Identification of Paramyxovirus V Protein Residues Essential for STAT Protein Degradation and Promotion of Virus Replication

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Some paramyxovirus V proteins induce STAT protein degradation, and the amino acids essential for this process in the human parainfluenza virus type 2 (hPIV2) V protein have been studied. Various recombinant hPIV2s and cell lines constitutively expressing various mutant V proteins were generated. We found that V proteins with replacement of Cys residues of the Cys cluster were still able to bind STATs but were unable to induce their degradation. The hPIV2 V protein binds STATs via a W-$(X)_3$-W-$(X)_9$-W Trp motif located just upstream of the Cys cluster. Replacements of two or more Trp residues in this motif resulted in a failure to form a V/STAT2 complex. We have also identified two Phe residues of the hPIV2 V protein that are essential for STAT degradation, namely, Phe207, lying within the Cys cluster, and Phe143, in the P/V common region of the protein. Interestingly, infection of BHK cells with hPIV2 led to the specific degradation of STAT1 and not STAT2. Other evidence for the cell species specificity of hPIV2-induced STAT degradation is presented. Finally, a V-minus hPIV2, which can express only the P protein from its P gene, was generated and partially characterized. In contrast to V-minus viruses of other paramyxovirus genera, this V-minus rubulavirus was highly debilitated, and its growth even in Vero cells was very limited. The structural rubulavirus V proteins, as expected, are thus clearly important in promoting virus growth, independent of their anti-interferon (IFN) activity. Interestingly, many of the residues that are essential for anti-IFN activity, e.g., the Cys of this cluster and Phe207 within this cluster, as well as the Trp of this motif, are also essential for promoting virus growth.

The interferon (IFN) system is the first line of host defense against virus infection. Viruses of the *Paramyxovirinae*, similarly to other viruses, have evolved proteins that specifically inhibit IFN-induced innate antiviral responses, at least in part through direct inhibition of cellular STAT proteins that are responsible for IFN signal transduction. The V proteins encoded by the rubulaviruses simian virus 5 (SV5), SV41, and mumps virus (MuV) and by Newcastle disease virus (NDV, an avulavirus) block IFN signaling by targeting STAT1 for degradation (1, 5, 6, 14, 20, 25, 33, 45, 46, 49, 51), whereas the V protein of human parainfluenza virus type 2 (hPIV2, a rubulavirus) targets STAT2 for degradation (25, 32). Moreover, the V proteins of measles virus (morbillivirus) and Nipah virus and Hendra virus (henipaviruses) have been shown to inhibit IFN signaling by preventing STAT1 and STAT2 nuclear accumulation (30, 37, 38, 41). Sendai virus (SeV) and hPIV3 also block IFN signaling, and this anti-IFN ability has been shown to be a property of these respirovirus C proteins (7, 8, 10, 11, 17, 19, 42). In cells that are highly IFN competent, the Sendai virus C protein also induces the intracellular loss of STAT1 (9). The rubulavirus V protein-dependent degradation of STAT proteins involves degradation complexes that contain the V protein, STAT1 and STAT2 (and STAT3 in the case of mumps virus), and a number of cellular proteins, including the UV-

damaged-DNA binding protein 1 (DDB1) and Cullin4A, which are subunits of an SCF-type ubiquitin ligase (45).

Viruses of the *Paramyxovirinae* subfamily are presently classified within five genera (respiro-, morbilli-, rubula-, avula-, and henipaviruses). This classification is based in part on the genetic organization and expression of their P genes, named for the P protein that is an essential component of the viral RNA-dependent RNA polymerase. Paramyxovirus P genes are divided in half by an mRNA editing site, at which pseudotemplated nucleotides are inserted during mRNA synthesis. Thus, all three open reading frames (ORFs) downstream of this site are now available for translation, yielding the P, V, and W/I proteins. In contrast to the other genera, in which the unedited P gene mRNA codes for the P protein, and in which (except for avulaviruses) a C protein(s) is expressed from an overlapping ORF upstream of the editing site, the unedited rubulavirus P gene mRNA codes for the V protein and rubulaviruses do not express C proteins. Except for the essential P protein, the other P gene products are referred to accessory proteins.

The accessory V and C proteins of the *Paramyxovirinae* are multifunctional and are associated not only with virus pathogenesis. V and C also participate directly in virus replication, and the SV5 V protein is known to modulate the cell cycle (22). Paramyxovirus V proteins are composed of an N-terminal domain that is shared with the P and W/I proteins and a unique, highly conserved C-terminal domain (due to P gene mRNA editing) that is approximately 50% identical in amino acid sequence between all these viruses. Paramyxovirus V proteins have no cellular homologues but are readily identifiable by seven conserved cysteines at their C termini that bind two

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atoms of zinc (13, 34, 40). These conserved Cys residues play a critical role in specifically binding to DDB1 (1, 23). However, the binding of MuV V to STAT proteins in vitro occurs via a tryptophan-rich motif that lies just upstream of the Cys cluster, and the cysteine residues are not required for this binding (24). A selective defect in STAT2 protein synthesis has also been suggested to play a role in lowering STAT2 levels by the hPIV2 V protein (25). In this study, we have used hPIV2 V protein-expressing HeLa cells, and recombinant hPIV2s (rPIV2s) carrying V protein mutations, to examine various regions of this V protein that are essential for STAT protein degradation and the inhibition of IFN signaling, as well as its ability to promote virus replication independent of its anti-IFN activity. We find that both the Trp motif and the Cys cluster are required for both IFN-dependent and IFN-independent activities. Furthermore, two Phe residues that are important for activity, one within the Cys cluster and one in the N-terminal domain, have been identified.

SV5 antagonizes alpha and beta IFN (IFN-α/β) in primate cells but not in mouse cells, effectively restricting SV5 host range (50). The basis for this species specificity was demonstrated to be differences between human and murine STAT2 (31). hPIV2 also shows host range restriction, and we have used our panel of rPIV2s to examine this process. We have found cell lines in which either STAT1 or STAT2 or both STAT1 and STAT2 are specifically degraded, and this specificity also appears to be related to the species of the host cell.

MATERIALS AND METHODS

Cells and viruses. HeLa, Vero, L929, DBT (murine astrocytoma cell line), BHK, MDCK (canine kidney cell line), and human 2fTGH (gift from I. M. Kerr, Imperial Cancer Research Fund, London, United Kingdom) cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum. Primary monkey kidney (PMK) cells were cultured from a kidney removed from a Japanese macaque (*Macaca fuscata*) in Eagle's MEM supplemented with 10% fetal calf serum. BSR T7/5 (2), HeLa-V, HeLa-SV41-V, and L929-V cells (24) were cultured in Eagle's MEM supplemented with 10% fetal calf serum and 1-mg/ml G418 (Geneticin; GIBCO). hPIV2 (Toshiba strain) and SV41 (Toshiba strain) were used in this study.

Antibodies. Monoclonal antibodies (MAbs) against hPIV2 P/V protein (85A), hPIV2 V protein (53V), and hPIV2 NP protein (20A) were as described previously (26, 27). MAb against hPIV2 P/V protein 85A has cross-reactivity with SV41 P/V protein (47). Anti-STAT1 MAb was purchased from BD Transduction Laboratories (Lexington, KY). Monoclonal antibody against actin and rabbit polyclonal antibodies against STAT2 (N17 or C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Construction of expression plasmids. Various V genes and the hPIV2 NP gene, P gene, and L gene were cloned into pTM1, which contains a T7 promoter and an encephalomyocarditis virus internal ribosome entry site (B. Moss, National Institutes of Health). Expression plasmids encoding substitution mutant and chimeric mutant V proteins were prepared by PCR mutagenesis, and all constructs were confirmed by nucleic acid sequencing.

The plasmids encoding N-terminal Flag-tagged STAT1 or STAT2 (pCI-neo-Flag-STAT1 or -STAT2) (49) were provided by N. Yokosawa, Sapporo Medical University.

Immunoprecipitation analysis. A monolayer of BSR T7/5 cells in six-well plates was transfected with 2 μg of pTM1-V or mutants, 2 μg of pCI-neo-Flag-STAT-1 or -2, and 7 μl of FuGENE 6 (Roche) according to the manufacturer's instructions. After 40 h of culture, the transfectants were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.6% NP-40, and 4 mM phenylmethylsulfonyl fluoride). The supernatants obtained by centrifugation were incubated with an anti-Flag M2 agarose affinity gel (Sigma, St. Louis, Mo.) for 6 h at room temperature. The agarose beads were washed three times with lysis buffer and then extracted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer for analysis by a Western blotting technique with anti-Flag MAb (Sigma) or anti-hPIV2 P/V MAb.

Reporter gene assay with luciferase. Various rubulavirus V protein genes and their chimeras were cloned into the episomal Epstein-Barr virus-based expression plasmid pEBS-PL. The IFN-α/β-responsive reporter plasmid p(9–27)4tk (−39) lecter, referred to here as pISRE-(f)-luc, contains four tandem repeats of the IFN-inducible gene 9-27 IFN-stimulated response element fused to the firefly luciferase gene. pTK-(r)-luc, used as a transfection standard, contains the herpes simplex virus thymidine kinase (TK) promoter region upstream of the *Renilla* luciferase gene (Promega). For the luciferase assays, 2fTGH cells were transfected with 1 μg of pEBS-V, 1 μg of pISRE-(f)-luc, 0.3 μg of pTK-(r)-luc, and 7.5 μl of FuGENE 6. At 24 h posttransfection, the cells were treated with or without 1,000 IU of recombinant IFN-$\alpha2/\alpha1$ per ml. At 14 h post-IFN treatment, the cells were harvested and assayed for firefly and *Renilla* luciferase activity (dual-luciferase reporter assay system; Promega). Relative expression levels were calculated by dividing the firefly luciferase values by those of the *Renilla* luciferase.

Establishment of cell lines constitutively expressing virus-specific proteins. A cDNA clone of the mutated hPIV2 V or chimera V gene was inserted into plasmid pcDL-SRα296 between PstI and KpnI sites. The cDNA fragment was inserted between the ClaI and SalI sites of the vector pkan2, which contains the G418 resistance gene. HeLa or L929 cells were transfected with each plasmid, and cell lines expressing virus-specific proteins were established as described previously (28). Briefly, after 2 days of transfection, the culture medium was changed to MEM containing 10% fetal calf serum, 1-mg/ml G418, and 0.1% agarose, and the cells were cultured for 3 weeks. When colonies resistant to G418 became visible, they were transferred into 24-well plates. Three independent clones that exhibited high expression levels of the V protein were analyzed.

Construction of hPIV2 full-length plasmids. pPIV2, containing the full-length cDNA (15,654 nucleotides [nt]; GenBank accession number AB176531) of the hPIV2 Toshiba strain, was constructed, and two additional restriction enzyme sites (EagI, nt 1977 at the NP/P intergenic region, and MluI, nt 3255 at the P/M intergenic region) were created as genetic tag sequences. To construct rPIV2/P-edit and various rPIV2 mutants, the EagI-SacI (nt 2673 of the full-length cDNA) fragment containing the P/V gene from pPIV2 was subcloned into plasmid pDrive (Qiagen). Mutations were introduced into the V gene by PCR. To construct rPIV2/P-edit, we introduced six nucleotide changes to disrupt the P/V gene editing site and added 2 nt to code for P protein. For maintaining the genome length as a multiple of 6 (3), we deleted 2 nt just before the MluI site at the P/M intergenic region by PCR. The mutation introduced with rPIV2/P-edit was silent with regard to the P reading frame.

Generation of rPIV2 mutants. BSR T7/5 cells in a six-well plate were transfected with each hPIV2 full-length plasmid (10 μg) together with the following expression plasmids: pTM1-NP (nucleoprotein) at 2 μg, pTM1-P (phosphoprotein) at 1 μg, and pTM1-L (RNA-dependent RNA polymerase) at 2 μg. The transfection was carried out using FuGENE 6, according to the manufacturer's instructions. After 2 days, the supernatants of the transfected cells were further amplified in Vero cells. Virus stock was propagated twice in Vero cells before sequence analysis. Total RNA from infected cells was prepared using Isogene (Nippon Gene, Toyama, Japan). The introduction of mutations was confirmed by reverse transcription-PCR followed by sequencing analysis.

Viral growth kinetics. Monolayers of Vero, HeLa, and PMK cells were infected with several viruses at a multiplicity of infection (MOI) of about 0.01 and incubated at 37°C in MEM with 2% fetal calf serum. Supernatants were harvested at appropriate intervals, and virus titers were determined by plaque assay on Vero cells. Briefly, serial 10-fold dilutions of the virus samples were prepared in serum-free medium, and 100 μl of each serial dilution was added to each well of a 12-well plate. After 1 h of adsorption, the cells were overlaid with MEM containing 2% fetal calf serum in 0.6% agarose and incubated for 6 days. The cells were stained with neutral red for examination of plaques. Since plaque formation by rPIV2/P-edit could scarcely be detected, 96-well plates containing Vero cells were infected with serial twofold dilutions of the sample, and the presence of the virus was determined by enzyme-linked immunosorbent assay using an anti-hPIV2 NP MAb at 2 days postinfection.

Infection, cell extraction, and Western blot assay. For examination of STAT degradation, cells were infected with or without rPIV2, mutant rPIV2, or SV41 at an MOI of 3, and the cell lysates were prepared for analysis at 48 or 72 h postinfection (hpi). Cytoplasmic extracts were analyzed by a Western blotting technique with appropriate antibodies as described previously (24).

Nucleotide sequence accession numbers. GenBank accession numbers for STAT1 and STAT2 of Vero and BHK cells are AB177382, AB177398, AB177396, and AB177399, respectively.

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                        193   197          209 211 214   218 221
PIV2      174-HRREWSIAWVGDQVKVFEWCNPRCAPVTASARKFTCTCGSCPSICGECEGDH-225
SV41      174-HRREWSIAWVGDEVKVYEWCNPTCAPVTATDRKFSCTCGTCPDRCGECEGDN-225
SV5       171-HRREYSIGWVGDEVKVTEWCNPSCSPITAAARRFECTCHQCPVTCSECERDT-225
MuV       170-HRREWSLSWVQGEVRVFEWCNPICSPITAAARFHSCKCGNCPAKCDQCERDYGPP-224
NDV       177-HRREHSISWTMGGVTTISWCNPSCSPIRAEPRQYSCTCGSCPATCRLCAGDD---239
SeV       318-HRREHIIYERDGYIVDESWCNPVCSRIRVIPRRELCVCKTCPKVCKLCRDDI---384
PIV2/SeV  174-HRREWIIYWRDGYIVDESWCNPVCSRIRVIPRRELCVCKTCPKVCKLCRDDI-225
-H178W/E182W   ■   ■         ■*   *          *   *   *   *   *
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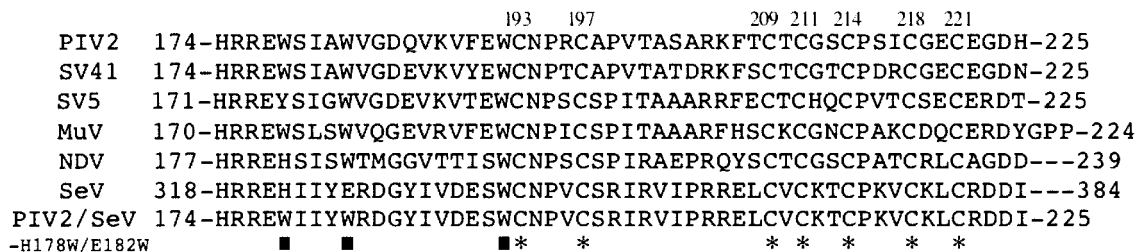
FIG. 1. V-specific regions of paramyxovirus V proteins. The V-specific regions of six paramyxoviruses and a chimeric virus, PIV2/SeV-H178W/E182W, were aligned. The amino acids are numbered from the amino terminus of each V protein. The asterisks indicate conserved cysteines; squares indicate conserved tryptophans.

RESULTS

The starting point for this study was the finding (i) that the MuV V protein bound to STAT1, STAT2, and STAT3 in vitro without specificity; (ii) that the cysteines of its Cys cluster, known to be important for rubulavirus V protein function, could be mutated to Ala without loss of binding; and (iii) that the MuV V/STAT association appeared to be due to a conserved W-$(X)_3$-W-$(X)_9$-W Trp motif located just upstream of the Cys cluster (Fig. 1). Only remnants of this motif are present in respiro- and morbilliviruses, and a chimeric V protein, containing the N-terminal portion of MuV and the C-terminal portion of SeV, MuV/SeV, which was unable to bind any STAT, could be converted to one which bound all three STATs simply by restoring the Trp motif, MuV/SeV-H5W/E9W (24). As we can carry out a more complete study with hPIV2, we examined the effects of mutation of these Cys and Trp residues on the various activities of hPIV2 V. We also examined other conserved residues within the C-terminal portion of hPIV2 V and a conserved Phe within the N-terminal portion of V that may bear a distant relationship to respirovirus C proteins (21). The various V genes were expressed by transfection, and these extracts were used to measure V association with STATs and their effect on IFN signaling. HeLa cell lines constitutively expressing V proteins were established to examine their effect on endogenous STAT1 and STAT2 protein levels. Finally, the mutated V genes were incorporated into full-length cDNAs, and infectious virus was recovered for characterization. The various properties of the mutant V proteins are summarized in Table 1.

hPIV2 V protein binds STAT1 and STAT2 via a Trp motif. To examine the role of the cysteines of the hPIV2 V protein Cys cluster in binding STAT proteins, three Cys/Ala substitution mutants (C193/197A, C209/211/214A, and C218/221A) were generated (Fig. 2A). The mutant hPIV2 V proteins and Flag-tagged STAT1 or STAT2 were then expressed in BSR T7/5 cells that contain cytoplasmic T7 RNA polymerase, via transfected plasmids with T7 promoters and internal ribosome entry sites (Fig. 2B). After immunoselection of cell lysates with an anti-Flag M2 agarose affinity gel, the precipitates were analyzed by Western blotting with anti-hPIV2 P/V. As shown in Fig. 2B, all three Cys-replacement V proteins were capable of binding to both STAT1 and STAT2, indicating that hPIV2 V/STAT complex formation does not require an intact Cys cluster.

The Trp motif upstream of the Cys cluster includes three

residues, W178, W182, and W192, in the hPIV2 V protein sequence (Fig. 1). For rubulaviruses, positions 182 and 192 are invariably W, whereas position 178 also contains Y (SV5) or H (NDV). To examine the role of these Trp residues in binding STAT proteins, seven Trp substitution mutants were generated. As the first and second Trp of this motif are His and Glu in SeV (Fig. 1), these two Trp residues were replaced with His and Glu, whereas the third Trp was mutated to Ala, as shown in Fig. 2C. When these Trp mutants were examined for STAT binding, we found that, whereas any single Trp residue could be replaced without loss of V/STAT2 complex formation, substitutions for two or more Trp residues eliminated this interaction (Fig. 2D). V/STAT1 complex formation was also assayed by coimmunoprecipitation, and the same results were obtained (data not shown). In addition, the F207H, F207E, and F143S mutants of hPIV2 V were examined by this test and found to bind STAT1 and STAT2 like the wild-type (wt) protein (data not shown). Thus, only V proteins in which two or

TABLE 1. Summary of the various properties of the hPIV2 V protein mutants

Mutation	STAT binding	IFN signaling block	STAT degradation		Virus growth in PMK cells[a]
			Cell lines	rPIV2	
wt	+	+	+	+	+++
F143S	+	−	−	−	++
W178Y	+	+	+	+	+++
W178H	+				
W182E	+				
W192A	+				
W178H/W182E	−	−	−	−	+
W182E/W192A	−	−	−	−	+[b]
W178H/W192A	−	−	−	−	+[b]
W178H/W182E/W192A	−	−	−	−	+[b]
C193/197A	+	−	−	−	+
C209/211/214A	+	−	−	−	+
C218A	+	−	−	−	+
C218/221A	+	−	−		
P199R	+	+	+	+	+++
F207E	+	−	−	−	+
F207H	+	+	+	+	++
V-minus					−[c]

[a] Virus growth in PMK cells is indicated as follows: +++, group 1; ++, group 2; +, group 3 in Fig. 5.

[b] The virus growth rate was examined only in HeLa cells under multiple-step growth conditions and found to be similar to that of W178H/W182E.
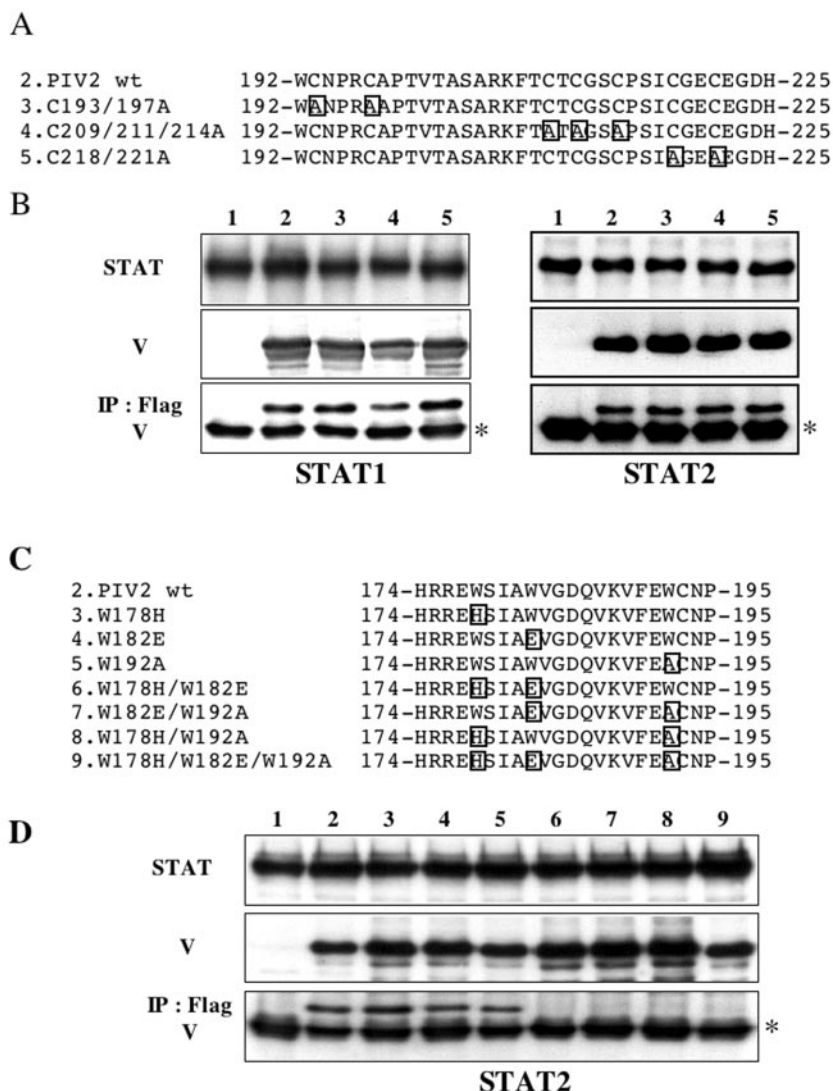
[c] −, no detectable virus growth.

A

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2.PIV2 wt      192-WCNPRCAPTVTASARKFTCTCGSCPSICGECEGDH-225
3.C193/197A    192-WANPRAAPTVTASARKFTCTCGSCPSICGECEGDH-225
4.C209/211/214A 192-WCNPRCAPTVTASARKFTATAGSAPSICGECEGDH-225
5.C218/221A    192-WCNPRCAPTVTASARKFTCTCGSCPSIAGEAFGDH-225
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B

STAT1   STAT2

C

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2.PIV2 wt          174-HRREWSIAWVGDQVKVFEWCNP-195
3.W178H            174-HRREHSIAWVGDQVKVFEWCNP-195
4.W182E            174-HRREWSIAEVGDQVKVFEWCNP-195
5.W192A            174-HRREWSIAWVGDQVKVFEACNP-195
6.W178H/W182E      174-HRREHSIAEVGDQVKVFEWCNP-195
7.W182E/W192A      174-HRREWSIAEVGDQVKVFEACNP-195
8.W178H/W192A      174-HRREHSIAWVGDQVKVFEACNP-195
9.W178H/W182E/W192A 174-HRREHSIAEVGDQVKVFEACNP-195
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D

STAT2

FIG. 2. V protein complex formation with STATs. (A and B) BSR T7/5 cells were transfected with either pFlag-STAT1 or pFlag-STAT2 plus pTM1 carrying one of the V genes indicated in panel A (lanes 2 to 5, panel B) or an empty pTM1 (lanes 1). Whole-cell extracts were prepared at 48 h posttransfection, and samples containing equal amounts of total protein were assayed by Western blotting for their levels of Flag-STAT1 or Flag-STAT2 (anti-Flag) and V proteins (anti-V). Other samples were first immunoprecipitated (IP) with Flag affinity gel (Sigma), and the selected materials were then assayed by Western blotting for their levels of V proteins. (C and D) The same experiment as above, except that tryptophan mutants are examined, and only the results of STAT2 complex formation are shown. The asterisks on the right indicate the immunoglobulin light chain.

more Trp residues of this motif are replaced with other residues have lost the ability to associate with STAT1 or STAT2.

STAT binding is necessary but insufficient to interdict IFN signaling. We next examined the abilities of the various MuV V and hPIV2 V proteins to interdict IFN-α-induced signaling to an IFN-responsive reporter gene. 2fTGH cells were transfected with a luciferase reporter construct and V protein expression plasmids and subsequently treated with IFN-α under the protocol indicated in Fig. 3. In cells transfected with a green fluorescent protein expression plasmid as an irrelevant control, firefly (f) luciferase activity was increased ca. threefold by treatment with IFN-α. Expression of the MuV V, hPIV2 V, and Sendai virus C proteins, as positive controls, all suppressed the activation of the IFN-α-responsive promoter. In contrast, all the mutant V proteins tested had lost the ability to interdict

IFN signaling. These included the MuV V Cys mutants C189/193A and C214/217A and hPIV2 V Cys mutants C193/197A, C209/211/214A, and C218/221A, as well as the MuV V and hPIV2 V Trp mutants in which the first and second Trp residues are mutated to His and Glu, respectively (Fig. 3). Mutation of the sole Phe examined within the N-terminal portion of V, namely, MuV V F139S and hPIV2 V F143S, also led to the loss of the antisignaling activity. As summarized in Table 1, although Cys/Ala substitution mutants of the V protein had the ability to associate with STAT proteins, all the V mutants tested, except for W178Y, had lost anti-IFN activity.

Endogenous STAT levels in HeLa cell lines constitutively expressing V proteins. To examine hPIV2 V protein-induced STAT degradation, HeLa cells constitutively expressing each of the mutant V proteins were established. As controls, we also
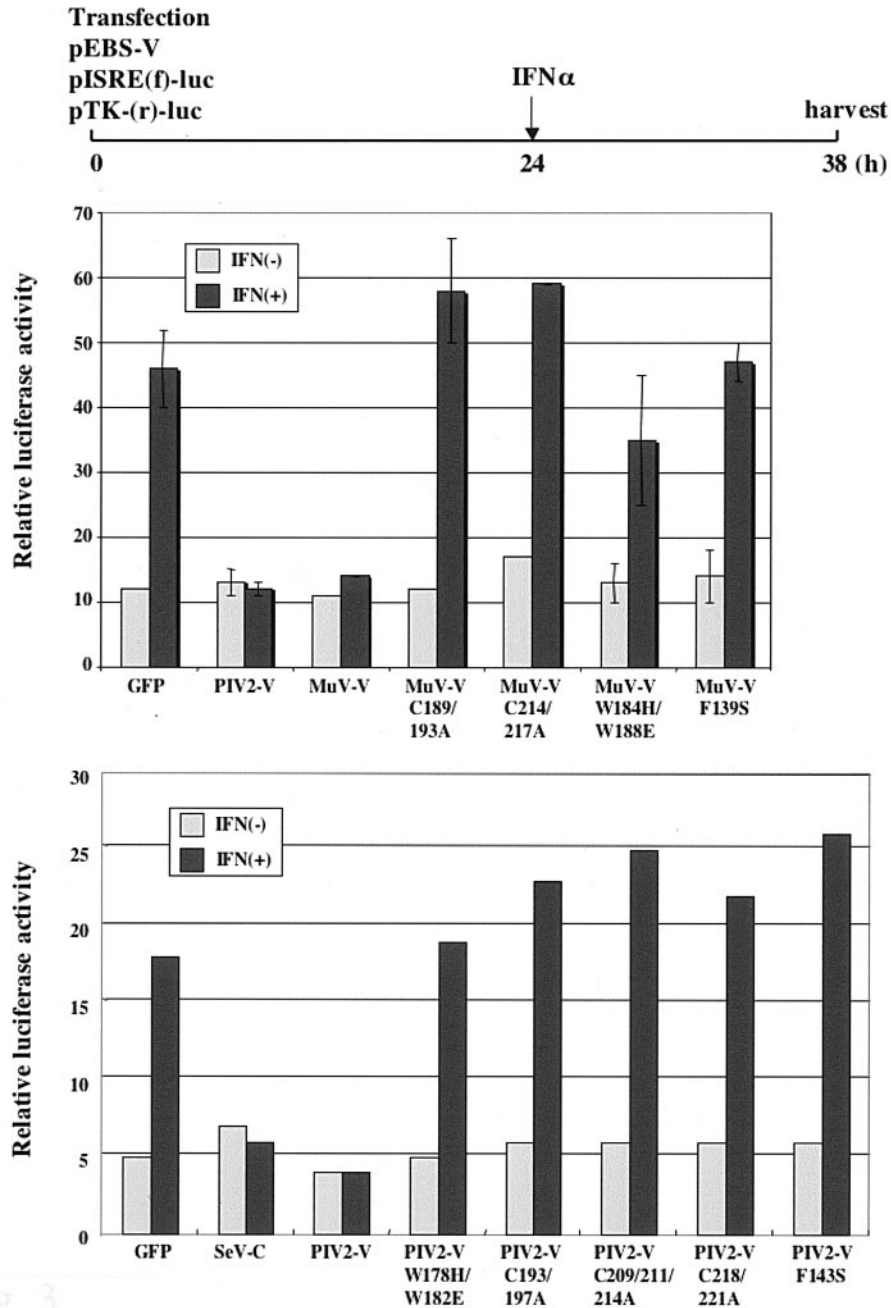
FIG. 3. Effects of V proteins on IFN-α-stimulated gene activation. This experiment was carried out as described in Materials and Methods. Briefly, 2fTGH cells were transfected with a reporter plasmid [pISRE-(f)-luc] and a V expression vector (pEBS-V) or relevant control (see text), along with pTK-(r)-luc as an internal reference for transfection efficiency. After incubation for 24 h, the cells were treated with or without IFN-α2/α1 (1,000 IU/ml) for 14 h and then lysed. The levels of both firefly (f) and *Renilla* (r) luciferase activities were determined. Data represent the mean values of the normalized firefly luciferase activities from triplicate samples. The V proteins in expression vectors used in this experiment are mumps virus (MuV) V, mutant MuV V (C189/193A, C214/217A, W184H/W188E, and F139S), hPIV2 V, and mutant hPIV2 V (W178H/W182E, C193/197A, C209/211/214A, C218/221A, and F143S) proteins. Green fluorescent protein alone and Sendai virus C protein (SeV C) were used as negative and positive controls, respectively.

examined SV41 V-expressing HeLa cells (HeLa-SV41-V) which contained specifically reduced levels of STAT1 (lane 3, Fig. 4A), while hPIV2 V-expressing HeLa cells (HeLa-V) contained specifically reduced levels of STAT2 (lane 2, Fig. 4A). In contrast, all the Cys-mutated V-expressing HeLa cells (C193/197A, C209/211/214A, and C218/221A) showed no re-

duction in their levels of either STAT1 or STAT2 (Fig. 4B, lanes 3 to 5). The presumed degradation of STAT2 in HeLa cell lines expressing the other mutant V proteins is summarized in Table 1 (data not shown). Except for W178Y, P199R, and F207H, all the other mutant V proteins had apparently lost their ability to induce STAT2 degradation.
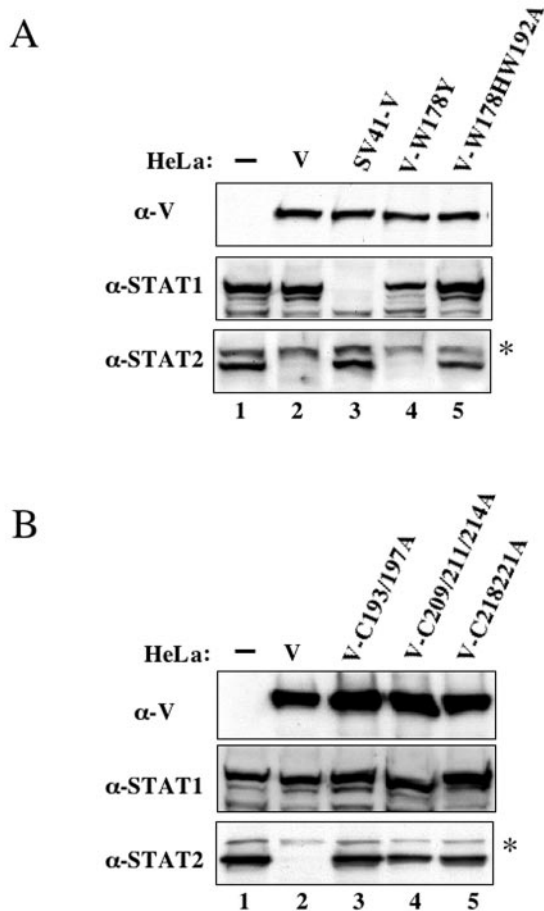
FIG. 4. STAT1 and STAT2 levels in HeLa cells constitutively expressing V proteins. Samples of cytoplasmic extracts containing equal amounts of total protein of HeLa cells constitutively expressing various V proteins (listed above each lane) or nonexpressing HeLa cells (lanes 1) were assayed by Western blotting for their levels of endogenous STAT1 and STAT2, as well as that of the various V proteins, using anti-P/V, anti-STAT1, and anti-STAT2, as indicated to the left of each panel. The asterisk on the right indicates a cross-reacting host band. These results, and those of other mutants (data not shown), are summarized in Table 1.

Growth of various rPIV2s with mutant V proteins in Vero, HeLa, and PMK cells. The various V genes were also incorporated into full-length hPIV2 cDNAs, and infectious virus was recovered so that the effects of these mutations could be characterized during a bona fide infection. These included mutations in the V-specific region that might be of interest other than Cys and Trp. For example, although the chimeric hPIV2/SeV-H178W/E182W protein (Fig. 1) associates with STAT1 and STAT2 like the wt hPIV2 V protein and MuV/SeV-H5W/E9W (24), it still fails to induce STAT1 or STAT2 degradation (data not shown). There may thus be other residues in the V-specific region that are important for STAT degradation and that are absent in the SeV V sequence. We therefore also examined Pro199 and Phe207, which are highly conserved among rubulaviruses, while these residues are Arg and Glu in SeV V protein, and amino acid 207 is His in the MuV V protein (Fig. 1).

All the rPIV2-expressing mutated V proteins were recov-

ered and are listed in Table 1 and Fig. 5A. Their growth characteristics, compared to those of rPIV2 wt, were investigated under multiple-step growth conditions, using the PMK, HeLa, and Vero cells (Fig. 5B). We note that the HeLa cells used in our laboratory produce extremely low levels of IFN upon virus infection (data not shown) and in this respect are closer to Vero cells. rPIV2s were classified by their growth patterns into three groups. (i) W178Y (amino acid 178 is Y in SV5) and P199R had growth rates comparable to that of wt rPIV2 in the three cell lines. (ii) F143S and F207H had growth rates in Vero and HeLa cells similar to that of wt rPIV2, but their virus yields in PMK cells were 20-fold lower. (iii) The strongest overall effects were found with the three Cys mutants C193/197A, C209/211/214A, and C218A, as well as with W178H/W182E and F207E. In Vero and HeLa cells, their yields were 100- and 1,000-fold lower than those of wt rPIV2. In PMK cells, the virus yields of these five mutant viruses were even lower, namely, 10,000-fold. Given that the growth of these latter rPIV2s is clearly debilitated even in Vero cells that do not secrete IFN, both the Trp motif and the Cys cluster (including F207) appear to also be important for promoting virus growth in an IFN-independent manner.

Recovery of a completely V-minus rPIV2. The rubulavirus V protein is reported to be multifunctional (22, 23, 36, 47, 48). To assess the extent to which the various mutations in V affected its global functions, we attempted the recovery of a recombinant hPIV2 which completely lacks expression of V protein, to provide the other point of reference with which the mutant rPIV2s can be compared. This virus was generated by simultaneously inactivating the P gene mRNA-editing signal and adding the 2 nt normally inserted during mRNA editing, such that the unedited P gene mRNA now directly codes for the P rather than the V protein (rPIV2/P-edit) (Fig. 6A). To maintain genome length as a multiple of 6 (2), 2 nt were removed from the noncoding region between P and M. rPIV2/P-edit was recovered, and this virus appeared to be extremely debilitated; rPIV2/P-edit showed no growth in HeLa and PMK cells and grew to very low levels in Vero cells. The V protein could not be detected in Vero cells infected with rPIV2/P-edit (Fig. 6B). When the growth rates of the rPIV2s with mutations in the Trp motif or Cys cluster are compared with that of rPIV2/P-edit, they are found to be considerably less debilitated in all three cell lines than the completely V-minus rPIV2. These results indicate that there may be other aspects of V function, which are not inactivated by mutations in the Trp motif or Cys cluster, that also play an important role in promoting virus replication.

V-induced degradation of STAT2 during rPIV2 infection. To examine the effect of the V protein mutations on STAT degradation during virus infection, HeLa cells were infected with wt rPIV2 and the various mutant rPIV2s at an MOI of 3. Cytoplasmic extracts were prepared (at 48 hpi) and examined by Western blotting for their levels of the viral P and V proteins, as well as the steady-state levels of STAT1 and STAT2. As shown in Fig. 7, whereas all the mutant rPIV2 infections had accumulated levels of the viral proteins roughly similar to those of rPIV2-wt, there were clear differences in their levels of STAT2. With one exception (F143S), all the mutations that strongly decreased virus propagation under multiple-step growth conditions (C193/197A, C209/211/214A, C218A, W178H/W182E, and F207E) had also lost the ability to de-
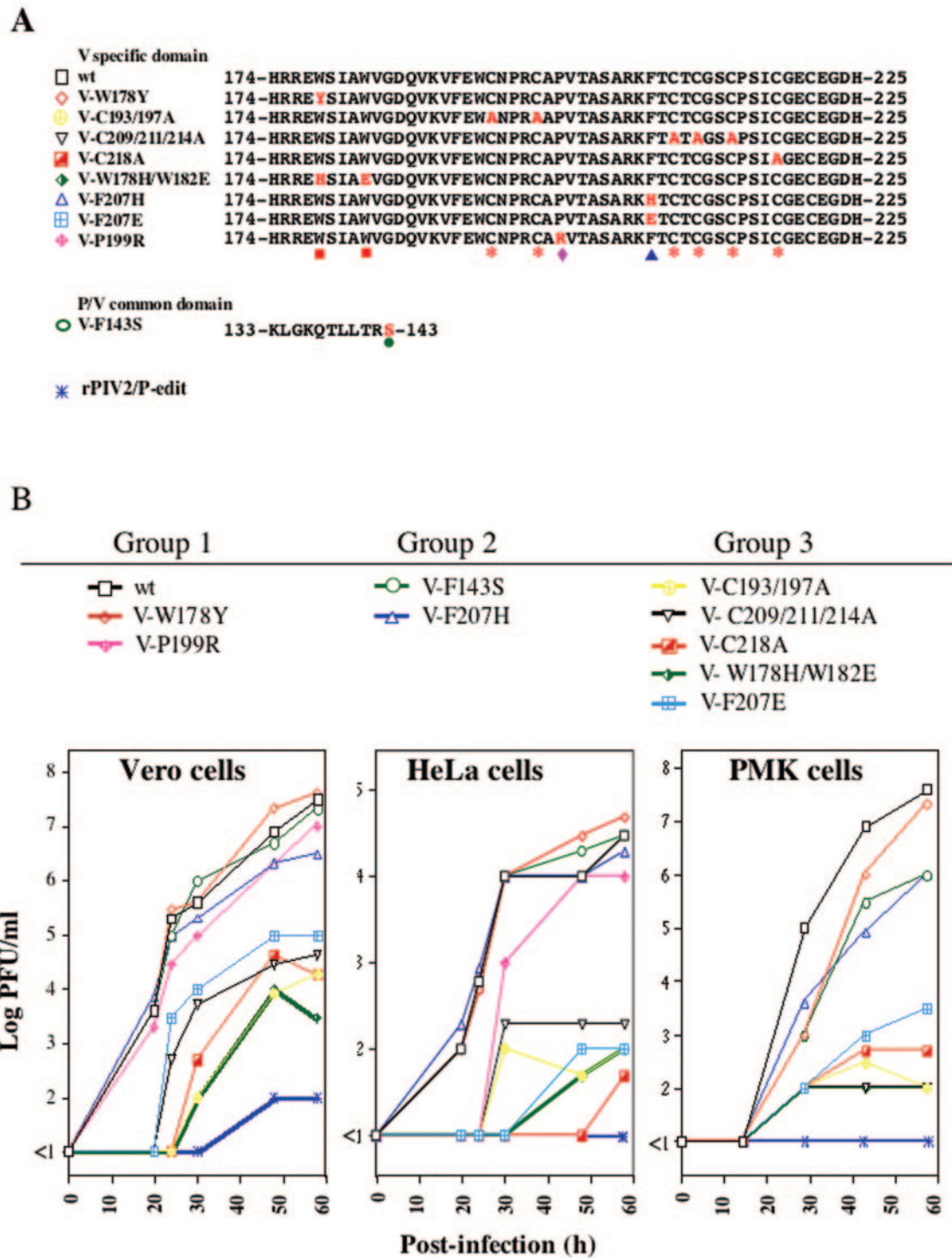
FIG. 5. Growth kinetics of rPIV2s carrying V protein mutations in tissue culture. Monolayers of Vero, HeLa, and PMK cells were infected with the various rPIV2s (A) at an MOI of ca. 0.01. Samples of the supernatants were harvested at various times postinfection, and their titers (PFU/ml) were determined by plaque formation on Vero monolayers (Materials and Methods). The symbols below the sequences indicate the positions of the mutations. (B). The three groups of virus mutants are listed (for details, see text).

grade STAT2, whereas those that had little or no effect on viral growth (W178Y, F207H, and P199R) had retained this ability. In contrast, the F143S mutant had lost the ability to degrade STAT2, even though its propagation rate in HeLa and Vero cells was similar to that of wt rPIV2. This one exception sup-

ports the notion that the ability of hPIV2 to grow well in these cells is related not only to its ability to degrade STAT2.

rPIV2-induced STAT degradation in monkey, hamster, murine, and canine cells. Parisien et al. (31) reported that STAT2 acts as a host range determinant for species-specific paramyxo-

A

F   K   R   G   G   A
V mRNA--TTTAAGAGGGGGGGGAGCT---

P mRNA--TTTAAGAGGGGGGG*GG*GAGCTA---
F   K   R   G   G   E   L

wt

V

mRNA

P mRNA(+2 frameshift)

V mRNA ----Not present

P mRNA--- TTcAAacGaGGaGGaGAGCTA---
F   K   R   G   G   E   L
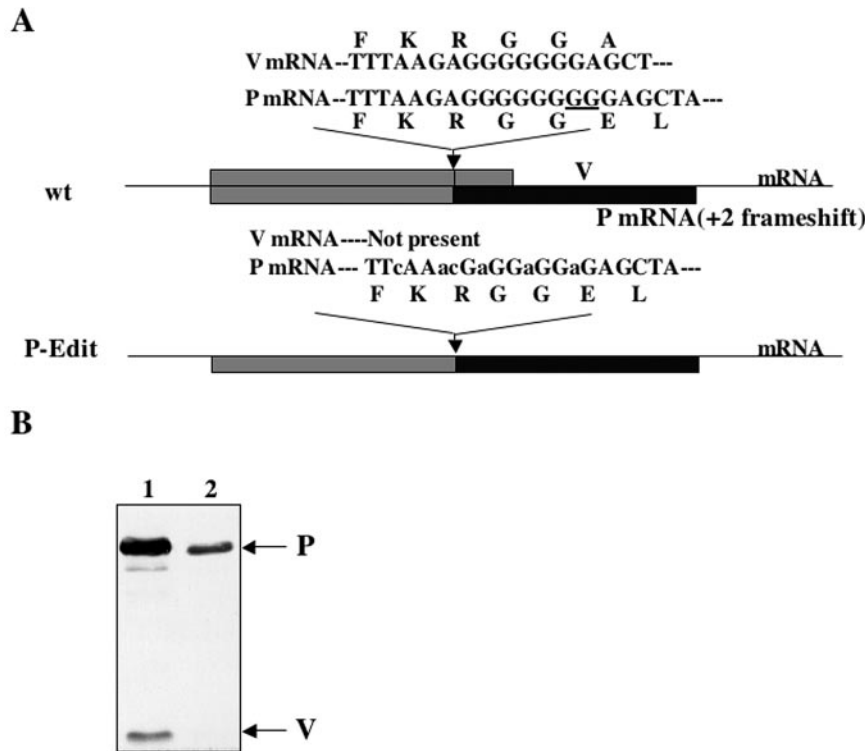
P-Edit

mRNA

B

1   2

← P

← V

FIG. 6. Generation of a recombinant hPIV2 which completely lacks expression of V protein (rPIV2/P-edit). (A) The expressed ORFs of the wt P and V mRNAs and the P-edit P mRNA are shown as boxes. The positive-sense nucleotide sequences around the editing site of each mRNA are shown above; the two pseudotemplated G's added during P mRNA synthesis are underlined. The encoded protein sequence is shown in single-letter code. Nucleotides in lowercase letters show the six silent mutations used to inactivate the mRNA editing signal (AAGAGGGGGGGG). The expected patterns of P gene expression are indicated. (B) Samples of cytoplasmic extracts of wt rPIV2 (lane 1)- and rPIV2/P-edit (lane 2)-infected Vero cells (72 hpi) were examined by Western blotting with anti-P/V MAb.

virus IFN antagonism and SV5 replication. hPIV2 infection also required the expression of human STAT2 to antagonize IFN-α/β signaling in mouse cells (31). We therefore examined STAT degradation during infection of various cells with our panel of rPIV2s. Infection of Vero (African green monkey kidney) cells with rPIV2s (48 hpi) showed the same pattern of STAT2 degradation as seen in HeLa cells (Fig. 8A), whereas STAT1 levels in Vero cells were not affected by any rPIV2 (Fig. 8A). Remarkably, infection of (hamster) BHK cells with the same panel of rPIV2s (48 hpi) showed precisely the same pattern of STAT degradation as in HeLa or Vero cells, except that in this case the rPIV2 infections reduced STAT1 but not STAT2 protein levels (Fig. 8B). Note that SV41, which lowers STAT1 in HeLa cells, also reduced STAT1 (but not STAT2) levels in BHK cells (Fig. 8B, lane 11). In murine L929 cells, rPIV2s (72 hpi) failed to degrade either STAT protein (Fig. 8C). The replication of hPIV2 is incomplete in murine cells (15). To examine the possibility that the absence of STAT degradation is due to lower growth of hPIV2 and less V protein expression, we analyzed the levels of STAT1 and STAT2 in two L929 cell lines constitutively expressing the hPIV2 V protein (L929-V cells). The expression of hPIV2 V protein did not result in a decrease in either the STAT1 or STAT2 level in these murine cells (Fig. 8D). In addition, infection of murine DBT cells similarly showed no reduction of STAT1 or STAT2 (data not shown). The hPIV2 V protein apparently cannot influence STAT levels in murine cells. Finally, to complete the
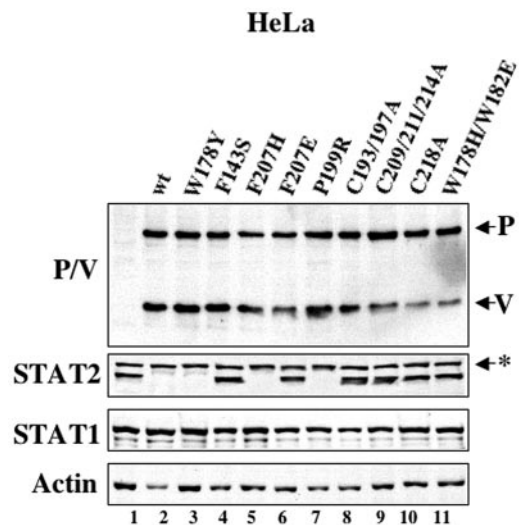

FIG. 7. STAT2 levels of HeLa cells infected with rPIV2s. HeLa cell monolayers were infected with the various rPIV2s listed above lanes 2 to 11, at an MOI of 3, or mock infected (lane 1). Cytoplasmic extracts were prepared at 48 hpi, and samples containing equal amounts of total protein were examined by Western blotting for their levels of P and V proteins and endogenous STAT1 and STAT2. Actin levels were also examined as a loading control. The asterisk beside the STAT2 blot indicates an unknown host band that also may serve as a loading control.
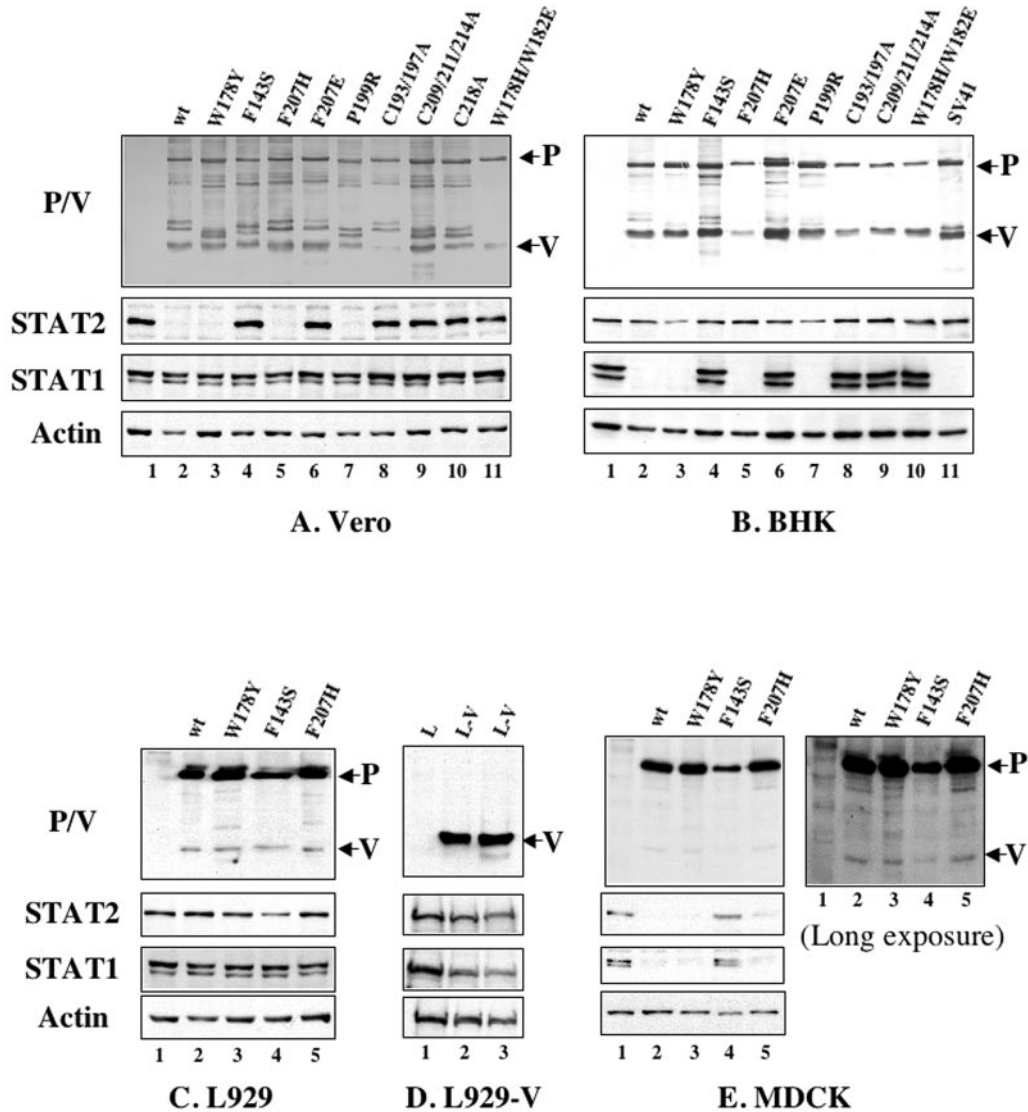
FIG. 8. STAT2 levels of Vero, BHK, L929, and MDCK cells infected with rPIV2s. Vero cell (monkey) (A), BHK cell (hamster) (B), L929 cell (murine) (C), and MDCK cell (canine) (E) monolayers were infected with the various rPIV2s listed above the lanes at an MOI of 3 or mock infected (lanes 1). Cytoplasmic extracts were prepared at 48 and 72 hpi, and samples containing equal amounts of total protein were examined by Western blotting for their levels of P and V proteins and endogenous STAT1 and STAT2. Actin levels were also examined as a loading control. Panel D shows Western blots of two independent L929 cell lines constitutively expressing hPIV2 V protein (lanes 2 and 3) and their progenitor cells (lane 1).

circle of possibilities, we found that wt rPIV2 and rPIV2/V-F207H diminished both STAT1 and STAT2 levels in canine MDCK cells (72 hpi) (Fig. 8E), even though hPIV2 grew to a lower level in MDCK cells (as in L929 cells) and V protein levels were significantly lower. Thus, the V protein of hPIV2 can induce the loss of STAT1 or STAT2, or both proteins, apparently in a cell-specific manner.

DISCUSSION

The most notable feature of the carboxyl domain of paramyxovirus V proteins is their cluster of seven Cys residues that bind two atoms of Zn^{2+}, and this region may thus resemble a RING finger, a known protein-protein interaction domain (29, 43). The V-specific carboxyl domains of rubulaviruses, which are

associated with STAT degradation and the inhibition of IFN signaling, also contain a Trp motif (W-X$_3$-W-X$_9$-W) that lies just upstream of the Cys cluster. Our previous studies showed that the V-specific domain of hPIV2 is required for its anti-IFN activity (25) and that MuV V protein associates with STAT proteins in vitro via its Trp motif, whereas the downstream Cys residues are not required for this binding (24). Here, we report that the hPIV2 V protein behaves similarly to that of MuV in this respect and, furthermore, that any two of the three Trp residues of this motif are sufficient for V association with STAT in vitro. However, hPIV2-induced STAT protein degradation and anti-IFN activity require (presumably all) the Cys residues as well as the Trp motif. Thus, V/STAT complex formation appears to be necessary, but not sufficient,

for the anti-IFN response. It has recently been shown that the SV5, hPIV2, and MuV V proteins bind to DDB1 and its associated Cullin4A, and this complex directly induces the polyubiquitination of STAT proteins (1, 45, 46). The interaction of SV5 V protein with DDB1 involves its carboxyl domain, and either deletion of this domain or replacement of its Cys with Ala eliminates DDB1/V/STAT complex formation, indicating that the Cys cluster is required for V binding to DDB1 (1, 23). Furthermore, an SV5 V protein deleted of the N-terminal 85 amino acids and that of the CPI⁻ strain (which has mutations at the N terminus of V) failed to bind DDB1 (1). The N terminus of V protein thus also affects DDB1 binding (1). The mutant hPIV2 V proteins C193/197A, C209/211/214A, C218/211A, and W178H/W182E, which are unable to lower STAT protein levels, also failed to bind DDB1 (data not shown). Thus, the ability of V to bind both STAT and DDB1 via adjacent Trp- and Cys-rich domains is required to form the ubiquitin-ligase complex that results in the reduction of STAT levels. Since the Cys cluster mutants still bind STAT whereas they do not bind DDB1, it seems reasonable that V/STAT complexes are formed first and that these then associate with the ubiquitin-ligase complex via DDB1.

These studies were motivated in part by finding that V bound to STAT1, STAT2, or STAT3 in vitro with little or no specificity via the Trp motif, whereas STAT degradation in vivo was highly specific. Our present finding that V/STAT complex formation via the Trp motif is necessary, but not sufficient, for STAT degradation helps explain this apparent discordance. It also suggests that these Trp side chains interact predominantly with surfaces of STAT1, -2, and -3 that are conserved and that the specificity of STAT degradation lies downstream, in the interaction of V and DDB1. This view is supported by the fact that V/DDB1 interactions involve not only the Trp motif but the Cys cluster and residues throughout the N-terminal domain as well. Assuming that the two V proteins are associated with each STAT dimer, the choice of which subunit of the dimer is targeted for ubiquitination presumably depends on which of the two V proteins preferentially interact with DDB1. This choice may depend not only on more subtle V/STAT interactions than can be measured in crude binding assays but also on direct contacts between the various STATs and DDB1 that are specific for each STAT protein (see below). Given that V presumably acts by connecting a STAT homo- or heterodimer to DDB1 and its associated ubiquitin-ligase and that V apparently does so via domains that are so very closely apposed, presumably only one ubiquitin-ligase complex can be bound to the V/STAT complex at any one time, for steric reasons, and this may contribute to the specificity of STAT degradation.

Parisien et al. (31) reported that STAT2 acts as a host range determinant for species-specific paramyxovirus IFN antagonism. Murine and human STAT1 proteins are 92.4% identical at the amino acid level, while murine and human STAT2 proteins are more divergent, with a modest 68.6% overall identity (35). hPIV2 infection induces STAT2 degradation in Vero (African green monkey kidney) cells, as in HeLa cells. Interestingly, hPIV2 infection induces STAT1 degradation in baby hamster kidney cells, similar to SV41 and SV5 infection in HeLa, Vero, and BHK cells. Thus, we sequenced STAT1 and STAT2 of Vero and BHK cells (GenBank accession numbers

AB177382, AB177398, AB177396, and AB177399, respectively). Monkey and human STAT1 proteins are 99.3% identical, and those STAT2 proteins are 97.8% identical in sequence (unpublished data). However, hamster and human STAT1 proteins are 93.9% identical, and those STAT2 proteins are only 73.6% identical (unpublished data). The differences between human and hamster STAT2 orthologues presumably determine which STAT protein is ubiquitinylated in cells of these different species.

Recombinant morbilliviruses (39), respiroviruses (4, 16), and NDV (14, 33) that cannot express their V and W proteins have been recovered, and all these viruses grow similarly to their respective wt virus at least in some cell lines, e.g., in Vero cells. NDV, the only nonmammalian virus, was previously classified as a rubulavirus because its proteins and genomic promoters most resemble those of mammalian rubulaviruses. However, the avian paramyxoviruses have recently been reclassified in the new *Avulavirus* genus, in part because their P genes are organized and their P gene mRNAs are edited, unlike those of mammalian rubulaviruses but like those of morbilli- and respiroviruses. rPIV2/P-edit is thus the only fully V-minus mammalian rubulavirus that has so far been recovered, and for good reason. In strong contrast to the equivalent viruses in the other three genera, rPIV2/P-edit barely grows relative to its wt parent even in Vero cells. rPIV2/VΔC, which expresses a W- or I-like protein in place of V, also grows very poorly in Vero cells (18), unlike SV5 VΔC (12). The growth defect of rPIV2/P-edit thus appears to be due to the absence of an intact V protein, and in this case this defect apparently cannot be compensated for by the overexpression of a W/I-like protein as in SV5 VΔC. Transient expression of the carboxyl portion of the NDV V protein enhanced the growth of NDVs in which the V or both the V and W proteins were not expressed (14). However, the growth rate of our rPIV2 mutant viruses did not improve in HeLa cells constitutively expressing the hPIV2 V protein (data not shown). Mammalian rubulavirus V proteins thus appear to act differently than NDV V and are unique among paramyxoviruses in that they are so clearly important in promoting virus growth, independent of their anti-IFN activity. The proposed role of rubulavirus V proteins in associating with unassembled NP protein, a process thought to be critical for genome replication, may be one reason for the defect in rPIV2/P-edit replication. However, rubulavirus V proteins are also unique in being incorporated into the virions, where they are associated with the N:RNA along with P and L (34, 36). The precise defect(s) in rPIV2/P-edit replication remains to be investigated.

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