Silencing STATs: Lessons from Paramyxovirus Interferon Evasion

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ABSTRACT

The Signal Transducer and Activator of Transcription (STAT) family proteins are essential mediators of cytokine and growth factor functions. The interferon (IFN) family of cytokines are well known as modulators of both innate and adaptive anti-microbial immunity. In response to the evolutionary struggle between host and pathogen, many viruses have developed strategies to bypass the IFN antiviral system. Uniquely, the Paramyxoviruses have developed the ability to efficiently inactivate STAT protein function, in many cases using a single virus-encoded protein called 'V'. The V protein plays a central role in STAT inhibition, but mechanistic studies have revealed great diversity in V-dependent STAT signaling evasion among Paramyxovirus species. These examples of IFN evasion by STAT protein inactivation can help define targets for antiviral drug design or improving vaccine regimens. Moreover, understanding these STAT inhibition mechanisms are likely to reveal strategic options for the design of STAT-directed therapeutics for treatment of diseases characterized by cytokine hyperactivity.

1 INTRODUCTION

Cytokines and growth factors control diverse cellular processes from embryonic development to immune regulation, and binding of these signaling proteins to their specific transmembrane receptors activates intracellular signal transduction systems that initiate the signal-specific phenotypic changes. Signal transducer and activator of transcription (STAT) family proteins are critical to the action of most cytokines and growth factors, as they directly regulate cellular gene expression (1). STAT signaling pathways normally function to precisely regulate cellular and organismal physiology, but defective or hyperactive STAT signaling has been associated with several human diseases such as chronic inflammation and cancer (2). Due to their essential roles in immediate signaling events, it has been postulated that STAT transcription factors represent ideal targets for rational therapeutic intervention for these diseases (3). The value of STAT-directed interference with cytokine signaling is well supported by the actions of some enveloped RNA viruses that have evolved STAT targeting as a means to escape the host immune response controlled by interferon (IFN). Recent investigations have revealed that paramyxoviruses encode IFN evasion molecules that are efficient STAT antagonists. While the viral strategies are phenotypically similar in targeting and inhibiting STAT protein functions, the molecular details of STAT-directed immune suppression are as diverse as the viruses themselves. Despite the idiosyncrasies of individual cytokine signaling events, the high degree of homology among the mammalian STAT pathways predicts that lessons learned from viral evasion of the IFN system will be instructive for identifying mechanisms of STAT inhibition that could be applied therapeutically.

2 PARAMYXOVIRUS V PROTEINS ARE STAT-TARGETING IFN INHIBITORS

Paramyxoviruses encompass a large family of enveloped, negative strand RNA viruses that cause myriad zoonotic diseases including significant human pathogens like measles virus, mumps virus, and Nipah virus. The large family Paramyxoviridae is subdivided into several genera, including the *Rubulavirus*, *Henipavirus*, and *Morbillivirus* groups (reviewed in (4)). All of these viruses share common genetic features including a polycistronic gene that encodes two or more viral proteins from overlapping open reading frames (ORFs). In most examples, the ORF encoding the phosphoprotein, P, overlaps partially with an ORF encoding a second protein, named V (Figure 1). Access to the hidden ORF is achieved by co-transcriptional insertion of non-templated guanine nucleotides at a precise location, or "editing site", to generate alternate mRNAs that differ only by the additional one or two nucleotides. Due to this unusual coding strategy, the paramyxovirus P and V proteins are amino co-terminal but have unique carboxyl termini (5).

Paramyxovirus V proteins are identifiable by a C-terminal domain (CTD) derived from the overlapping ORF coding for a cysteine-rich domain (4-6). This CTD is approximately 50% identical among all paramyxovirus V proteins (Figure 1C) and contains seven invariant cysteine residues capable of binding 2 atoms of zinc (6, 7). Aside from this stoichiometry that is similar to that of some cellular metalloproteins, the spacing of CTD cysteine residues is not consistent with that of known zinc-binding domains and no cellular V protein homologues have been described. Paramyxovirus host evasion activities, including IFN signaling inhibition (8), prevention of apoptosis (9, 10), cell cycle alterations (11), inhibition of double-stranded RNA signaling (10, 12), and prevention of IFN biosynthesis (9, 10, 12) have been ascribed to V

proteins. A growing number of the paramyxovirus V proteins have been found to directly interfere with STAT protein stability and/or function (summarized in Table 1). Recent mechanistic studies have demonstrated that these viruses exhibit great diversity in their abilities to achieve the common outcome of STAT signaling inhibition.

3 PARAMYXOVIRUS-INDUCED STAT DEGRADATION

3.1 RUBULAVIRUSES ASSEMBLE STAT-TARGETING UBIQUITIN LIGASE ENZYMES

The dynamic control of STAT protein activity in uninfected cells is regulated primarily at the level of nuclear-cytoplasmic distribution. STATs are activated by receptor-induced tyrosine phosphorylation, dimerize via intermolecular SH2-phosphotyrosine interactions, and accumulate in the nucleus where they bind to target gene promoters. The STAT dimers are inactivated in the nucleus by tyrosine phosphatase-mediated dephosphorylation rather than by changes in protein stability (13-15). The estimated half lives of STAT1 and STAT2 are on the order of days rather than hours (15-17), but these long half lives can be greatly reduced upon infection with paramyxoviruses in the *Rubulavirus* species. In the first characterized example, STAT1 abundance was found to be markedly reduced by infection of cells with simian virus 5 (SV5) (8). This STAT1 elimination was conferred by the individual expression of the SV5 V protein, and similar abilities to induce STAT protein degradation were soon reported for a number of viruses in the *Rubulavirus* genus (8, 17-24). Chemical proteasome inhibitors were found to prevent the V-induced STAT degradation (8, 17), and expression of the *Rubulavirus* V proteins were later

demonstrated to induce polyubiquitylation of their target STATs (17, 23-25). Characterization of the activity of bacterially-expressed SV5 and type II human parainfluenza virus (HPIV2) V proteins revealed their intrinsic abilities to catalyze the transfer of ubiquitin (Ub) in an *in vitro* reaction containing ATP, Ub-activating enzyme (E1), and Ub-conjugating enzyme (E2) (25). This enzymatic activity satisfies the definition of a Ub ligase enzyme (E3), but the observed substrate-independent monoubiquitylation did not fully recapitulate a native STAT-targeting reaction (25). However, expression of SV5, HPIV2, or mumps virus V protein in human cells by cDNA transfection results in complete STAT targeting by poly-ubiquitylation resulting in efficient proteasomal degradation (24, 25).

3.2 STATS, BUT NOT IFN SIGNALING, ARE NEEDED FOR STAT DEGRADATION

Despite the high amino acid sequence identity between V proteins and their similar abilities to target highly homologous STAT family proteins for proteasomal degradation, *Rubulavirus* species differ in their substrate specificity (Figure 2). While the SV5 V protein can target STAT1 for polyubiquitylation and proteasomal degradation, HPIV2 V protein favors ubiquitylation of STAT2 (17), and mumps virus V protein can eliminate both STAT1 (24, 26) and STAT3 (24) while leaving STAT2 intact.

A series of human cell lines deficient in IFN signaling proteins (27, 28) was used to evaluate whether components of the IFN signaling system were required for STAT degradation by SV5 or HPIV2. Results indicated that neither the type I IFN receptor, their associated tyrosine kinases Jak1 or Tyk2, nor the ISGF3 DNA binding subunit, IRF9, are required for STAT protein

targeting induced by either virus (19). Nonetheless, both STAT1 and STAT2 were observed to be strictly required in the host cell to establish a degradation-permissive environment enabling SV5 or HPIV2 to target their respective STAT protein. STAT protein N-terminal regions are known to mediate several protein:protein interactions via a lengthy coiled-coil while the C-terminal domains participate in signal transduction, dimerization, DNA binding, and transcriptional activation (29, 30); reviewed in (31). Complementation of the degradation-incompetent (i.e., STAT-deficient) cell lines with STAT cDNAs revealed that STAT protein activating tyrosine phosphorylation and SH2 domain function are both dispensable for recreating a permissive STAT degradation environment, reinforcing that IFN signal transduction is not needed for V-dependent STAT targeting. Expression of a fusion protein consisting of the N-terminus of STAT2 (amino acids 1-315) linked to the C-terminal domains of STAT1β (amino acids 306-712) (32) complemented the intrinsic degradation defect due to the STAT2 deficiency, and a STAT2 fragment consisting of only amino acids 1-578 (the STAT2 N-terminus through the linker domain) was sufficient to complement SV5 V-dependent IFNβ antagonism in STAT2-deficient cells (19). These data indicated that STAT protein N-terminal regions are necessary and sufficient to re-establish a degradation-competent environment for *Rubulavirus*-induced STAT targeting and IFN signaling evasion. Altogether, these results implicated the N-terminal residues of the confederate STAT protein as key components in destruction of the target STAT, and constituted genetic evidence suggesting the existence of a virus-induced, IFN-independent, STAT protein degradation complex that contains (at least) STAT1 and STAT2 (19). Biochemical analysis has confirmed this model of protein complex formation and accumulated evidence has revealed that SV5 and HPIV2 V proteins must form multi-subunit complexes with non-target STATs for their full E3 Ub ligase activity toward STAT1 or STAT2. In other words,

SV5 can only target STAT1 in cells that express STAT2, while HPIV2-mediated STAT2 degradation fails in the absence of STAT1 (19).

3.3 CELLULAR PROTEINS AND THE VDC UBIQUITIN LIGASE

Although STAT proteins are both target and collaborator for V-dependent IFN evasion, additional cellular proteins are also required to assemble the E3 enzyme. Affinity purification of V proteins from host cells identified overall similar patterns of V-interaction protein (VIP) partners for each virus, but in detail each species also co-purified unique superimposed VIP patterns. The current evidence favors a model where the VIP composition represents a core degradation complex, but the differential use of cellular cofactors by individual V proteins accounts for the observed variations in V protein target specificities (Figure 3) (24, 25).

The STAT-targeting machinery consists of V protein-assembled E3 complexes that contain the V protein and cellular VIPs including STAT1 and STAT2 (and STAT3 in the case of mumps virus, see below). A number of additional cellular proteins have been identified in these complexes, including DDB1, a UV-damaged DNA binding protein (24, 25, 33, 34), and several members of the Cullin family of SCF ubiquitin ligase subunits, prominently including Cullin 4A (summarized in Table 2) (24, 25). RNA interference experiments demonstrate that both DDB1 (25, 33) and Cullin 4A (25) are required for STAT1 degradation by SV5, lending support to the model that the STAT-targeting activity is a combination of virus-encoded and host-derived factors that together function as a STAT-directed E3 ubiquitin ligase enzyme (25). By analogy to the nomenclature conventions adopted for the multi-subunit SCF ubiquitin ligase complexes

(referring to components Skp1/CUL1/F-box (35)), these virus-assembled ubiquitin ligase machines were named VDC for V/DDB1/CUL4A which is also an acronym for 'Virus Degradation Complex' and 'V-dependent Degradation Complex'. The DDB1 and Cul4A proteins are known to regulate the activity of the transcription factor E2F and control the stability of DDB2, a protein important for double-stranded break repair in xeroderma pigmentosum group E (36, 37). A cellular ubiquitin ligase complex that also contains DDB1 and CUL4A has been recently implicated in regulating the half life of the transcription factor, c-jun, in combination with partner proteins COP1 and hDET (38). Although the current data suggest that the VDC may be assembled *de novo* from cellular components, it will be interesting to learn of potential connections between the cellular E3 complexes, the VDC, and *Rubulavirus* biology.

3.3 STAT2 RESTRICTS SV5 HOST RANGE

A compelling confirmation of the role of STAT2 in STAT1 destruction by SV5 was provided by the discovery that STAT2 acts as a host range determinant for this virus (18). It has been demonstrated that while SV5 can enter murine cells and initiate viral protein synthesis, the infection is rapidly cleared by the endogenous innate immune system. Neutralizing antibodies to IFN or genetic IFN receptor deficiency enable SV5 replication in mouse cells, indicating the importance of IFN antiviral responses for controlling SV5 infection (39), but SV5 does not replicate efficiently or cause STAT1 degradation in the mouse or murine cell cultures (40, 41), The cellular basis for the differential ability of murine and human cells to support SV5-mediated STAT1 destruction was not immediately apparent from analysis of the proteolytic target. Amino acid sequence comparisons illustrate murine and human STAT1 orthologues to be 92.4%

identical (42), and IFN responses in STAT1-deficient human cells complemented with murine STAT1 were efficiently suppressed by the SV5 V protein (18). As STAT2 was found to be required for STAT1 degradation by SV5 in human cell lines (19), it seemed reasonable to hypothesize that the STAT2 orthologues might influence the murine host range restriction of SV5-induced STAT1 degradation. In contrast to STAT1, mouse and human STAT2 proteins are quite divergent, exhibition only 68.6% amino acid identity overall due in large part to a minisatellite insertion that disrupts the 3' exon structure, but also reflecting the presence of numerous amino acid substitutions throughout the protein (42-44).

Expression of human STAT2 or human STAT2 fragment 1-578 in mouse fibroblasts enabled SV5 to effectively disrupt murine IFN signaling and to support specific degradation of the endogenous (murine) STAT1. This acquired IFN antagonism allowed SV5 to replicate more efficiently in the "humanized" mouse cells, conferring a selective advantage for SV5 growth across the species barrier (18). These findings demonstrated a unique role for STAT2 as species-specific host range determinant and corroborated its importance as a cofactor for STAT1 targeting by SV5. The extent of STAT2-dependent host range restriction is not yet known, but it is perhaps relevant to note that Newcastle disease virus (NDV), a member of the avian-specific *Avulovirus* genus, also encodes a V protein that can antagonize the avian IFN system (45) via STAT1 destabilization (46). Like SV5, the NDV V-dependent IFN signaling inhibition is species-restricted, and while the molecular basis has not yet been revealed, it is interesting to speculate that avian STAT2 might be involved.

3.4 MUMPS VIRUS TARGETS BOTH STAT1 AND STAT3

Mumps virus is a common infectious agent of humans causing parotitis, meningitis, encephalitis, and orchitis. Like other *Rubulavirus* species, mumps virus V protein catalyzes the proteasomal degradation of STAT1 (21, 23, 24, 47, 48). Unexpectedly, mumps virus infection or V protein expression was found to also eliminate cellular STAT3, a protein that mediates transcriptional responses to cytokines, growth factors, non-receptor tyrosine kinases, and a variety of oncogenic stimuli (24). Evidence from somatic cell genetics indicated that STAT1 and STAT3 are targeted independently by the mumps virus V protein, as the STAT1 targeting required the participation of STAT2 while the STAT3 targeting activity is STAT2-independent (24). Affinity purification of mumps VIPs revealed a pattern of associated proteins that resembles the SV5 and HPIV2 VDCs, with common components including STAT1, STAT2, DDB1, and Cullin4A (24). Additional associated proteins were also detected by silver stain or immunoblotting and include the alternate degradation target, STAT3. While both SV5 and mumps V proteins induced polyubiquitylation of STAT1, only mumps V protein induced polyubiquitylation of STAT3, consistent with the unique targeting specificity (24).

3.5 STAT3 DESTRUCTION AND ANTIVIRAL IMMUNITY

Viruses target STAT1 or STAT2 to evade the IFN antiviral responses, but the reason for STAT3 antagonism by mumps virus is not immediately apparent from the perspective of innate antiviral immunity. STAT3 has not been generally considered to be a major component of the IFN-induced antiviral system, but STAT3 activation by IFN has been widely reported (49-54), implying a potential antiviral role for STAT3 that has yet to be fully elucidated. An additional

benefit from STAT3 evasion might be avoidance of adaptive immune responses that occur downstream of inflammatory cytokines, and mumps-induced STAT3 inhibition will also antagonize mitogenic growth factors, tyrosine kinases, G proteins, and other STAT3 inducers. Mumps V protein was shown to prevent the activity of two well-characterized STAT3 activators, IL6 and v-Src (24), and destruction of STAT3 is predicted to provide a much broader spectrum of cytokine and growth factor suppression (55). Theoretically, the STAT3 destruction could allow the virus to effectively inhibit diverse cellular responses providing both general and tissue-specific replication advantages that might play a role in the ability of mumps virus to invade and replicate in activated T lymphocytes (56, 57).

3.6 MUMPS V PROTEIN AS AN ONCOLYTIC AGENT

It is attractive to speculate that the ability of mumps virus or mumps V protein to irreversibly remove STAT3 from cells might have practical therapeutic applications for a range of human illnesses characterized by overactive STAT3, such as cancer, arthritis, lupus, autoimmunity, dwarfism, cardiac hypertrophy, obesity, and kidney disease (55). A leading target for such a therapy is the oncogenic potential of STAT3 that has been associated with a large number of cancers including head and neck, lung, ovarian, prostate, renal, breast, skin, blood, and kidney (58, 59). In most cases, the inducer of STAT3 activity is unknown, but inhibition of STAT3 leads to tumor regression and apoptosis (3, 58). Oncolytic viruses have a high potential for use in cancer treatment (60, 61), and the use of live mumps virus as a cancer treatment was described 40 years ago ((62), reviewed in (61)). These studies demonstrated that treatment with live mumps virus reduced tumor size and improved clinical presentation in a high percentage of patients with advanced cancers including gastric, mammary, cutaneous, uterine, and pulmonary

carcinomas (61, 63, 64). More recent investigation demonstrated the ability of mumps virus to initiate programmed cell death in renal cell carcinoma (65). As secreted proteins like IL6 and EGF act as autocrine or paracrine growth factors for many cancer types and intracellular stimuli like oncogenic tyrosine kinases are associated with neoplastic transformation, it is likely that destruction of STAT3 will have a broad range of anti-cancer effects (65-67). Rather than using live mumps virus, a more contemporary approach might involve delivery of the mumps V protein via a stealth vector system, and similar approaches could be adapted for treatment of other diseases resulting from STAT3 hyperactivity. As proof of principle, mumps V protein expression was shown to induce apoptosis in STAT3-dependent multiple myeloma cells and transformed murine fibroblasts (24). Together, these findings of cytokine signaling inhibition and oncogene evasion properties of mumps V protein provide a molecular basis for its previously observed oncolytic properties. Accordingly, the mumps virus V protein represents an efficient STAT3 inhibitory reagent from which targeted therapies may be derived.

4. STAT INHIBITION WITHOUT DEGRADATION

4.1 HENIPAVIRUSES SEQUESTER STATS IN HIGH MOLECULAR WEIGHT COMPLEXES

Nipah virus and Hendra virus are the two known species of a recently-emerged deadly Paramyxovirus genus, *Henipavirus,* that was responsible for outbreaks of zoonotic respiratory disease and fatal encephalitis in humans and livestock in Malaysia and Australia (68, 69). Both *Henipavirus* species share V-dependent IFN signaling evasion properties with other

paramyxoviruses (45, 70-72), but unlike the *Rubulavirus* V proteins do not induce STAT destabilization.

Nucleotide sequencing of the *Henipavirus* genomes revealed many similarities with other paramyxoviruses, including a polycistronic gene encoding a V protein CTD (69, 73). The *Henipavirus* V proteins are highly homologous to each other and share approximately ~50% amino acid identity to SV5 V protein within the CTD (Figure 1). However, the *Henipavirus* V protein N-terminus is not homologous to other paramyxovirus proteomes or any cellular protein. This sequence divergence between *Henipavirus* and *Rubulavirus* V proteins hinted at an alternate mechanism of IFN signaling inhibition. Both *Henipavirus* V proteins have been demonstrated to subvert IFN responses by sequestering STAT1 and STAT2 in high molecular weight cytoplasmic complexes without inducing their degradation (70, 71), a distinct mechanism of IFN signaling inhibition. However, it was surprising that despite the sequence conservation within the CTD, this domain is entirely dispensable for IFN signaling inhibition by *Henipavirus* V proteins (45, 72).

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. 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. STAT1 shuttling between the nucleus and cytoplasm results in a steady-state accumulation in both compartments (70, 74). However, in cells expressing a *Henipavirus* V protein, STAT1 is completely relocalized to the cytoplasm (70,

71), indicating that the V protein can enter the nucleus, bind to STAT1, and carry it back to the cytoplasm.

4.2 DISSECTION OF THE NIPAH VIRUS V PROTEIN FUNCTIONAL DOMAINS

Functional dissection of the Nipah V protein indicated that this shuttling behavior is peripheral to the biological activity of STAT interference, and also explained the dispensable role of the conserved CTD region (72). Three V protein activities map to the N-terminal portion: nuclear export, STAT protein interaction, and IFN signaling inhibition (Figure 4). A novel nuclear export signal (NES) was identified within Nipah V amino acids 174-192 that is necessary for V protein cytoplasmic accumulation and sufficient to direct nuclear export of a reporter protein. Further, deletion or substitution within the NES prevented redistribution of latent STAT1 to the cytoplasm. Nonetheless, the ability to thwart IFN-dependent STAT1 and STAT2 nuclear accumulation and IFN-induced transcriptional responses remained intact regardless of NES mutation, suggesting that the shuttling behavior of the V protein has a distinct role in *Henipavirus* biology unrelated to IFN evasion.

Dissection of the V protein domains involved in IFN signaling inhibition and STAT protein interactions revealed that these functions also map within Nipah V amino acid residues 100-300. STAT1 binds independently to residues 100-160, and this interaction site is the primary evasion motif, sufficient to block IFN signaling responses. Moreover, STAT1 binding is a prerequisite for STAT2 binding, and association with STAT2 consequently requires a large overlapping binding site between residues 100-300. Evidence from site-directed mutagenesis also suggested

that contact between STAT2 and Nipah V requires a conserved peptide including amino acids 230-237. Hence, in intact cells, a coordinately-assembled trimeric V-STAT1-STAT2 complex forms that inhibits IFN signal transduction. As these protein interaction domains are absolutely required for V protein IFN evasion activity, they are ideal candidates as targets for therapeutic intervention with *Henipavirus* outbreaks.

The region of STAT1 bound by Nipah V was also determined. Nipah V binds to STAT1 but not to STAT3, and only binds to STAT1-STAT3 hybrid fusion proteins when the C-terminal region was derived from STAT1. The results implicated a STAT1 fragment containing the linker domain and SH2 domain as the target site for *Henipavirus* V protein interaction. These domains are well known to be important for STAT activation, dimerization, and DNA binding, illustrating the efficiency of virus-designed STAT inhibitors.

4.3 MEASLES VIRUS INHIBITS STAT NUCLEAR TRANSLOCATION

Measles virus, a prototype species of the *Morbillivirus* genus is well known as a highly infectious agent that causes a common acute childhood illness. Measles virus infection can induce adaptive immune suppression that can lead to opportunistic infections or neurological complications and, despite effective vaccines, causes millions of fatalities annually. Measles virus encodes a V protein distinct from both the *Rubulavirus* and *Henipavirus* genera, sharing only ~20% overall amino acid sequence identity. Despite the divergence, measles virus V protein is an efficient inhibitor of IFN signal transduction in both human and mouse cells, but acts via a mechanism distinct from either *Rubulavirus* or *Henipavirus* V proteins (75). Measles virus V protein expression effectively prevents both IFN□/□ and IFN□-induced transcriptional

responses. The measles virus V protein does not degrade STATs or prevent IFN-induced STAT protein activating tyrosine phosphorylation, but effectively blocks IFN-induced STAT1 and STAT2 nuclear import. Unlike the *Henipaviruses*, measles V does not shuttle between nucleus and cytoplasm, and consequently does not alter the distribution pattern of latent STAT1.

Affinity chromatography to purify the measles VIP partners demonstrated that the measles V protein co-purifies STAT1, STAT2, STAT3, and IRF9, but not the cellular components required for *Rubulavirus* VDC ubiquitin ligase function, in agreement with its distinct mechanism of action. In addition, measles V binds to an IFN receptor subunit (IFNAR2.2 or $\square_{Long}$ (76)) and a signaling adaptor, RACK1 (77), possibly indicating multivalent receptor interactions.

The reason for STAT1, STAT2, and IRF9 in the evasion complex(es) may be rationalized from the evolutionary pressure from IFN antiviral effects, similar to the evasive activities demonstrated for other paramyxoviruses. Interaction with IRF9 and interferon receptor is a unique feature for measles virus not shared with *Rubulaviruses*, and might ensure a more complete ISGF3 inactivation. As with mumps virus, the discovery of STAT3 as a component of the measles V affinity preparation was not as easily explained. However, IL6 biosynthesis has been previously reported for measles virus infections (78, 79), and STAT3–responsive transcriptional assays revealed that the measles virus V protein can inhibit the transcriptional activity induced by IL6 or by v-Src. It is important to note that the effect on STAT3 was partial for the Edmonston strain-derived V protein, but it is conceivable that strain-specific differences in STAT3 interference might result in different degrees of measles-induced immune suppression, possibly correlating with different degrees of virulence among strains. Nonetheless, in

conjunction with the mumps virus STAT3 degradation, these findings further suggest a role for STAT3 in antiviral responses that has yet to be appreciated.

4.4 MEASLES VIRUS PACKAGES STATS IN CYTOPLASMIC BODIES

The ability to prevent STAT nuclear import was also observed in measles virus-infected cells. In addition to providing confirmation of the STAT translocation blockade, these experiments revealed an additional phenotype of STAT protein redistribution by measles virus (75). The measles virus nucleocapsid protein, used as a marker for infected cells, was found to accumulate in punctate cytoplasmic bodies. In measles-infected cells, a portion of the STAT1 and STAT2 proteins were also redistributed to cytoplasmic aggregates that co-localized not only with the nucleocapsid protein, but also contained nucleic acids (Figure 5) (75). Similar granular structures are produced by other paramyxovirus infections, and, in some cases, by simple expression of the nucleocapsid protein along with the P, V, or C proteins. These intracellular aggregates have been observed for measles (80), SV5 (81-83), HPIV2 (84, 85), respiratory syncytial virus (86), Sendai virus (87), and mumps virus (24), but the exact function of these structures is still unknown. Further, it has been observed that the IFN regulatory factor, IRF3, can localize to similar bodies induced during SV5 infection (10), and that STAT2 localizes to cytoplasmic bodies induced by mumps virus infection (24). These results provide intriguing new information that STAT proteins and nucleic acids are components of virus-induced cytoplasmic bodies, and suggest a possibility that these bodies may represent active subcellular sites for paramyxovirus replication, or macromolecular assemblies of viral and cellular components required for both host immune evasion and virus replication.

5. CONCLUSIONS

The diverse mechanisms that have evolved for V protein-dependent IFN evasion provide many insights into STAT protein inhibition that could not be easily discerned by laboratory investigations and are relevant to the analysis of virus-host interaction, vaccine production, and cytokine biology. Discovery of new paramyxoviruses and their IFN evasion properties will almost certainly reveal novel mechanisms of STAT protein-directed or other means of IFN antagonism, and may also uncover new functions for STAT proteins in viral replication or immune responses. Probing the molecular details of virus-designed STAT inhibitors will not only yield new therapeutic targets for the control of infectious outbreaks, but will also provide numerous insights into the regulation of hyperactive cytokine-JAK-STAT signaling characteristic of neoplastic and inflammatory diseases.

ACKNOWLEDGEMENTS

The help and support of all current and former members of the Horvath laboratory is gratefully acknowledged, as is the continued advice and friendship of many mentors, collaborators and colleagues who have supported our work in STAT biology and Paramyxovirology. Research in the Horvath laboratory is sponsored by the National Institutes of Health and the American Cancer Society.

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TABLE 1. CYTOKINE SIGNALING INHIBITION BY PARAMYXOVIRUS V PROTEINS

V PROTEIN	INHIBITS	TARGET	MECHANISM
SV5	IFN□,□,□	STAT1	Ubiquitin Ligase
HPIV2	IFN□,□	STAT2	Ubiquitin Ligase
MUMPS	IFN□,□,□, IL6, v-Src	STAT1, STAT3	Ubiquitin Ligase
MEASLES	IFN□,□,□, IL6, v-Src	STAT1, STAT2, STAT3	Nuclear Import
NIPAH	IFN□,□,□	STAT1, STAT2	Sequestration
HENDRA	IFN□,□,□	STAT1, STAT2	Sequestration

TABLE 2. CELLULAR V-INTERACTING PROTEIN (VIP) PARTNERS

V PROTEIN	STAT1	STAT2	STAT3	DDB1	CULLINS	RACK1	IRF9	IFN□R
SV5	+	+	-	+	+	-	-	-
HPIV2	+	+	-	+	+	-	-	-
MUMPS	+	+	+	+	+	+	-	-
MEASLES	+	+	+	-	-	+	+	+
NIPAH	+	+	-	-	-	NT	NT	NT
HENDRA	+	+	-	-	-	NT	NT	NT

+ = Detected in co-immunoprecipitation/immunoblot experiment
- = Not detected in co-immunoprecipitation/immunoblot experiment
NT = Not Tested

Figure Legends

Figure 1. Gene structure at the polycistronic P/V locus and sequence conservation of the V protein C-terminus. Box diagrams on top represent the open reading frame (ORF) organization encoded in the single strand viral RNA of the *Rubulavirus* genus (A.) and the *Henipavirus* and *Morbillivirus* genera (B.). Arrows point to two alternative mRNAs derived by either faithful transcription or the co-transcriptional addition of non-templated guanine (G) nucleotides. Alternate ORFs are indicated by yellow or blue shading, and the "editing site" illustrated by a dotted line. (C.) Alignment of amino acids in the conserved V protein C-terminal domain (CTD) for the six viruses described in the text. Numbers indicate the conserved cysteine residues.

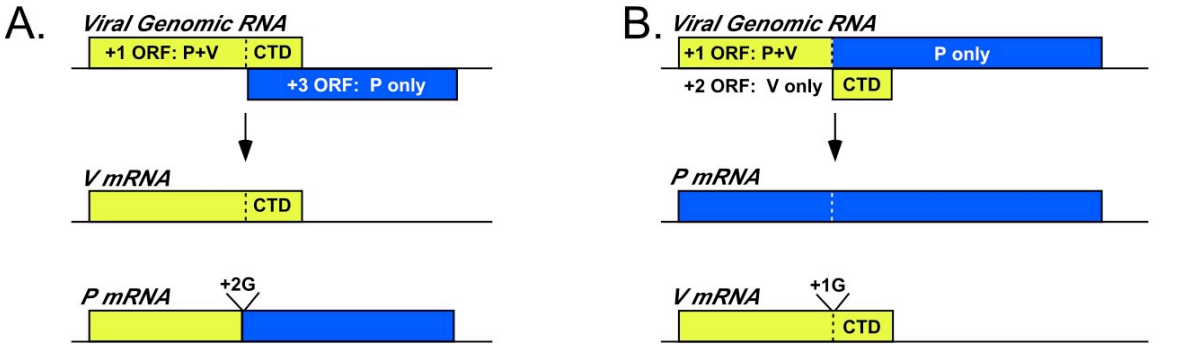
Figure 2. Rubulavirus V proteins induce specific and selective STAT targeting for proteasome-mediated degradation. (A.) Indirect immunofluorescence of human fibrosarcoma 2fTGH cells expressing V proteins from SV5, HPIV2, and mumps viruses by cDNA transfection. Cells are stained for V protein in red, STAT1, STAT2, or STAT3 in green, and nucleic acids in blue. Arrows indicate the positions of V-expressing cells. TOTO3 is a nucleic acid stain. (B.) and (C.) Cells were infected with SV5 (B.) or HPIV2 (C.) in the presence (+) or absence (-) of the proteasome inhibitor epoxomycin, and cell lysates processed for immunoblot with STAT1 or STAT2 antisera.

Figure 3. Models of *Rubulavirus* V-dependent degradation complexes. Yellow box represents the V protein and colored ovals indicate cellular V-interacting proteins that comprise the VDC ubiquitin (Ub) ligase. (A.) SV5 VDC targets STAT1 using a STAT2, DDB1, and Cullin 4A (Cul4A) –dependent complex. (B.) HPIV2 targets STAT2 using a similar STAT1-dependent complex. (C. and D.) Mumps V protein can target both STAT1 and STAT3 using complexes that differentially require participation of STAT2. E1= Ub activating enzyme, E2= Ub conjugating enzyme. Not drawn to scale or representative of actual regions of contact.

Figure 4. Summary of the functional domains of Nipah Virus V protein. Nipah V protein is depicted as grey box, STAT1 and STAT2 as colored ovals. Interferon evasion activity is associated with amino acids 100-160. STAT1 (blue) interacts with residues 100-160 of the Nipah virus V protein via its SH2 and linker domains, while STAT2 (red) binds to V residues between 100 and 300 in a reaction that requires STAT1. The nuclear export signal (NES, black) maps to amino acids 174-192. Position of dispensable CTD is indicated.

Figure 5. Measles virus infection prevents IFN-induced nuclear transport of STAT1 and STAT2, and induces STAT relocalization to cytoplasmic bodies. Measles-infected 2fTGH cells were treated with IFN for 1 hour, then stained for viral N protein (red), STAT1 or STAT2 (green), and nucleic acids (TOTO3, blue). Merged images for STAT and N protein or nucleic acid and N protein demonstrate co-localization of STATs, N, and nucleic acids. Note that IFN-induced STAT nuclear import is defective in measles-infected cells, but intact in neighboring uninfected cells.

Figure 1.



C.

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SV5       161.FKRG----RDTGGFHRREYSIGWVGDEVKVTEWCNPSCSPITAAARRFECICHQCPVTCSECERDT
HPIV2     160.FKRGGANRERARGNHRREWSIAWVGDQVKVFEWCNPRCAPVTASARKFTCTCGSCPSICGECEGDH
Mumps     156.AGSGCSRPDNPRGGHRREWSLSWVQGEVRVFEWCNPICSPITAAARFHSCKCGNCPAKCDQCERDYGPP
Nipah     394.TPMPKSRGIPIKKGHRREISICWDGKRAWVEEWCNPACSRITPLPRRQECQCGECPTECFHCG
Hendra    392.TPMPKSRIMPIKKGHRREVSICWDGRRAWVEEWCNPVCSRITPQPRKQECYCGECPTECSQCCHEE
Measles   218.PGRASTSGTPIKKGHRREISLIWNGDRVFIDRWCNPMCSKVTLGTIRARCTCGECPRVCEQCRTDTG(+15)
                                  1   2          3 4  5   6  7
```

Figure 2.

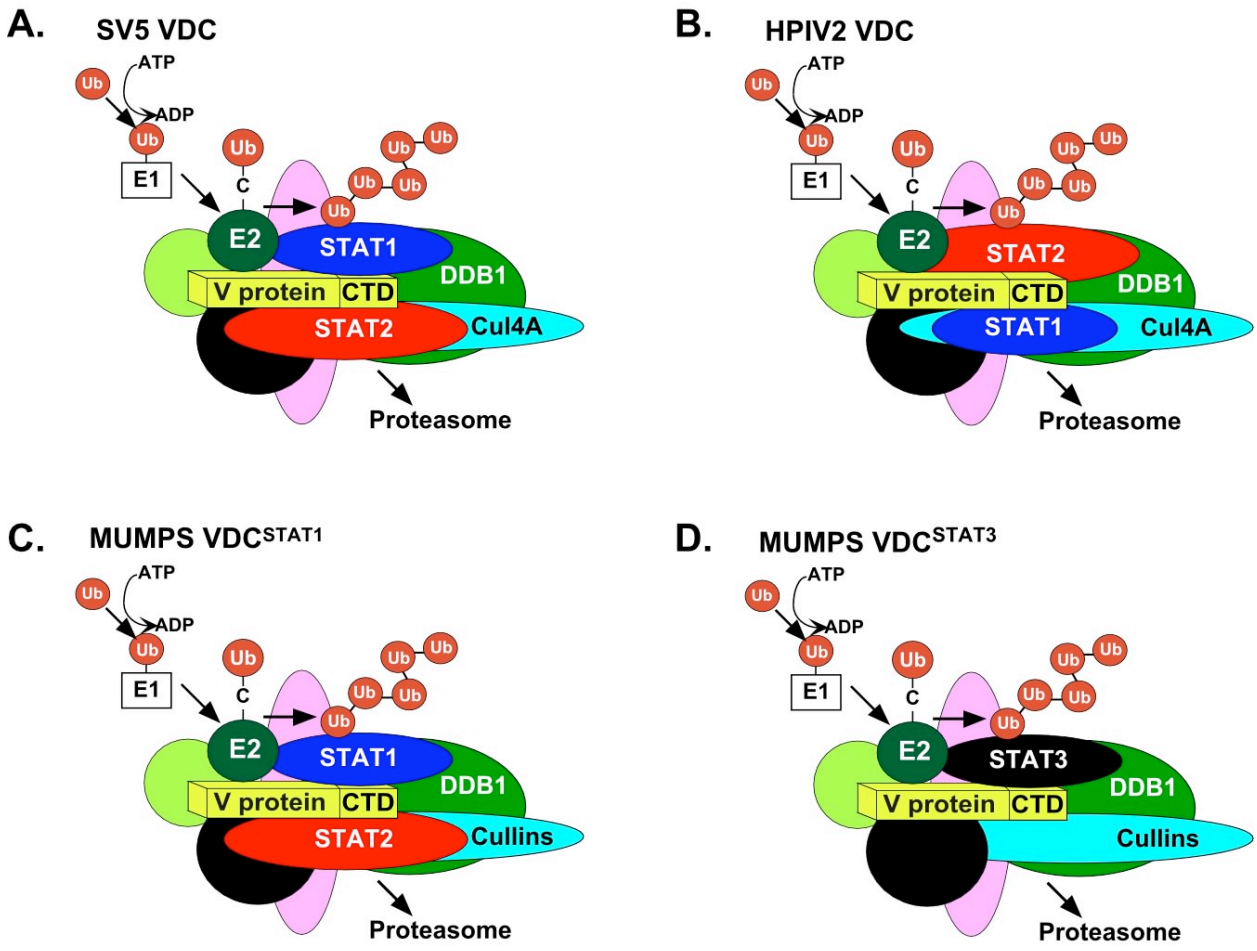

A.
SV5 VDC
ATP
ADP
Ub
E1
E2
STAT1
STAT2
V protein
CTD
DDB1
Cul4A
Proteasome
B.
HPIV2 VDC
STAT2
STAT1
C.
MUMPS VDCSTAT1
Cullins
D.
MUMPS VDCSTAT3
STAT3

Figure 3.

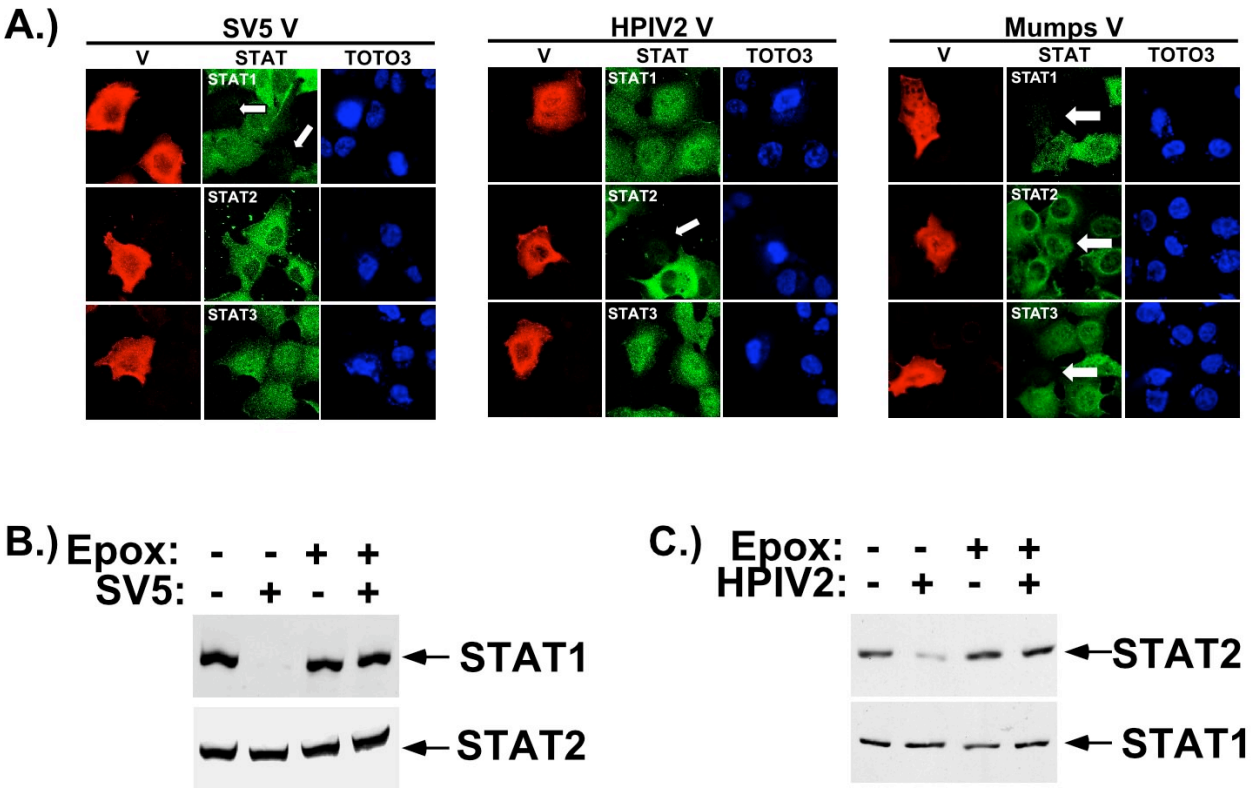

A.)
SV5 V
HPIV2 V
Mumps V
V
STAT
TOTO3
STAT1
STAT2
STAT3
B.)
Epox: - - + +
SV5: - + - +
STAT1
STAT2
C.)
Epox: - - + +
HPIV2: - + - +
STAT2
STAT1

Figure 4.

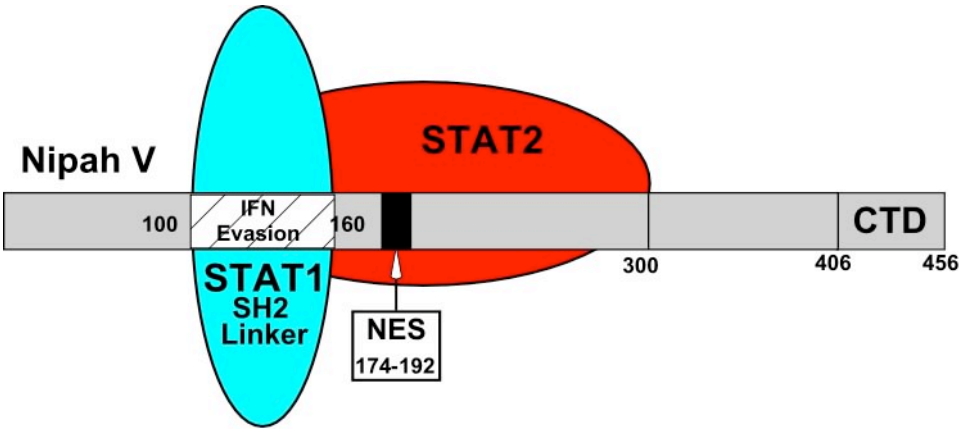
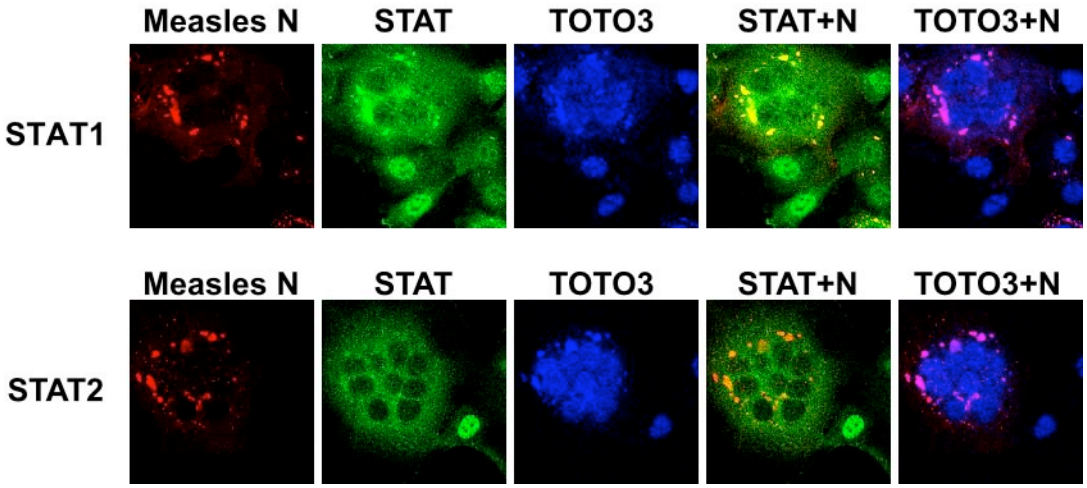

Nipah V
STAT2
100
IFN
Evasion
160
CTD
300
406
456
STAT1
SH2
Linker
NES
174-192

Figure 5.


Measles N
STAT
TOTO3
STAT+N
TOTO3+N
STAT1
Measles N
STAT
TOTO3
STAT+N
TOTO3+N
STAT2