Identification of the Nuclear Export Signal and STAT-Binding Domains of the Nipah Virus V Protein Reveals Mechanisms Underlying Interferon Evasion

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Received 8 November 2003/Accepted 19 December 2003

The V proteins of Nipah virus and Hendra virus have been demonstrated to bind to cellular STAT1 and STAT2 proteins to form high-molecular-weight complexes that inhibit interferon (IFN)-induced antiviral transcription by preventing STAT nuclear accumulation. Analysis of the Nipah virus V protein has revealed a region between amino acids 174 and 192 that functions as a CRM1-dependent nuclear export signal (NES). This peptide is sufficient to complement an export-defective human immunodeficiency virus Rev protein, and deletion and substitution mutagenesis revealed that this peptide is necessary for both V protein shuttling and cytoplasmic retention of STAT1 and STAT2 proteins. However, the NES is not required for V-dependent IFN signaling inhibition. IFN signaling is blocked primarily by interaction between Nipah virus V residues 100 to 160 and STAT1 residues 509 to 712. Interaction with STAT2 requires a larger Nipah virus V segment between amino acids 100 and 300, but deletion of residues 230 to 237 greatly reduced STAT2 coprecipitation. Further, V protein interactions with cellular STAT1 is a prerequisite for STAT2 binding, and sequential immunoprecipitations demonstrate that V, STAT1, and STAT2 can form a tripartite complex. These findings characterize essential regions for *Henipavirus* V proteins that represent potential targets for therapeutic intervention.

The biological effects of alpha and beta interferon (IFN-α and -β) are mediated by a transcription factor complex, ISGF3, that is comprised of the signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT2, in association with a DNA-binding subunit, an IFN regulatory factor, IRF9 (1, 12). IFN-induced, ISGF3-mediated transcription results in a cellular antiviral state that provides protection against a broad range of virus types. In response to this negative selection, most viruses have evolved adaptations that allow them to evade IFN-induced innate antiviral responses (13, 26).

In the case of the paramyxovirus family of negative-strand RNA viruses, IFN signaling to ISGF3 is disrupted by direct targeting of STAT proteins (4). Current evidence from the study of several family members indicates that the common outcome of STAT targeting and IFN evasion is achieved by individual paramyxovirus species through a diverse range of molecular mechanisms. For example, the *Rubulaviruses*, simian virus 5 (SV5), human parainfluenza virus 2, and mumps virus all encode STAT-directed E3 ubiquitin ligase activities that function in combination with cellular proteins to target STAT1, STAT2, or STAT3 for proteasomal degradation (5, 18, 19, 28, 29). Distinctly, measles virus, a prototype *Morbillivirus*, does not induce STAT ubiquitylation and degradation but instead prevents IFN-induced ISGF3 assembly and STAT protein nuclear translocation (17, 27). These IFN evasion mechanisms rely on protein-protein interactions between STATs and the paramyxovirus-encoded V protein. The paramyxovirus V protein is characterized by a highly conserved cysteine-rich C-terminal domain (CTD), a zinc-binding domain that is essential for virus replication in IFN-competent systems and for diverse V protein activities including cell cycle arrest, restriction of apoptosis, suppression of IRF3 activity, and protein-protein interactions (6, 9, 14, 15, 22, 23, 28). As an IFN response modifier, the V protein is a virulence and pathogenesis-determining factor. In the case of SV5, species-specific restriction of STAT1 degradation has been shown to be a determinant of host species tropism, restricting SV5 replication in IFN-competent murine cells (20, 31).

A new genus of recently emerged paramyxoviruses, *Henipavirus*, was demonstrated to share V-dependent IFN signaling evasion properties with other paramyxoviruses (21, 24, 25). Encoded by a polycistronic gene, the 405-amino-acid V protein N terminus is shared with the co-amino-terminal P and W proteins (3). In addition, a C protein is encoded in an alternate open reading frame. In comparison to the STAT-degrading *Rhabdovirus* V proteins, the V proteins of Nipah virus and Hendra virus, the two known *Henipavirus* species, share approximately 55% amino acid identity within the CTD. Despite this CTD conservation, it is dispensable for IFN signaling inhibition (21). The *Henipavirus* V protein N terminus is entirely unique compared to other paramyxovirus proteomes, and the V proteins have no obvious homology to any cellular protein. The *Henipavirus* V proteins are overall 58% identical in amino acid sequence, with 81% identity between amino acids (aa) 1 to 140, 44% identity between aa 141 to 405, and 83% identity within the CTD (aa 406 to 456). This sequence conservation accounts for the functional similarity in the IFN evasion activities of the Nipah virus and Hendra virus V proteins. Both V proteins have been demonstrated to subvert IFN responses by sequestering STAT1 and STAT2 in high-molecular-weight cytoplasmic complexes (24, 25). In addition to the ability to bind to both STAT1 and STAT2, the *Henipavirus* V proteins exhibit nuclear-cytoplasmic shuttling behavior that depends on...
CRM1-dependent nuclear export signals (NES). Not only does this shuttling affect the steady-state subcellular distribution of the V protein, but it also alters the distribution of the latent STAT1, relocating the protein to the cytoplasm (24, 25).

To decipher the molecular mechanisms underlying Henipavirus IFN evasion activities, the functional domains of the Nipah virus V protein involved in nuclear cytoplasmic distribution and STAT protein binding were examined experimentally. The NES and minimal interaction domains for both STAT1 and STAT2 were identified as discrete regions within Nipah virus V protein aa 100 to 300. The Nipah virus NES maps within aa 174 to 192, a peptide that is necessary and sufficient for directing protein subcellular distribution. IFN evasion activity is independent of nuclear export but correlates exactly with the ability of Nipah virus V amino acid residues 100 to 160 to interact with STAT1 residues 509 and 712. Cellular STAT1 is required for V protein interactions with STAT2, and therefore, the STAT2 interaction region is constrained to a larger overlapping region between aa 100 and 300, but evidence suggests that contact between STAT2 and Nipah virus V requires aa 230 to 237. Moreover, a tripartite protein complex between Nipah virus V, STAT1, and STAT2 is demonstrated.

MATERIALS AND METHODS

Cell culture. Human 2fTGH, 293T, U3A (STAT1 deficient), and U6A (STAT2 deficient) cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% cosmic cell serum (HyClone) and 1% penicillin-streptomycin (Gibco-BRL). U3A cells stably expressing either STAT1 or STAT1-STAT3 chimeric fusion proteins (18) were maintained in the above-mentioned medium supplemented with G418 (Calbiochem).

Plasmids and reagents. Expression vectors for FLAG epitope-tagged Nipah virus and Hendra virus V proteins were obtained as described previously (24, 25). To create truncated Nipah virus V proteins, PCR products were generated with BglII and NotI restriction sites and were ligated into a pEFN expression vector containing an in-frame N-terminal FLAG epitope tag. Truncations were made at amino acid positions 100 (Δ4, Δ5), 220 (Δ3, Δ6), 300 (Δ2), and 406 (Δ1). To generate Rev1.4-green fluorescent protein (GFP) Nipah virus V fusion proteins, PCR products flanked by BamHI and AgeI restriction sites were ligated into a Rev1.4-GFP expression vector (7) to place the Nipah virus V peptide segment in frame and between the Rev1.4 and GFP. Nipah virus V deletion mutants (ΔNES, ΔSS) and Nipah virus V NES point mutants (L174A, V175A, and L186A) were generated by a three-step PCR. Final PCR products were ligated into pEFN FLAG epitope expression vector as described above. All constructs were verified by DNA sequencing.

Luciferase and Rev1.4 reporter gene assays. Luciferase reporter gene assays were performed as described previously (24, 25). 2fTGH cells were transfected with expression vectors for an ISRE-luciferase reporter gene, empty vectors, or different Nipah virus V peptides and Hendra virus V expression vectors described above. Cells were treated with 1,000 U of IFN-α/ml for 6 h prior to lysis and luciferase assays. All conditions represent the average values from triplicate samples, normalized to cotransfected Renilla luciferase and expressed as percentages of IFN-stimulated controls.

Rev1.4-GFP reporter gene assays were performed essentially as described in reference 7. 2fTGH cells were transfected with expression vectors for Rev1.4-GFP, Rev1.4-NES-GFP, or Rev1.4-GFP fused to full-length Nipah virus V or various Nipah virus V peptide segments. Five hours prior to fixation, cells were treated with either 5 μg of actinomycin D (ACTD)/ml or 10 ng of leptinomin B (LMB)/ml. All samples were treated with 20 μg of cycloheximide/ml to prevent new protein synthesis. After 24 h, cells were fixed and permeabilized and GFP fluorescence was visualized by a confocal scanning microscope as described previously (24, 25). Quantitative analysis was done by using visually scoring ACTD-treated cells (n = 100) for cytoplasmic GFP fluorescence in a blind comparison.

Immunofluorescence microscopy. Immunofluorescence was carried out exactly as described (24, 25). Samples were stained with either 100 μl of IFN-α/ml or 5 ng of IFN-γ/ml for 30 min or 10 ng of LMB/ml for 5 h prior to fixation. Fixed and permeabilized cells were stained with antisera to STAT1 (6.6 μg/ml; Santa Cruz), STAT2 (6.0 μg/ml; Santa Cruz), and FLAG epitope tag (24.5 μg/ml; Sigma). Images were visualized and collected with a Leica TCSSP confocal microscope.

Cell extracts, immunoblotting, and immunoprecipitation. For preparation of cell extracts, cells were lysed in whole-cell extract buffer supplemented with 1 mM dithiothreitol, 1 mM NaVO4, and protease inhibitors (Roche) exactly as described previously (24). For immunoblotting, cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, probed with antisera to STAT1α, STAT1β, STAT2, STAT3 (Santa Cruz), and FLAG epitope tag (Sigma), and visualized with chemiluminescence (NEN Life Sciences). For immunoprecipitation of FLAG epitope-tagged proteins, cell extracts were incubated with M2 affinity gel exactly as described previously (28). Immunoprecipitates were then separated by SDS-PAGE and analyzed by immunoblotting for coprecipitated proteins. For immunoprecipitation of GFP fusion proteins, lysates (2 μg) were first precleared with protein G-agarose for 1 h and incubated with antisera to GFP for 4 h with rocking at 4°C. Immunoprecipitated complexes were then incubated with protein G-agarose for 12 h, washed five times with whole-cell extract buffer, separated by SDS-PAGE, and analyzed by immunoblotting with specific antisera.

RESULTS

Subcellular distribution of Nipah virus N-terminal fragments. Protein shuttling between nucleus and cytoplasm involves competitive interactions between nuclear localization signals and NES. Many NES sequences have been characterized as having regularly spaced leucine or hydrophobic residues, similar to that of the Rev protein from human immunodeficiency virus type 1 (HIV-1). However, export signals often do not conform to the Rev paradigm and must be identified by experimental approaches (11).

To test for functional domains directing cytoplasmic accumulation of Nipah virus V, truncated FLAG epitope-tagged Nipah virus V protein fragments were constructed. The largest fragment, Δ1, is truncated at the P/V/W RNA editing site and, hence, contains aa 1 to 406 in common with the P, V, and W proteins but none of the CTD residues. The remaining constructs are truncated within lower-homology regions in between highly conserved Henipavirus V protein domains: the C-terminal boundaries of Δ2, Δ3, and Δ4 are residues 300, 220, and 100, respectively, while the Δ5 and Δ6 N-terminal boundaries are residues 100 and 220, respectively (Fig. 1A). These fragments were expressed by transfection of human 2fTGH cells, and the distribution of the expressed proteins was analyzed by indirect immunofluorescence with antisera to the N-terminal FLAG epitope tag (Fig. 1B). The steady-state distribution of the largest N-terminal fragment, Δ1, or a shorter piece, Δ2, were indistinguishable from that of full-length Nipah virus V, exhibiting exclusive cytoplasmic accumulation (Fig. 1B). Truncation to aa 220 (Δ3) resulted in a clearly cytoplasmic accumulation but with a slightly increased nuclear staining, and further truncation to residue 100 (Δ4) produced a greater nuclear accumulation pattern (Fig. 1B). N-terminal truncation of Nipah virus V to residue 100 (Δ5) had no effect on the cytoplasmic accumulation, whereas further N-terminal truncation to residue 220 (Δ6) demonstrated a nuclear cytoplasmic distribution. Like the full-length protein, all of the truncated V proteins accumulated in the nucleus when exposed to LMB, indicating that nuclear export is driven by a CRM1-dependent mechanism. The nuclear cytoplasmic accumulation patterns of these truncated V proteins suggest that signals controlling V protein distribution map to the N terminus and that an LMB-resistant Nipah virus V NES lies between aa 100 and 220.
Dissection of the Nipah virus V protein NES. Inspection of the Nipah virus V protein primary amino acid sequence did not clearly identify a region similar to other well-defined NES peptides. To experimentally define the NES region, a Rev complementation strategy devised by Henderson and Eleftheriou was utilized (7). The HIV-1 Rev protein shuttles between the nucleus and cytoplasm due to a well-characterized NES and nuclear localization signal. Rev import into the nucleus and nucleolus is sensitive to the drug ACTD, whereas export from the nucleus is CRM1 dependent and sensitive to LMB. In this system, an export-defective HIV-1 Rev protein mutant, Rev1.4, which contains an inactivated NES, is fused to GFP. Putative Nipah virus V NES sequences were subcloned between Rev1.4 and GFP, expressed as tripartite fusions, and tested for their nuclear export activity.

A series of Rev1.4-Nipah virus V-GFP fusions were constructed as illustrated in Fig. 2A, and the use of this reporter with Nipah virus V is demonstrated in Fig. 2B. The Rev1.4 mutant remains in the nucleus regardless of drug treatments, but addition of the Rev NES reconstitutes a shuttling protein, as revealed by cytoplasmic distribution in the presence of ACTD and nuclear accumulation in response to LMB treatment (all panels include cycloheximide to prevent new protein synthesis that might obscure localization patterns). Similarly, fusion with the full-length Nipah virus V protein produces a shuttling protein, consistent with CRM1-dependent export (24). Treatment with ACTD or LMB confirms the shuttling behavior of Nipah virus V in the Rev1.4-GFP fusion system.

The Nipah virus V aa 100 to 300 also complemented Rev1.4 to produce a shuttling protein (Fig. 2C), supporting the localization of a Nipah virus V NES to this region. Analysis of a series of subfragments reveals that aa 100 to 220, but not aa 220 to 300, carry NES activity. Nuclear export activity was also associated with both aa 130 to 190 and aa 160 to 220 but not aa 100 to 160. Quantification of the distribution patterns of ACTD-treated cells confirms that the nuclear export activity maps clearly to aa 100 to 220. The smaller fragments were somewhat attenuated in their ability to relocate Rev1.4 to the cytoplasm, but a statistically significant number of cells exhibited a cytoplasmic distribution pattern, suggesting that an NES sufficient for Rev1.4 complementation lies near or within aa 160 to 220 (Fig. 2C).

Mutation of the Nipah virus V NES. NES peptides often contain regularly spaced leucine residues, although some well-characterized NES peptides do not conform to this archetype (7). Examination of the Nipah virus and Hendra virus V protein sequences within aa 160 to 220 reveals a highly conserved candidate NES peptide between aa 174 and 192 that contains several leucine residues (Fig. 3A). To test if this candidate NES was needed to establish the Nipah virus V protein cytoplasmic distribution pattern, this peptide was deleted from the full-length protein to generate a deletion mutant, ΔNES (Fig. 3A). The subcellular distribution of ΔNES is substantially different from that of wild-type Nipah virus V protein, as it lacks cytoplasmic accumulation (Fig. 3B). Treatment with LMB resulted in redistribution of the wild-type Nipah virus V protein to the nucleus but did not change the distribution pattern of the mutant protein, indicating that the deletion eliminates the CRM1-dependent shuttling.

To determine the ability of ΔNES to alter STAT subcellular distribution, the STAT1 and STAT2 accumulation patterns were also analyzed in ΔNES-expressing cells (Fig. 3B). STAT1 was found in both the nucleus and cytoplasm of ΔNES-expressing cells, and there was no redistribution of STAT1 to the cytoplasm as observed with wild-type Nipah virus V (24) (Fig. 4C). However, STAT1 did not relocate to the nucleus in IFN-stimulated ΔNES-expressing cells, and it retained the distribution pattern typical of unstimulated cells. Similarly, IFN-dependent redistribution of STAT2 was prevented by ΔNES. These findings indicate that NES activity and STAT interference are not strictly linked.

To determine the amino acid residues within the NES region required for nuclear export, conserved leucines at positions 174 and 186 or a nonconserved valine at position 175 was individually mutated to alanine, and the subcellular distribution patterns of the mutant proteins were analyzed (Fig. 3C). Mutant V175A was indistinguishable from the wild type and had no effect on the cytoplasmic accumulation of Nipah virus V. In contrast, both L174A and L186A mutants failed to ac-
The two leucine residues are necessary elements of the Nipah virus V protein NES. The effect of the mutant V proteins on STAT distribution was also analyzed by immunofluorescence. Like wild-type Nipah virus V, mutant V175A caused a redistribution of STAT1 to the cytoplasm of unstimulated cells (data not shown) and also prevented IFN-induced nuclear redistribution of STAT1 and STAT2 (Fig. 3C), indicating that this mutation does not alter the activity of Nipah virus V protein. Analysis of Nipah virus V mutants L174A and L186A revealed a different STAT localization phenotype. Neither leucine mutant induced relocalization of latent STAT1 to the cytoplasm (data not shown), consistent with a loss of shuttling activity. Both leucine substitutions prevented IFN-induced STAT1 and STAT2 nuclear redistribution, resulting in a pattern similar to that of cells not stimulated with IFN (Fig. 3C). Together, these data indicate that within the NES, amino acid residues 174 and 186 are required for the export of Nipah virus V and basal STAT1 redistribution. Since the export-deficient Nipah virus V proteins prevented IFN-induced STAT nuclear accumulation, export activity is not strictly required for IFN signaling inhibition.

To directly test whether the NES has a role in IFN signaling evasion, assays for both IFN inhibition and STAT protein interactions were carried out. The ΔNES protein and leucine substitution mutants retained the ability to disengage IFN-dependent gene regulation (Fig. 3D). Deletion of the NES does not disrupt the interaction with STAT1 and STAT2 (Fig. 3E). These functional assays confirm that the Nipah virus V NES mutants are not grossly malfolded and suggest that the cellular distribution of the V protein is not a formal determinant of IFN evasion activity, a result consistent with earlier

FIG. 2. Localization of a CRM1-dependent NES in the Nipah virus V (NiV) protein. (A) Illustration of the Rev1.4-GFP fusions analyzed. (B) Use of Rev1.4-Nipah V-GFP fusions. (Top) 2TGH cells were transfected with expression vectors for Rev1.4-GFP, Rev1.4-NES-GFP, and full-length Nipah virus V (NiV) fused to Rev1.4-GFP and fixed and permeabilized 24 h later for confocal microscopy. CHX, cycloheximide; ACTD, actinomycin D; LMB, leptomycin B. (Bottom) Quantitation of NES activity. ACTD-treated cells were scored for cytoplasmic GFP (n = 100). Images were inverted in grayscale to better visualize cytoplasmic fluorescence. (C, Top) Localization of Nipah virus V NES. Same is in panel B but using Nipah virus V fragments fused to Rev1.4-GFP. (Bottom) Quantitation of NES activity (n = 100).
conclusions based on experiments using the nuclear export inhibitor compound LMB (24).

**IFN signaling inhibition and STAT binding by Nipah virus V fragments.** Prior analysis of the Nipah virus V protein demonstrated its ability to inhibit IFN signaling through binding and cytoplasmic retention of both STAT1 and STAT2 (24). The ability of the truncated V proteins to interfere with STAT signaling was tested with an ISRE-luciferase reporter gene.
assay. All of the truncated V proteins effectively prevented induction of the ISRE reporter gene except /H9004 (aa 1 to 100) and /H9004 (aa 220 to 456), indicating that a peptide segment between aa 100 to 220, the same region containing the NES, is important for inhibition of IFN signaling (Fig. 4A). To analyze whether Nipah virus V protein fragments interact with STAT1 and STAT2, FLAG-tagged V protein fragments were expressed and immunoprecipitated, and then the immune complexes were probed for STAT1, STAT2, and STAT3 (Fig. 4B). Full-length Nipah virus V, the two largest N-terminal fragments (Δ1 and Δ2), and the larger C-terminal fragment (Δ5) exhibited similar levels of association with STAT1 and STAT2. In contrast, fragment Δ3 (aa 1 to 220) coprecipitated STAT1 but not STAT2. Fragment Δ4 (aa 1 to 100) bound to neither STAT protein. N-terminal truncation to amino acid residue 220 (Δ6) lost the ability to bind STAT1 but retained a weak,
Cells were stimulated with IFN-porter gene and the Rev1.4-Nipah V-GFP fusion proteins indicated. STAT1 binding. Cells were transfected with an ISRE-luciferase re-expression in total extracts. (B) IFN signaling inhibition correlates with STAT1 or STAT2. Immunoblotting for GFP demonstrates similar serum to GFP. Immune complexes were probed with antisera for fusion vectors indicated and lysates immunoprecipitated with anti-virus V fragments. 293T cells were transfected with the Rev1.4-GFP Nipah virus V (NiV) protein. (A) Selective STAT binding by Nipah STAT1, STAT2, or FLAG epitope tag. (D) Nipah virus V binds STAT2 nuclear accumulation (Fig. 4C). Fragment Δ6 (aa 220 to 456) was slightly capable of preventing STAT2 nuclear accumulation in response to IFN-α, but the phenotype was not completely penetrant, possibly the result of the observed weak interaction with STAT2. These data indicate that the region of Nipah virus V protein specifying STAT interference lies between aa 100 and 220.

Localization of STAT protein binding domains. To further delineate the site(s) of STAT association, the set of Rev1.4-GFP fusion proteins was used for coimmunoprecipitation experiments. Lysates from transfected cells were immunoprecipitated with GFP-specific antiserum, and the immune complexes were tested for the presence of STAT1 or STAT2 by immunoblotting. The Rev1.4-GFP protein did not interact with either STAT protein, but both STAT1 and STAT2 were present in immune complexes containing the full-length Nipah virus V protein or aa 100 to 300 (Fig. 5A), but smaller fragments differentially coprecipitated the two STATs. STAT1 was co-precipitated by aa 100 to 220 and aa 100 to 160, mapping STAT1 interactions to a 60-aa peptide. In contrast, none of the shorter fragments coprecipitated STAT2. In conjunction with differential STAT binding of truncated Nipah virus V proteins, these data indicate separate but overlapping binding sites for STAT1 and STAT2.

To verify that these tripartite fusion proteins could imitate IFN evasion by Nipah virus V protein, the Rev1.4-GFP series were tested for IFN-α signaling inhibition. All of the fragments encompassing aa 100 to 160 were tested for the presence of STAT1 or STAT2 by immunoblotting. The Rev1.4-GFP protein did not interact with either STAT protein, but both STAT1 and STAT2 were present in immune complexes containing the full-length Nipah virus V protein or aa 100 to 300 (Fig. 5A), but smaller fragments differentially coprecipitated the two STATs. STAT1 was co-precipitated by aa 100 to 220 and aa 100 to 160, mapping STAT1 interactions to a 60-aa peptide. In contrast, none of the shorter fragments coprecipitated STAT2. In conjunction with differential STAT binding of truncated Nipah virus V proteins, these data indicate separate but overlapping binding sites for STAT1 and STAT2.

Because Δ6 retained weak STAT2 binding in the absence of a STAT1 interaction site, the presence of a STAT2 interaction site downstream of aa 220 was inferred. In this region, a peptide that is highly conserved between Nipah virus and Hendra virus V proteins was identified between amino acid residues 230 and 271. Deletion of 7 aa from the N terminus of this conserved peptide generated a Nipah virus V protein (ΔS2) that was capable of coprecipitating STAT1 (Fig. 5C) but deficient for STAT2 interaction. This finding verifies the presence of individual STAT1 and STAT2 binding sites on the Nipah virus V protein.

FIG. 5. Localization of STAT1 and STAT2 binding sites on the Nipah virus V (NiV) protein. (A) Selective STAT binding by Nipah virus V fragments. 293T cells were transfected with the Rev1.4-GFP fusion vectors indicated and lysates immunoprecipitated with antisera to GFP. Immune complexes were probed with antisera for STAT1 or STAT2. Immunoblotting for GFP demonstrates similar expression in total extracts. (B) IFN signaling inhibition correlates with STAT1 binding. Cells were transfected with an ISRE-luciferase reporter gene and the Rev1.4-Nipah V-GFP fusion proteins indicated. Cells were stimulated with IFN-α (1,000 U/ml) (+) or not stimulated (−). (C) STAT2 binding requires Nipah virus V residues 230 to 237. Whole-cell extracts from cells expressing Nipah virus V (N) or deletion of residues 230 to 237 (ΔS2) were immunoprecipitated (IP) with FLAG affinity gel. Total extracts (left panels) and immunoprecipitates (right panels) were analyzed by immunoblotting with antisera to STAT1α/β, STAT2, or FLAG epitope tag. (D) Nipah virus V binds STAT1 residues 509 to 712. STAT1-deficient U3A cells stably expressing complementary STAT1-STAT3 fusion proteins 13F and 31F described in the text were transfected with either empty vector (C) or HA-tagged Nipah virus V (N), and extracts were immunoprecipitated with FLAG affinity gel and processed by immunoblotting for HA epitope tag, STAT1α, or STAT3. Numbers to the left of immunoblots indicate positions of prestained molecular weight standards.
To determine the STAT protein region that interacts with Nipah virus V protein, U3A cell lines stably expressing FLAG-tagged complementary STAT1-STAT3 fusion proteins were used (8, 19). Expression vectors for these fusions, 13F (aa 1 to 508 of STAT1 fused to aa 514 to 770 of STAT3) and 31F (aa 1 to 514 of STAT3 fused to aa 509 to 750 of STAT1) were transfected with hemagglutinin (HA)-tagged Nipah virus V expression vector, and lysates were subjected to FLAG immunoprecipitation (Fig. 5D). Immunoblotting demonstrates coprecipitation of Nipah virus V protein only in the cells expressing the C-terminal region (aa 509 to 750) of STAT1, a fragment that encompasses the STAT protein linker domain, SH2 domain, and transcriptional activation domain. STAT1/H9252, a splice variant lacking the STAT1 C-terminal 38 aa, also was found to coprecipitate with Nipah virus V (Fig. 5C), narrowing the Nipah virus V contact region to STAT1 residues 509 to 712.

### STAT2 binding requires STAT1

Since the minimal interaction sites for STAT1 and STAT2 overlap, the ability of the STATs to independently bind to Henipavirus V proteins was tested (Fig. 6A). Parental 2fTGH cells, STAT2-deficient U6A cells, STAT1-deficient U3A cells, and U3A cells stably complemented with STAT1 were transfected with either empty vector (C) or expression vectors for FLAG epitope-tagged Nipah virus V (N) or Hendra virus V (H). After 24 h posttransfection, cells were lysed and extracts were immunoprecipitated with FLAG affinity gel. Coprecipitation of STAT1 and STAT2 was analyzed by immunoblotting. (B) Henipavirus V proteins can interact with STAT1 and STAT2 in the same protein complex. Cell extracts expressing FLAG-tagged GFP (G) or Nipah virus (N) or Hendra virus (H) V proteins were affinity purified with FLAG M2 affinity gel (left panel), and peptide-eluted protein complexes were immunoprecipitated (IP) a second time (right panels) with antisera for STAT1 or STAT2. Immune complexes were separated by SDS-PAGE and tested for the presence of STAT1 and STAT2 by immunoblotting with specific antisera. Numbers to the left of immunoblots in panels A and B indicate positions of prestained molecular weight standards. (C) Sequence alignment of amino acids 100 to 300 of Nipah virus (NiV) and Hendra virus (HeV) V proteins. The STAT binding sites and NES are indicated by overlining. (D) Schematic diagram summarizing the functional domains and protein interactions mapped in this study.

To determine the STAT protein region that interacts with Nipah virus V protein, U3A cell lines stably expressing FLAG-tagged complementary STAT1-STAT3 fusion proteins were used (8, 19). Expression vectors for these fusions, 13F (aa 1 to 508 of STAT1 fused to aa 514 to 770 of STAT3) and 31F (aa 1 to 514 of STAT3 fused to aa 509 to 750 of STAT1) were transfected with hemagglutinin (HA)-tagged Nipah virus V expression vector, and lysates were subjected to FLAG immunoprecipitation (Fig. 5D). Immunoblotting demonstrates coprecipitation of Nipah virus V protein only in the cells expressing the C-terminal region (aa 509 to 750) of STAT1, a fragment that encompasses the STAT protein linker domain, SH2 domain, and transcriptional activation domain. STAT1β, a splice variant lacking the STAT1 C-terminal 38 aa, also was found to coprecipitate with Nipah virus V (Fig. 5C), narrowing the Nipah virus V contact region to STAT1 residues 509 to 712.

### DISCUSSION

The Henipavirus V protein encodes an intrinsic nuclear export activity and is able to mediate IFN signaling evasion through interaction with cellular STAT proteins. In this study, discrete V protein sequences mediating nuclear export and...
STAT interaction were delineated. Results indicate that all of these activities map within Nipah virus V protein amino acids 100 to 300.

The Nipah virus V protein NES that is required for its CRM1-dependent cytoplasmic accumulation was identified. This NES encompasses aa 174 to 192, a peptide that is highly conserved between Nipah virus and Hendra virus not only in the V protein but also in the P and W proteins. NES peptides frequently contain 10 to 30 aa with indispensable leucine residues at irregularly spaced intervals (7). Sequence comparisons indicate that the Henipavirus NES is distinct from other known cellular and viral NES peptides that mediate CRM1-dependent export. This NES was demonstrated to be required for the observed V-dependent redistribution and retention of latent STAT1 from the nucleus to the cytoplasm. However, in spite of its essential role in controlling V protein shuttling behavior, the NES is dispensable for interaction with either STAT1 or STAT2 and for the execution of V-dependent IFN signaling inhibition. In fact, results from deletion and substitution mutagenesis indicate that the NES and IFN evasion activities are separable functions of a similar protein region encompassing amino acid residues 100 to 300.

STAT1 and STAT2 are bound by distinct regions of the Nipah virus V protein. STAT1 contacts Nipah virus V through interaction with a 10 to 30 aa peptide between Nipah virus V residues 100 and 160 and STAT1 residues 509 to 712 (Fig. 6D). This STAT1 interaction site expressed in cells as a heterologous Rev1.4-GFP fusion protein is sufficient to mediate IFN signaling inhibition, suggesting that contact with STAT1 is sufficient to block the antiviral response. The domain structure of STAT1 has been well characterized, and the V proteins interact with the SH2 and Linker domains of the STAT1 protein core (2). The SH2 domain is the primary receptor recognition motif and also an essential component of phosphoryrosine-mediated STAT dimerization. The function of the linker domain is not well understood, but sequence perturbations alter STAT1 DNA binding affinity (30). Apparently, the Henipavirus V protein has evolved to contact these domains to prevent STAT activation, dimerization, and transcriptional activity. Furthermore, it was observed that Nipah virus V segments lacking this STAT1-binding peptide are also unable to efficiently bind to STAT2. The STAT2 interaction region of Nipah virus V maps to a larger segment, requiring aa 100 to 300. Deletion of aa 230 to 237 greatly impaired STAT2 coprecipitation, but it is important to note that this is merely the extreme N terminus of a longer peptide (aa 230 to 271) that is highly conserved (67% identical) between Nipah virus and Hendra virus.

It was also observed that cellular expression of STAT1 is absolutely required for Henipavirus V proteins to bind to STAT2, as precipitation of V proteins from cells deficient in STAT1 did not copurify STAT2. This phenotype was complemented by STAT1 expression, indicating it is a direct consequence of STAT1 availability and not the result of additional unidentified alterations in the STAT1-deficient cells. In contrast, STAT1 was readily detected in V protein precipitations from STAT2-deficient cells, in agreement with previously reported Nipah virus V-induced IFN-γ signaling inhibition in the absence of cellular STAT2 (24). The requirement for STAT1 to efficiently bind STAT2 in part explains the observation that STAT2 requires a larger interaction site; it reflects the need to accommodate the STAT1-binding region. This codependent relationship between STAT1 and STAT2 is somewhat similar to the requirement for accessory STAT proteins in IFN signaling evasion by the Rubulavirus V protein-dependent ubiquitin ligases. Both SV5 and mumps virus V proteins strictly require cellular STAT2 expression to target STAT1, whereas human parainfluenza virus 2 requires cellular STAT1 for STAT2 targeting (19, 29). In fact, for SV5, the requirement for STAT2 provides the molecular basis for host range restriction, as the murine STAT2 orthologue fails to support STAT1 degradation and IFN evasion (20). The exact role for this STAT codependence is currently unknown, but available evidence suggests that V proteins form multisubunit targeting complexes that utilize both cellular and viral partners, and the confederate STAT may provide contact sites for the recruitment of essential components (28).

Sequential immunoprecipitation demonstrates that in intact cells, the Henipavirus V proteins can form tripartite complexes with both STAT1 and STAT2. It is likely that independent STAT1-V complexes are also formed, supported by the higher abundance of STAT1 found in STAT1 immune complexes compared to STAT2 immune complexes (Fig. 6B). Based on the known molecular mass of each protein, the predicted mass of this tripartite complex would be in the range of ~300 kDa. This figure is in agreement with earlier gel filtration estimates of 300 to 500 kDa (24), but it is reasonable to assume that additional cellular proteins may also be associated with the expressed Henipavirus V proteins, as this has been demonstrated for several other paramyxovirus V proteins.

The results of this study indicate that the highly conserved cysteine-rich CTD of the Henipavirus V proteins is entirely dispensable for all aspects of STAT-directed IFN signaling inhibition. Instead, the minimal peptide region capable of STAT1 binding and IFN evasion maps to aa 100 to 160 and provides a mechanistic explanation for the observed rescue of an IFN-sensitive reporter virus and IFN signaling suppression conferred by the common N-termini of V and W proteins (21). The Henipavirus proteins, P and W, which are translated from partially overlapping open reading frames, share the same 406-aa N terminus prior to the editing site. Clearly, all proteins encoded by this reading frame include the STAT binding sites and NES defined in this report. It has yet to be demonstrated whether the Henipavirus P protein is capable of attenuating IFN responses, but considering that the P protein shares the N terminus, it is predicted that, if accessible to STAT proteins, the P protein would in principle block IFN signal transduction. The W protein consists of the same N terminus linked to a different C terminus, and its ability to block IFN signaling has been demonstrated (21). The presence of an NES in P and W proteins also predicts that they might have an intrinsic ability to shuttle between cytoplasm and nucleus. Interestingly, examination of the primary amino acid sequence of the W protein C-terminal extension reveals two pairs of basic residues that are suggestive of a bipartite nuclear localization signal. The roles for shuttling proteins in Henipavirus physiology is yet to be determined.

Given that the only homology between the Henipavirus V proteins and other paramyxovirus V proteins is within the conserved CTD, a significant question remains regarding
the function of the zinc-binding region. For some paramyxoviruses, the CTD has been demonstrated to be required for V protein function in IFN antagonism (6, 9, 16). Furthermore, for several paramyxoviruses, expression of the isolated CTD alone has been reported to evade IFN responses (10, 16, 21). However, expression of the CTD alone is not sufficient for IFN evasion by all species tested (e.g., compare results for Newcastle disease virus and Nipah virus in reference 21) and notably fails to copurify proteins important for Rubulavirus V protein-dependent ubiquitin ligase complexes (28). Therefore, this domain may play a role in additional aspects of IFN evasion, including the prevention of de novo IFN biosynthesis (6, 23). It is also likely that Henipavirus V proteins have evolved to use different peptide regions to counteract IFN signaling, while the conservation of the CTD is reflective of a broader function in the context of virus infection. It may be relevant that other paramyxoviruses have been demonstrated to form intracytoplasmic inclusions that contain viral proteins, nucleic acids, IRF3, and STATs (6, 17, 29), but more detailed analysis of V protein functions in the context of intact viruses will be required to shed light on the precise role of the CTD.

Further studies encompassing Henipavirus infection must be carried out to examine the potential for a common role for the N termini of V, W, and P proteins in the inhibition of IFN signaling and viral pathogenesis. Although the precise contributions of all protein products generated from the polycistronic V/P/W/C locus towards Henipavirus replication and pathogenesis are yet to be determined, the functional domains defined here represent logical targets for the development of specific Henipavirus antiviral compounds.

ACKNOWLEDGMENTS

We are grateful to Beric Henderson for providing the Rev1.4-GFP reagents, Christina Ulane for assistance with FLAG purification methods, and members of the Horvath laboratory for helpful discussions and comments on the manuscript.

This study was supported by research grants to C.M.H. from the American Cancer Society (Research Scholar Grant 103079) and the NIH (AI-48722, AI-50707, and AI-55733). J.J.R. is a trainee of the integrated training program in pharmacological sciences (GM-62754).

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