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NISHIO, Machiko, et al.

Abstract
Viruses of the Paramyxovirinae, similar to other viruses, have evolved specific proteins that interdict IFN action as part of a general strategy to counteract host innate immunity. In many (but not all) cases, this interdiction is accompanied by a lowering of the intracellular levels of the STAT proteins. Among rubulaviruses, there is a notable variation in how they interfere with IFN action. Whereas SV41, SV5, and MuV all act by lowering Stat1, hPIV2 acts by lowering Stat2. Here, we show that the mumps and hPIV2 V proteins both form a complex with several Stat proteins in a mixed-extract assay. This suggests that the specific degradation of these Stat proteins is not determined by complex formation, but presumably at some later stage of the degradation pathway. V/Stat complex formation requires a specific carboxyl segment of V. However, a previously unrecognized trp-rich motif, rather than the Zn(++)-binding cys-cluster of this segment, appears to be required for V/Stat interaction. The C protein of Sendai (respiro-) virus, another P gene encoded protein, also forms a complex with Stat1, and prebinding of MuV V to Stat1 [...]
The Carboxyl Segment of the Mumps Virus V Protein Associates with Stat Proteins in Vitro Via a Tryptophan-Rich Motif

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Viruses of the Paramyxovirinae, similar to other viruses, have evolved specific proteins that interdict IFN action as part of a general strategy to counteract host innate immunity. In many (but not all) cases, this interdiction is accompanied by a lowering of the intracellular levels of the STAT proteins. Among rubulaviruses, there is a notable variation in how they interfere with IFN action. Whereas SV40, SV5, and MuV all act by lowering Stat1, hPIV2 acts by lowering Stat2. Here, we show that the mumps and hPIV2 V proteins both form a complex with several Stat proteins in a mixed-extract assay. This suggests that the specific degradation of these Stat proteins is not determined by complex formation, but presumably at some later stage of the degradation pathway. V/Stat complex formation requires a specific carboxyl segment of V. However, a previously unrecognized trp-rich motif, rather than the Zn\(^{2+}\)-binding cysteine cluster of this segment, appears to be required for V/Stat interaction. The C protein of Sendai (respirovirus), another P gene encoded protein, also forms a complex with Stat1, and prebinding of MuV V to Stat1 prevents the subsequent binding of SeV C. Our results suggest that rubulavirus V proteins may be related to both the C and the V proteins of respiroviruses.

INTRODUCTION

The Paramyxovirinae are a large subfamily of important human and animal pathogens that are currently classified in three genera: respiro-, morbilli-, and rubulaviruses. This classification reflects in part the genetic organization of their P genes (Fig. 1). Paramyxovirus P genes express not only P proteins that are essential subunits of the viral RNA polymerase, they also express "accessory" proteins such as C and V that are not expressed by all P genes of this subfamily. These accessory proteins are not essential for virus replication in some cell culture conditions, and they appear to act primarily to counteract the innate defenses of host cells (for review, see Lamb and Kolakofsky, 2001).

All viruses of the Paramyxovirinae (with the notable exception of hPIV1) contain an mRNA-editing site at which G residues are inserted into the P mRNA in a programmed manner during its synthesis (Fig. 1; dotted vertical line) (Cattaneo et al., 1989; Thomas et al., 1988; Vidal et al., 1990). The number of G residues inserted varies between the different viruses and appears to be matched to the organization of their alternate ORFs. The editing site separates each P gene into two segments, and it generates three different mRNAs in which the three possible ORFs downstream of the editing site (regardless of their size) are fused to a unique upstream ORF (Fig. 1). These mRNAs are translated into three polypeptides with common N-terminal segments and different C-terminal segments, called P (polymerase cofactor module), V, and W/D/I. In respiroviruses and morbilviruses, additional P gene complexity is provided by the presence of two (overlapping) ORFs upstream of the editing site (a longer ORF that is in acidic in nature, and a shorter basic C ORF), and only the longer ORF extends to this site. For Sendai virus and hPIV1, the C ORF that terminates before the editing site can also code for a nested set of up to four polypeptides, due to the use of four ribosomal initiation sites. For rubulaviruses, there is but a single ORF upstream of the editing site. The P genes of the Paramyxovirinae are thus composed of a series of modules that are combined in various ways via mRNA editing, or as additional modules expressed via unusual translational initiation (Boeck et al., 1992; Curran and Kolakofsky, 1988; Latorre et al., 1998). However, the P protein itself is the only P gene product that is essential for viral RNA synthesis (Curran et al., 1995; Murphy and Parks, 1997). For SeV, C and V have negative effects on viral RNA synthesis when this property is examined in isolation (Curran et al., 1991, 1992). However, at least the C proteins act in a positive manner in promoting early steps in intracellular replication, when they are present at low concentrations (Latorre et al., 1998).

Viruses of the Paramyxovirinae, similar to other viruses, have evolved specific proteins that interdict IFN signaling as part of a general strategy to counteract host innate immunity. In many (but not all) cases, this inter-
Among rubulaviruses, there is also a notable variation in how they interfere with IFN action. Whereas SV41, SV5, and MuV all act by lowering Stat1, hPIV2 acts by lowering Stat2, and this difference is mirrored in how these rubulaviruses counteract IFNα and IFNγ signaling (Young et al., 2000; Nishio et al., 2001; Parisien et al., 2001). Although V/Stat complex formation has not as yet been directly demonstrated for a rubulavirus V protein, we hoped this could be carried out by an in vitro approach that demonstrated SeV C protein/Stat1 complex formation (Garcin et al., 2002). If so, we could then examine where this specificity for Stat degradation was located, by constructing chimeric V proteins consisting of the amino-terminal and carboxyl segments of MuV and hPIV2 proteins fused at the editing site. This article reports that the MuV and hPIV2 V proteins both form a complex with both Stat1 and Stat2 in vitro. This suggests that the specific degradation of these Stat proteins is not determined by complex formation, but presumably at some later stage of the degradation pathway. Moreover, MuV V protein may interact with a similar surface of Stat1 as the SeV C protein, as prebinding of V prevents the subsequent binding of C.

RESULTS

SeV C protein/Stat1 complexes can be coimmunoprecipitated from infected human and murine cell extracts (Takeuchi et al., 2001; Garcin et al., 2002). This complex can also be formed in vitro by mixing MEF cell extracts that are naturally rich in Stat1 with extracts of BSR T7 cells (Buchholz et al., 1999), in which the C proteins were expressed by transfection, and which naturally contain low levels of Stat1 and are IFN-incompetent, i.e., they do not respond to IFN treatment (data not shown). Complex formation in vitro is rapid, appears not to require energy, and is specific in that a natural-point mutation in C (F170S) responsible for the loss of anti-IFN activity also abrogates C/Stat1 complex formation (Garcin et al., 2002). We therefore first examined whether the MEF extracts also contained detectable amounts of Stat2 and Stat3 (the latter as a prospective negative control) by Western blotting with anti-Stat1, 2, or 3. These MEFs appear to be in a permanent antiviral state. Highly IFN-sensitive viruses such as VSV or SeV-C (F170S) do not
grow in these cultures (Garcin et al., 2000), and their supernatants contain high levels of IFN (D. Garcin, unpublished results). These MEFs might then contain elevated levels of many ISGs, including several Stat proteins. Lanes "none" of Fig. 2 show that these extracts contained detectable amounts of Stat2 and Stat3 as well as Stat1. To determine whether our antibodies were acting in a specific manner, i.e., that they did not cross-select the other Stat proteins, MEF extracts were first precipitated with anti-Stat1, 2, or 3, and the individual precipitates were then Western blotted with anti-Stat1, anti-Stat2, or anti-Stat3, as indicated. Ten percent of the samples used for immunoprecipitation were separated directly on the gel and not immunoprecipitated (lanes "none"). None lanes indicate the relative levels of Stat proteins in the starting extract and also serve as electrophoretic mobility markers.

The MuV V, I (or NS2), and P proteins were then expressed by transfection in BSR T7 cells (Fig. 3, top panel, "extract") and their extracts were mixed with MEF extracts. Possible complexes with Stat1, 2, or 3 were precipitated with anti-Stat1, 2, or 3, and the individual precipitates were then Western blotted with anti-Stat1, 2, or 3. All three anti-Stat antibodies were found to precipitate only their cognate Stat proteins (Fig. 2). Preexisting Stat protein complexes that may be present in the MEF extracts (such as Stat1/Stat2 and Stat1/Stat3 heterodimers) are presumably below the level of detection with this method.

The MuV V, I (or NS2), and P proteins were then expressed by transfection in BSR T7 cells (Fig. 3, top panel, "extract") and their extracts were mixed with MEF extracts. Possible complexes with Stat1, 2, or 3 were precipitated with their respective antibodies, and these precipitates were then Western blotted with an antibody (T61) to the common amino-terminal segment of V, NS2, and P. Unexpectedly, all three anti-Stat antibodies were found to coprecipitate V, whereas NS2 and P/Stat complexes were not detected. Moreover, the reciprocal precipitation of the mixed extracts with T61 followed by Western blotting with anti-Stat1 and anti-Stat3 also showed specific V/Stat complex formation (Fig. 3). A similar experiment was then carried out with the hPIV2 V and P proteins, using mAb 84-1A to the common amino-terminal domain of hPIV2 V and P. As before, all three anti-Stat antibodies coprecipitated the V protein, but not the P protein (Fig. 4). Thus, both the MuV and the hPIV2 V proteins form complexes in vitro with all three Stat proteins examined.

The carboxyl, cys-rich, Zn$^{2+}$-binding segment of the V proteins

The carboxyl segment of the V ORF is by far the best conserved of the various P gene ORFs (Fig. 5A). There are seven perfectly conserved cysteine residues, and this domain binds two atoms of Zn$^{2+}$ (Liston and Briedis, 1994; Paterson, 1995). Similar to the carboxyl pol-cofactor module of the P protein, the cys-rich V ORF is found only as the carboxyl segment of a fusion protein; this module is never expressed by itself. As rubulavirus P proteins share the amino-terminal segments of their V proteins
but do not prevent IFN signaling, the carboxyl segment of the V protein is thought to be primarily responsible for this effect (Didcock et al., 1999). To examine the importance of the Zn++-binding cys cluster for MuV V protein/Stat interaction in vitro, two pairs of closely spaced cys residues (C20/C24 and C36/C38) were individually mutated to ala (residues numbered from start of the expressed V ORF, Fig. 5A). These mutant V proteins were then tested for their interaction with either Stat1 or Stat2 by coimmunoprecipitation. The C20A/C24A and C36A/C38A double mutants consistently interacted well with both Stat1 and 2 (Fig. 5B, lanes MuV-C20A/C24A and lanes MuV-C36A/C38A, and data not shown). Thus, either a completely intact cys cluster is not critical for Stat1 or Stat2 interaction, and/or the mixed-extract assay is particularly permissive for this complex formation. As the MuV NS2 and P proteins do not associate with Stat proteins in vitro, we further examined the specificity of this complex formation using chimeric MuV V proteins (fused at the editing site). To examine whether any paramyxovirus carboxyl segment will suffice for Stat1 binding, the MuV V ORF was replaced with that of SeV (the SeVV protein does not appear to bind Stat1; Fig. 6A). The chimeric MuV/SeV V protein was found not to interact with Stat1, 2, or 3 (lane SeV, Fig. 5B). MuVV/Stat interaction in vitro thus requires a specific carboxyl segment.

The above results suggest that residues in the carboxyl segment other than those conserved for Zn++-binding are important for Stat interaction. The V ORF can itself be divided into two parts; the downstream cys-rich
Zn$^{+2}$-binding domain that begins with the conserved tetrapeptide $^{19}$WCNP$^{22}$ (where cys to ala double mutations have little or no effect on Stat interaction) and the region upstream that extends to the fusion site, beginning with the conserved tetrapeptide $^{1}$HRRE$^{4}$. Within this upstream region, there are two trp residues that are notable ($W^5$ and $W^9$); they are partially conserved, and trp residues are normally very rare. $W^9$ is conserved among all rubulavirus (and morbillivirus), but this position is occupied by a charged residue in the respiroviruses. Position 5 is always occupied by an aromatic residue, but $W$, $Y$, and $H$ are all found here. Together with $^{19}$WCNP$^{25}$, this region vaguely resembles the well-studied WW protein domain that is notorious for protein/protein interaction (Sudol and Hunter, 2000). WW domains contain two strictly conserved trp residues that are spaced 20–22 aa apart, and there is a highly conserved proline that follows the downstream trp by three residues (Freemont, 1993; Jiang et al., 2001). $W^5$ and $W^9$ are only 10 residues apart, but $W^9$ is indeed followed by P$^{22}$.

To examine whether $W^5$ and $W^9$ of the carboxyl segment of MuV V are important for V/Stat1 interaction, both trp residues were mutated to those found at the same location in the SeV V protein, i.e., $W^5$ to H and $W^9$ to E. This MuV V(W5H/W9E) protein was found to be inactive in Stat complex formation (lanes MuV-W5H/W9E, Fig. 5B). Thus, in contrast to mutations of the cys cluster, mutation of the upstream trp residues inactivated Stat interaction, and at least one of these two trp are thus essential for this interaction in vitro. To examine whether the absence of these two W by themselves could possibly account for the inability of the MuV/SeV V protein chimeras to interact with Stat1 in this assay, we constructed the reciprocal mutant MuV/SeV (H5W/E9W). These mutations that restore the $W^5$/P$^{22}$/WCNP$^{22}$ motif to the chimeric MuV/SeV V protein also restore the ability of this chimera to interact with Stat (lanes SeV-H5W/E9W, Fig. 5B).

Although the available evidence indicates that the carboxyl segment of V is primarily responsible for V/Stat interaction, we nevertheless also examined a single residue in the amino-terminal segment that could be involved in this interaction. There is circumstantial evidence that the amino-terminal modules of the rubulavirus V proteins are related to the respirovirus C proteins; they are all ca. 170 residues long and are basic (pl ~ 10.5) and there is a small region of apparent sequence conservation that includes F170 of SeV C that is critical for its interaction with Stat1 (Fig. 5C). However, when the MuV V (F139S) protein was tested, this phe-to-ser mutation did not abolish V interaction with Stat1 (lanes MuV-F139S, Fig. 5B).

**MuV V and SeV C proteins may interact with the same region of Stat1**

As both the MuV V proteins and the SeV C proteins coimmunoprecipitate with Stat1 in our mixed-extract assay, we next determined whether this interaction was mutually exclusive, i.e., whether these two proteins could compete for interaction with a limiting amount of Stat1. This experiment was carried out with GFP-C fusion proteins whose binding to Stat1 is also sensitive to the F170S mutation (Fig. 6A, top). From numerous assays, the SeV C proteins appear to bind Stat1 as avidly as the MuV V protein. A strong test of competitive binding was therefore to determine whether the MuV V protein could prevent subsequent C protein interaction with Stat1. MEF extracts were mixed with BSR T7 extracts containing either none, a 1X, or a 2X amount of MuV V protein for 1 h at 20°C to allow V/Stat1 complex formation. Other BSR T7 extracts containing GFP-C (or GFP-C$^{F170S}$ as a
negative control) were then added and after a further 1 h, the samples were precipitated with anti-GFP and the amounts of Stat1 recovered were estimated by Western blotting. As shown in Fig. 6B, increasing amounts of the MuV V protein containing BSR T7 extract increasingly interfered with the subsequent C protein interaction with Stat1. The MuV V and SeV C proteins thus appear to interact with a similar region of Stat1, as the prebinding of one prevents the subsequent binding of the other. At this stage, however, one cannot rule out the possibility that that this interference is indirect, i.e., that V binding to Stat1 induces a conformational change in Stat1 that is responsible for the loss of subsequent C/Stat interaction.

DISCUSSION

SV5, SV41, and MuV infections all lower Stat1 levels, whereas hPIV2 lowers Stat2, and this difference is consistent with how these rubulavirus infections interfere with IFNα and IFNγ signaling. However, when the MuV and hPIV2 V proteins were examined for V/Stat complex formation by coimmunoprecipitation, both V proteins unexpectedly associated with both Stat1 and Stat2. Moreover, both V proteins also associated with Stat3, which was included in the study as prospective negative control. The association of the MuV V protein with the three Stat proteins nevertheless appears to be specific, in that it requires two trp residues of a trp-rich motif (W9/W9/ WCNP9) that vaguely resembles a WW protein/protein interaction domain (Sudol and Hunter, 2000). In contrast, V/Stat complex formation in vitro does not appear to require an intact Zn2⁺-binding cys cluster that vaguely resembles a RING domain (both contain seven cys that coordinate two Zn2⁺ atoms) that is also notorious as a protein/protein interaction domain (Zheng et al., 2000). Many RING finger proteins have recently been found to be components of ubiquitin-ligase complexes and to function by controlled degradation of specific target protein (Freemont, 1993, 2000). There may be two different protein interaction domains in the carboxyl segment of the rubulavirus V proteins: one that mediates Stat protein interaction and the other that mediates Stat degradation. For SeV, a respirovirus, the V protein does not counteract IFN signaling (Garcin et al., 1999), nor interact with Stat1 in vitro (Fig. 6A). SeV V nevertheless counteracts some aspect of the innate immune response during infection of mice (Kato et al., 1997; Delenda et al., 1998), and mutational analysis has shown that the cys cluster is clearly critical for this effect (Huang et al., 2000). The SV5 V protein cys cluster is similarly thought to be required for V interaction with UV/DBP and possible cell-cycle control (Lin and Lamb, 2000). It will now be of interest to examine the relative importance of the trp- and cys-rich motifs of the rubulavirus V proteins in preventing IFN signaling and Stat protein degradation.

Although MuV V/Stat1 interaction in vitro requires a specific carboxyl segment, i.e., one that contains the W9/W9/WCNP9 motif, we were surprised that the V protein appeared to interact equally with Stat1, 2, and 3. The SeV C proteins also associate with Stat1 in the mixed-extract assay, but their association with Stat2 or Stat3, in contrast, is just above the level of detection in some experiments, and absent in others (data not shown). Stat1, 2, and 3 are part of a family of seven latent mammalian transcription factors that are structurally and functionally related (Darnell, 1997). They are activated by phosphorylation of a single tyrosine in the carboxyl end of the molecule, homo- or heterodimerized via reciprocal SH2 domains, and migrate to the nucleus where they recognize specific elements in the promoters of genes and activate transcription. Stat proteins are subsequently thought to be inactivated by dephosphorylation and returned to the cytoplasm; their activity is not normally thought to be controlled by degradation (Haspel et al., 1996). The SH2 domains of Stat1, 2, and 3 are also functionally compatible, as Stat1 can heterodimerize with both Stat2 and Stat3 (Bromberg, 2001). It is not impossible that common features of Stat1, 2, and 3 are recognized by the rubulavirus V proteins, at least in the mixed-extract assay. Nmi-1 and CBP/p300, two cellular regulators of transcription, bind to the same region of most Stats (Horvath, 2000). If the amino-terminal segment of the rubulavirus V proteins also binds to Stat (the carboxyl segment is never expressed by itself), then V might interact with Stat via both amino-terminal and carboxyl segments. However, whether these interactions also take place in vivo remains to be determined, e.g., whether there is evidence of impaired Stat3 signaling during rubulavirus infections.

If the MuV and hPIV2 V proteins associate with both Stat1 and Stat2, then why does only the former only lower Stat1 levels and vice-versa? How this might occur is unknown, but there is a precedent close at hand that may be germane. All four SeV C proteins bind Stat1 and interdict IFN signaling in reporter gene assays. However, only the longer C proteins prevent the IFN-mediated antiviral state and virus-induced apoptosis during infection (Garcin et al., 2002). Moreover, only the longer C proteins induce Stat1 ubiquitination and instability during infection (Garcin et al., 2002). This suggests that the binding of Stat1 and its turnover are separable events for SeV C protein, and this may also be the case for the rubulavirus V proteins. As the determining step in protein degradation/ubiquitination is generally its association with an ubiquitin ligase (Herskho and Ciechanover, 1998), we suggested that only the longer C proteins might act as connectors to a cellular ubiquitin ligase, presumably via their amino-terminal extensions. By analogy, it is possible that the MuV and hPIV2 V proteins bind both Stat1 and Stat2, but only the MuV V/Stat1 interaction leads to further interaction with an ubiquitin ligase, and vice-versa, thus accounting for the specificity of Stat
degradation. Part of this hypothetical ubiquitin–ligase connection site might also be located in the amino-terminal segment, as a mutation in this region of the SV5 V protein (N100D) determines anti-IFN activity in murine cells (Young et al., 2001).

As mentioned above, respirovirus C proteins and the amino-terminal segments of rubulavirus V proteins (up to the editing site) are similar in size and overall charge, and there is also very limited sequence conservation. However, there are no obvious similarities between these related C-like polypeptides and the carboxyl segment of V. Nevertheless, the MuV V protein and SeV C proteins both bind to Stat1 and the binding of MuV V to Stat1 can interfere with the subsequent binding of SeV C. There are three possible explanations for this interference: (1) SeV C and the carboxyl segment of V bind to different surfaces of Stat1, but V interaction alters subsequent C interaction allosterically. (2) C and V bind to the same surface of Stat1, even though these polypeptides appear to be unrelated structurally. (3) It is also possible that the carboxyl segment of V binds to a site adjacent to that bound by SeV C, and that the “C-like” amino-terminal segment of V, once tethered to Stat1 via the carboxyl segment, interacts with the adjacent SeV C-binding site and interferes with subsequent SeV C association with Stat1. This latter possibility is appealing, as the presence of Stat binding domains on both the amino-terminal and the carboxyl segments of the rubulavirus V proteins, together with the advent of mRNA editing, would have facilitated the evolution of these paramyxoviruses into their present genera. As MuV NS2 does not associate with Stat, V/Stat interaction in vitro, similar to anti-IFN signaling activity in vivo, requires the carboxyl segment of V. A recombinant hPIV2 in which the carboxyl segment of V is closed by a stop codon (and thus expresses NS2 instead of V) is hypersensitive to IFN action (Kawano et al., 2001).

MATERIALS AND METHODS

Expression plasmids and transient transfections.

The MuV (Belfast strain) V, NS2, and P genes were obtained from Bert Rima (Belfast) and the previously described hPIV2 V and P genes (Nishio et al., 1996) were cloned into the NcoI (start codon) and EcoRI sites of pTM1, which contains a T7 promoter and an EMCV IRES (Moss et al., 1990). Mutant and chimeric V proteins were prepared by PCR mutagenesis, and all constructs were confirmed by sequencing. For transfections, 200,000 BSR T7 cells (that express T7 RNAP) (Buchholz et al., 1999) were plated in 3.5-cm six-well plates 20 h before transfection with 3 μg pTM1-V DNA and 5 μl of Fugene (Roche) according to the manufacturer’s instructions. After 24 h, the cells were harvested and treated as described below.

Cell extracts

MEFs in 10-cm dishes were scraped into their medium and pelleted by centrifugation (5 min at 5000 rpm in Eppendorf). Cell extracts were prepared by vortexing the cell pellet with 600 μl lysis buffer containing 0.5% NP-40, 50 mM Tris–Cl, pH 7.4, 150 to 250 mM NaCl, 10 mM EDTA, 3 μg/ml aprotinin, and 1 mM AEBSF. The disrupted cells were then centrifuged at 13,000 g for 2 min and the cytoplasmic supernatant was used for immunoprecipitation.

Antibodies and coimmunoprecipitation

Equal volumes (150 μl) of MEF and BSR T7 cytoplasmic extracts were combined and incubated for 1 h at 20°C. One of the following antibodies (1 μl or 1/300 dilution) was then added; a mouse monoclonal antibody to Stat1 carboxyl-terminus (Transduction Laboratories, S21120); Stat2 (Santa Cruz, sc-1668), Stat3 (Santa Cruz, sc-7179), a mAb (84-1A) to the amino-terminal region of hPIV2 P protein (Nishio et al., 1997), a rabbit polyclonal antibody (T61) to a peptide from the amino-terminal region of MuV P protein (K. Takeuchi, Tokyo), rabbit polyclonal antibody to GFP (Clontech), or anti-HA. After a further 1 h at 20°C, protein A-Sepharose beads were added and the mixture was kept at 4°C overnight. The beads were then washed three times in lysis buffer containing 250 mM NaCl, and the remaining proteins were solubilized in protein sample buffer and separated by SDS–PAGE (10 or 15%). The gels were then blotted (semidry) onto nylon membranes and detected using one of the various primary antibodies listed above. The secondary antibodies used were alkaline phosphatase conjugated goat antibodies specific for either rabbit or mouse immunoglobulin G (Bio-Rad). The immobilized proteins were detected by light-enhanced chemiluminescence (Bio-Rad).

Note added in proof. Parisien et al. (J. Virol. 76, 4190–4198, 2002) have recently reported that the SV5 and hPIV2 V protein both bind Stat1 and Stat2, and that the selective degradation of each Stat protein is dependent on the presence of the other Stat protein.

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