0001) reduced in cells expressing RABV P-WT compared with cells expressing a control P-protein lacking the C-terminal 30 residues (P-ΔC30) that does not bind STATs [10] (Figure 1A). Intriguingly, although DUVV P-WT also inhibited IFNα-signaling, it did so to a significantly (P < .0001) lesser extent than RABV P-protein (Figure 1A).

To examine whether this difference related to differing STAT interaction, we performed IP of GFP-fused P-proteins from cell lysates used in the luciferase reporter assays and analyzed by IB. RABV P-protein interacts selectively with IFN-activated STATs, such that STAT1/2 are only precipitated from IFN-treated cells, while P-ΔC30 shows no interaction with STATs [12, 22] (Figure 1B). Importantly, although DUVV P-WT interacted with STAT1/2 in IFN-treated cells, it precipitated lower amounts of both compared with RABV P-WT (Figure 1B). Comparable results were obtained from luciferase reporter and IP assays using HEK293T cells (not shown). Thus, IFN antagonism by DUVV P-protein was impaired compared with that of RABV P-protein, and this correlated with differing interaction with STATs.

**W-hole Residues W265 and M287 are Essential for STAT1/2-binding**

Structural analysis of the RABV and MOKV P-protein CTDs has identified a conserved fold forming a “half-pear” structure
with two putative molecular interfaces: a positive patch on the
round face, implicated in N-protein-binding, and the W-hole
on the flat face, of unknown function [13, 14, 23] (Figure 2A
and 2B).

Analysis of P-protein sequences from 120 field isolates of
RABV, DUVV, and 12 other lyssaviruses indicated that positive
patch residues K211, K214 and R260 are 100% con-
served, and W-hole residues C261 and M287 show nearly
100% identity (not shown), consistent with important func-
tions. Interestingly however, W265 of RABV P-protein is sub-
stituted for glycine in DUVV. To examine the potential role of
this substitution in P-protein-STAT interaction, we substituted
W265 for glycine in RABV P-protein (P-W265G, denoted as
W in figures), identifying substantially impaired STAT1/2 in-
teraction and antagonism in IP and luciferase reporter assays
(Figure 2C).

To further assess the role of the W-hole, we selected for muta-
tion the residue M287, which is 100% conserved among lyssavi-
rus P-proteins except those of MOKV and Ikoma virus where it
is substituted for isoleucine. STAT-binding and antagonism by
the P-protein of MOKV is comparable to those of RABV and
ABLV [12], suggesting that the methionine sulphur atom is not
critical, but that size/structure of the residue might be important.
We thus mutated M287 of DUVV and RABV P-proteins to
valine (P-M287V, denoted as M in figures), thereby retaining hy-
drophobicity at this position, but introducing a shorter, bulkier
residue. This substantially reduced IFNα antagonism and STAT-
binding by RABV P-protein in IP and luciferase reporter assays,
and entirely prevented these functions in DUVV P-protein
(Figure 2C), perhaps relating to a requirement for conformational
flexibility in STAT-binding that is restricted due to the presence
of the branched β-carbon atom in the side chain of valine. This

Figure 2. W-hole residues 265 and 287 are critical to STAT antagonism by P-protein. Surface representations showing the side (A) and flat face (B) of the RABV P-protein CTD (residues 186–297, PDB file 1VYI) [23] with W-hole residues W265, M287 and C261 in red and residues K211, K214 and R260 of the positive patch (corresponding to the N-binding site) in blue. C and D, Luciferase reporter assays (upper panels) and IP analysis of corresponding samples (lower panels) from IFNα-treated (1000 U/mL, 16 hours) Cos-7 cells expressing the indicated GFP-fused P-proteins were performed as described in the legend to Figure 1 (mean relative luciferase activity ± SEM, n = 4; data are from a single assay representative of 3 independent assays; P-M287V, M; P-W265G, W; P-W265G/M287V, W/M); ns, non-significant; **P ≤ .01; ***P ≤ .001; ****P ≤ .0001. The Western blots are representative of 3 independent assays.
suggested that combined mutation of W265G and M287V would strongly impact STAT-binding/antagonism, and this was confirmed in IP and luciferase reporter assays using Cos-7 and HEK293T cells expressing RABV P-protein containing both mutations (P-W265G/M287V, denoted as W/M in figures) (Figure 2D, not shown). In addition qRT-PCR analysis indicated that W265G/M287V mutation entirely prevents P-protein antagonism of IFN-dependent activation of the ISGs ISG15 and MxA (Figure 3), which have been implicated in negative strand RNA virus infection [8, 24, 25]. The difference observed between P-WT and P-W265G/M287V was reproduced in 3 separate qRT-PCR assays.

P-protein expression or RABV infection also inhibits dephosphorylation of IFN-activated pY-STAT1, potentially by inhibiting interaction with nuclear phosphatases, and thereby impairing STAT-recycling [22]. IB analysis of Cos-7 cell lysates revealed that pY-STAT1 was clearly present in RABV P-WT-expressing cells at 16 hours post-IFN treatment, as expected [12], but was undetectable in cells expressing P-ΔC30 or P-W265G/M287V (Supplementary Figure 1A), consistent with defective STAT1 interaction. Thus, W265G/M287V mutation strongly impairs P-protein interaction with and functional modification of STATs.

**W265G/M287V Mutation Does Not Impair P-protein Functions in Genome Replication or Antagonism of IFN Induction**

Since the W-hole and N-protein-binding sites are spatially distinct in the CTD (Figure 2A and 2B) [14, 23], we hypothesized that STAT-binding/antagonism and N-binding/replication functions of P-protein might be separable. Consistent with this, IP assays using Cos-7 cells coexpressing GFP-fused CTD regions of WT or mutant RABV P-protein with mCherry-fused N-protein revealed that N-protein interacts with P-W265G, P-M287V, and P-W265G/M287V CTDs, but not with a control CTD in which the N-binding site is mutated (P-K214A/R260A, denoted as K/R in figures) (Figure 4A). Comparable results were obtained using HEK293T cells (not shown) as well as by Y2H analysis (Figure 4B). P-ΔC30, previously used as a STAT-binding deficient P-protein, lacks N-binding function through the CTD [26], indicative of broad effects due to deletion of 30 residues from the globular domain [14]. Thus, W265G/M287V mutation appears to affect STAT-binding selectively.

To confirm that P-W265G/M287V is functional in genome replication, we used a minigenome system encoding a luciferase reporter (Supplementary Information). RABV P-W265G, P-M287V
and P-W265G/M287V induced luciferase expression to the same extent as P-WT, indicative of unimpaired polymerase co-factor activity; as expected P-K214A/R260A and P-ΔC30 lacked this function (Figure 4C). Together these data indicated that P-protein interactions with N-protein and STATs are separable, and likely to be mediated by discrete independent interfaces (the positive patch and W-hole, respectively) on opposite faces of the CTD (Figure 2A and 2B). Consistent with this, although K214A/R260A mutations prevented P-N-protein interaction, they did not prevent P-protein-STAT-binding (Supplementary Figure 1B).

In addition to roles in STAT antagonism and genome replication, P-protein can inhibit viral induction of IFNβ [27]. To examine potential effects thereon of W265G/M287V mutation, we used RIG-I overexpression to induce IFNβ expression [15, 28, 29], detecting clear induction by qRT-PCR (Figure 4D). This was strongly inhibited by P-WT and, importantly, P-W265G/M287V caused inhibition to the same extent (Figure 4D). Thus, the effects of the mutations appear to be highly specific to antagonism of STAT1/2.

**RABV Carrying W265G and M287V Mutations is Viable But Highly Sensitive to IFN**

To examine the effect of W265G/M287V mutation in RABV, we used the CE-NiP strain (hereon referred to as CE-NiP-WT), which showed can efficiently bind STAT1 via the P-protein and inhibit STAT1/2-dependent signaling, and causes neurological symptoms and death in infected mice [17]. Introduction of W265G/M287V mutations generated the CE-NiP-STAT(-) virus, which was clearly viable, producing titers of >10^7 ffu/mL in NA cells. RT-PCR/sequencing confirmed that the rescued virus retained the W265G/M287V mutations.

To compare the growth kinetics of CE-NiP-STAT(-) and CE-NiP-WT, we infected Vero cells, which do not produce IFN [30], and monitored growth by focus formation assays, with results indicating identical growth (Figure 5A). Importantly, however, in cells treated with 500 U/mL IFNα for 2 days, growth of CE-NiP-STAT(-) was substantially impaired compared with CE-NiP-WT (a decrease of ca. 3 log vs ca. 1 log, respectively), indicative of greatly increased IFN sensitivity of the mutant strain (Figure 5B).

To examine effects of the mutations on viral inhibition of STAT responses, we infected human neuroblastoma SK-N-SH cells and treated without or with IFNα (4000 U/mL, 0.5 hours) before fixation and immunostaining for N-protein and STAT1 (Figure 6A) or P-protein and pY-STAT1 (not shown), and analysis by confocal microscopy. Consistent with conservation of N-binding and replication function in P-W265G/M287V protein, viral N- and P-antigen was clearly detectable in cells infected by CE-NiP-WT and CE-NiP-STAT(-), and showed a typical distribution, with accumulation in cytoplasmic Negri bodies (the major sites of genome transcription/replication [31]) (Figure 6A, not shown). Calculation of the nucleocytoplasmic fluorescence ratio for P-protein [12] also indicated that its nucleocytoplasmic localization was equivalent in CE-NiP-WT and CE-NiP-STAT(-)-infected cells (not shown), demonstrating that the mutations do not affect nuclear export of P-protein, which was previously implicated in STAT antagonist function [17]. By contrast, STAT1 localization differed between IFN-treated cells infected with CE-NiP-WT and CE-NiP-STAT(-), with clearly greater levels of nuclear STAT1 in the latter (Figure 6A, not shown). Determination of the nucleocytoplasmic fluorescence ratio confirmed significantly (P < .0001) greater localization of IFN-activated STAT1 to the nucleus of CE-NiP-STAT(-)-infected cells compared with CE-NiP-WT-infected cells (Figure 6B, not shown). Importantly, the nuclear accumulation of IFN-activated STAT1 in CE-NiP-STAT(-)-infected cells was not different to that in mock-infected

![Figure 5](image-url)

**Figure 5.** CE-NiP-STAT(-) virus is more sensitive to IFN than CE-NiP-WT virus. A, Vero cells were infected with CE-NiP-WT or CE-NiP-STAT(-) viruses (MOI of 0.001) and titers (ffu/mL) were determined every 24 hours for 4 dpi by focus formation assays (data are from a single assay representative of 3 independent assays). B, Vero cells infected as in A were treated at 1 dpi without or with 500 U/mL IFNα and titers were determined at 3 dpi (data are from a single assay representative of 2 independent assays).
cells, consistent with a strong defect in P-protein-STAT complex formation.

**CE-NiP-STAT(-) Virus is Strongly Attenuated In Vivo**

To examine viral pathogenicity, we i.c. inoculated 12 ddY mice with 10^4 ffu of CE-NiP-WT or CE-NiP-STAT(-), and monitored symptoms over 21 dpi (Figure 7A and 7B). CE-NiP-WT infection caused marked weight loss and severe neurological symptoms in all mice by 7 dpi (Figure 7A and 7B), similar to previous observations [35], and all mice succumbed to infection or reached a non-responsive end-point between 6 and 13 dpi (Figure 7A). By contrast, the only symptoms of CE-NiP-STAT(-) infection were temporary weight loss (11/12 mice) and mild ataxia in one mouse (Figure 7A and 7B). No neurological symptoms were observed by 21 dpi, and weight loss/ataxia was no longer evident by 18 dpi (Figure 7A and 7B), indicating recovery from infection. Comparable results were obtained in two independent assays (Supplementary Movie 1). Importantly, the infectious virus load of mouse brains infected with CE-NiP-STAT(-) (5 dpi) was 10^3-10^6-fold lower than that of mouse brains infected with CE-NiP-WT (Figure 7C), indicating that the different symptoms relate to the levels of virus in target tissues of the central nervous system (CNS).

**DISCUSSION**

In this study, we investigated the mechanism by which P-protein coordinates multiple interactions important to roles in the basic viral life cycle and in viral immune evasion, and developed the first mutant lyssavirus specifically deficient for interaction with STATs. Using this virus, we showed that P-protein-STAT1/2 interaction is critical to pathogenicity, identifying the P-protein-STAT complex as a key pathogen-host interface in the development of rabies. Importantly, the finding that P-WT but not P-W265G/M287V can inhibit IFNα-dependent expression of ISGs, but that both proteins mediate genome replication and

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**Figure 6.** CE-NiP-STAT(-) virus cannot prevent IFNα-induced nuclear accumulation of STAT1. A. SK-N-SH cells were mock-infected or infected with CE-NiP-WT or CE-NiP-STAT(-) (MOI of 0.01, 18 hours) and treated without or with 4000 U/ml IFNα for 0.5 hours before fixation, immunostaining using anti-N-protein and anti-STAT1 antibodies with Alexa-568- and Alexa-488-conjugated secondary antibodies, and analysis by confocal microscopy. Open arrowheads indicate N-positive cells; filled white arrowheads in STAT1-immunostained cells indicate the nucleus. B. Confocal microscopic images such as those shown in A were used to determine the nucleocytoplasmic fluorescence ratio (Fn/c) for STAT1 as previously [32–34] (mean ± SEM, n > 30 cells; data are from a single assay representative of 2 independent assays); ****P ≤ .0001.
antagonism of IFNβ induction, enabled delineation of the specific importance of P-protein targeting of STATs in disease, with the finding that W265G/M287V mutation profoundly impairs RABV pathogenicity in vivo indicative of a major contribution. Notably, the fact that CE-NiP-STAT(-) caused no major neurological symptoms in mice in spite of inoculation into the brain indicates a critical role for P-STAT interaction in infection of the CNS, consistent with the importance of IFN-mediated innate immunity in these tissues, from which cells of the adaptive response are excluded by the blood-brain barrier [36, 37]. This is further supported by the observation of a >3 log difference in infectious virus load of WT and STAT(-) virus in the CNS within 5 dpi. Thus our data support a central role for P-protein-STAT interactions in the principal target organs of lyssavirus infection. However, we cannot discount the possibility that other as yet unidentified functions of P-protein might be affected by W265G/M287V mutations, and that this might contribute to the reduced pathogenicity. Future analysis of infection of IFNAR-knockout mice and mice deficient for specific STATs will provide further insights into the precise role of P-protein-STAT targeting in pathogenicity.

In common with other viruses that use P-gene products for IFN antagonism, the role of lyssavirus-STAT interaction in disease has proven elusive [38, 39], largely because of the complex roles of P-gene products in both IFN antagonism and genome replication. As a result, previously identified mutations that inhibit STAT-binding (e.g., RABV PΔC30) have also prevented the formation of a functional replication complex (Figure 4C), precluding the generation of fully viable virus deficient for STAT interaction. Point mutations that inhibit STAT-binding by the P-gene-encoded V-protein of measles virus have been described, and were used to generate mutant virus that showed moderately reduced disease symptoms, but also showed defective growth in IFN-deficient cells, indicating effects distinct from IFN antagonism [7]. Studies of Nipah virus P/V/W-proteins also suggested that genome replication and STAT-binding/mislocalization functions are separable in vitro, but effects of mutations on antagonism of STAT-signaling, viral IFN resistance, and disease progression are yet to be reported [40]. Thus the present study is to our knowledge the first to separate STAT antagonism and replication functions of a P-gene product, with significance to understanding of the mechanisms of infection by lyssaviruses.

Figure 7. CE-NiP-STAT(-) virus is strongly attenuated in vivo compared with CE-NiP-WT. A, 10⁴ ffu of the indicated virus was inoculated i.c. into mice (12 per group) and disease symptoms were monitored for 21 dpi. All CE-NiP-WT-infected mice succumbed to infection or reached a non-responsive endpoint and were sacrificed by 12 dpi, but no lethality was observed for mock- or CE-NiP-STAT(-)-infected mice. B, Mean body weight changes of the infected mice described in A are shown. C, Viral titers in brain emulsions (ffu/g) from 5 mice infected with the indicated viruses were measured at 5 dpi by focus formation assays.
and paramyxoviruses, and potential significance to analogous multifunctional STAT antagonists, such as Ebola virus VP35, dengue and West Nile virus NS5, and hepatitis C virus NS5A [3, 41, 42] for which the importance of IFN antagonist function in vivo is yet to be demonstrated.

Critical to this study was the finding that key residues for STAT-binding reside within the P-protein W-hole enabling direct analysis of the localization of interfaces for STAT and N-protein interaction in the globular CTD structure. This provided indications that these sites are spatially and functionally separable, a finding which has important implications for our understanding of basic lyssavirus biology. In particular, it has not previously been understood how P-protein could efficiently mediate IFN/STAT-antagonism, as most P-protein is likely to be engaged in P-N complexes in infected cells, where N is produced in excess over P [43]. Our findings suggest that P-protein might form interactions with N- and STAT proteins simultaneously through interfaces on opposite sides of the CTD, such that STAT antagonism would not be restricted to limited amounts of free P-protein, but could also involve P-N complexes.

Rabies remains a major threat to human and animal health worldwide due to the absence of effective therapeutics, and the limitations of current inactivated vaccines. The potential of targeting viral IFN antagonism for the development of vaccines and antivirals is well appreciated [3, 4, 44], and the data in this study now provide evidence that IFN antagonist-STAT interactions specifically represent viable targets. The identification of novel attenuating mutations in P-protein, and of the W-hole as the interface of P-STAT interaction, is thus of potential significance to efforts to develop new attenuated lyssavirus vaccine strains and novel therapeutics for currently incurable rabies disease. Future work will include analysis of the effect of these mutations in live RABV vaccine strains to examine both induction of immune responses, including ISG expression, and establishment of protective immunity.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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References


