STAT3 Ubiquitylation and Degradation by Mumps Virus Suppress Cytokine and Oncogene Signaling

Christina M. Ulane, Jason J. Rodriguez, Jean-Patrick Parisien, and Curt M. Horvath*

Immunobiology Center, Mount Sinai School of Medicine, New York, New York 10029

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Mumps virus is a common infectious agent of humans, causing parotitis, meningitis, encephalitis, and orchitis. Like other paramyxoviruses in the genus Rubulavirus, mumps virus catalyzes the proteasomal degradation of cellular STAT1 protein, a means for escaping antiviral responses initiated by alpha/beta and gamma interferons. We demonstrate that mumps virus also eliminates cellular STAT3, a protein that mediates transcriptional responses to cytokines, growth factors, nonreceptor tyrosine kinases, and a variety of oncogenic stimuli. STAT1 and STAT3 are independently targeted by a single mumps virus protein, called V, that assembles STAT-directed ubiquitylation complexes from cellular components, including STAT1, STAT2, STAT3, DDB1, and Cullin4A. Consequently, mumps virus V protein prevents responses to interleukin-6 and v-Src signals and can induce apoptosis in STAT3-dependent multiple myeloma cells and transformed murine fibroblasts. These findings demonstrate a unique cytokine and oncogene evasion property of mumps virus that provides a molecular basis for its observed oncolytic properties.

Gene expression and biological responses to cytokines and polypeptide growth factors are often mediated by signal transducer and activator of transcription (STAT) proteins (12). The activities of various STAT proteins control cell growth and differentiation, organogenesis, embryonic development, and host responses to cancer and infection. Inappropriate activation of STAT signaling is frequently observed in human diseases, including inflammation, asthma, autoimmunity, and cancer, suggesting that inhibitors of STAT factors might be of wide-ranging therapeutic value (reviewed in reference 55 and references therein).

Of the seven mammalian STAT proteins, STAT1, STAT2, and STAT3 exhibit the broadest expression profiles and respond to activating stimuli in most cell types (70, 71). A transcription factor complex, ISGF3, containing activated STAT1 and STAT2 is essential for induction of alpha/beta interferon (IFN-α/β) target genes that establish an innate cellular antiviral state (54). Similarly, the transcriptional response to IFN-γ is mediated by a STAT1 homodimer that is required for IFN-γ-dependent innate and adaptive immune responses (31). The third widely expressed STAT protein, STAT3, is activated by many cytokine systems, including interleukin 6 (IL-6), leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, and leptin (reviewed in reference 28). STAT3 is also activated by growth factor receptors with intrinsic protein tyrosine kinase activity (e.g., platelet-derived and epidermal growth factor receptors [53, 61]) as well as cellular and viral cytoplasmic tyrosine kinases (e.g., c-Src and v-Src [6, 68]).

Targeted disruption of STAT3 in mice results in early embryonic lethality (57). Tissue-specific disruption has revealed diverse STAT3 functions in mammary gland, liver, keratinocytes, thymus, blood, and neurons that are involved in growth and differentiation, inflammation, liver regeneration, acute-phase responses, and other processes (reviewed in references 1 and 37). Activated STAT3 regulates the transcription of genes involved in growth regulation and apoptosis inhibition, and STAT3 is frequently found hyperactivated in human cancers and cancer cell lines (11). Several studies have implicated STAT3 as an oncogene involved in malignant transformation (6, 7, 66, 68), and STAT3 also plays an important role in tumor maintenance, as illustrated by the spontaneous programmed cell death that follows STAT3 inhibition in cancer cells (10, 20, 22–24, 44–46, 68, 69). The overwhelming evidence for STAT3 functions in normal and disease processes underscores the potential therapeutic importance of STAT3 inhibitors (8, 11).

STAT proteins typically exhibit long half-lives (26, 35, 48), but their stability can be dramatically reduced by infection of cells with certain negative-strand RNA viruses (Paramyxovirinae) from the genus Rubulavirus (13, 21, 34, 48–50, 65, 67). The ability to reduce the steady-state level of STAT1 or STAT2 is the property of a single viral gene product, the V protein (13, 48). The IFN evasion activities of simian virus 5 (SV5) and human parainfluenza virus type 2 (HPIV2) have been best characterized: infection with SV5 or expression of the SV5 V protein can target STAT1 for ubiquitin-mediated proteasome degradation, and infection with HPIV2 or expression of the HPIV2 V protein can target STAT2 (13, 21, 34, 48–50). Despite their different STAT substrate specificities, the targeting mechanisms for SV5 and HPIV2 are remarkably similar. These viruses assemble a V-dependent degradation complex that includes V, both STAT1 and STAT2 (49), and V-interacting cellular proteins, including the damaged-DNA-binding protein DDB1 (2, 38, 59) and the cullin family member Cul4A (59). The V-dependent degradation complex VDC (an acronym for V, DDB1, Cullin) is an amalgam of virus-encoded and cell-derived activities that functions as a STAT-specific E3 ubiquitin ligase enzyme, efficiently inducing STAT protein polyubiquitylation and proteasomal degradation (59). The reported IFN suppression, reduction in STAT1 accumulation, and STAT1 polyubiquitylation in cells stably expressing the mumps
virus V protein (17–19, 34, 64, 65) are consistent with a similar mechanism of STAT destruction.

The distinct STAT-targeting activities of SV5 and HPIV2 and the divergent nonproteasomal targeting of STAT1 and STAT2 reported for the V protein from a more distantly related paramyxovirus, Nilpah virus (52), support a hypothesis that evolutionary diversification could produce V protein variants capable of targeting proteins other than STAT1 or STAT2. As STAT3 is highly homologous to STAT1 (70, 71), the ability of V proteins to target STAT3 was examined. The results demonstrate that in addition to its ability to target STAT1, mumps virus infection or mumps virus V protein expression results in degradation of cellular STAT3 protein. The mumps virus V protein induces the assembly of a protein complex(es) similar to but distinct from the VDC ubiquitin-ligase complexes assembled by SV5 and catalyzes polyubiquitylation of both STAT1 and STAT3. As a consequence, STAT3-dependent transcription is disengaged in cells expressing mumps virus V protein.

Comparison of the STAT1 and STAT3 targeting requirements indicates that separate mechanisms are used for each STAT, suggesting that independent complexes are formed. We demonstrate that the STAT3 interference is equally effective for suppressing transcription initiated by either cytokine (IL-6) or oncogene (v-Src) signals. Moreover, mumps virus V protein oncolytic potential is revealed by mumps virus V-dependent apoptosis induced in human multiple myeloma cells and transformed murine fibroblasts. STAT3 interference by mumps virus or the mumps virus V protein represents a previously unrecognized STAT3 targeting agent with therapeutic potential.

**MATERIALS AND METHODS**

**Cells and viruses.** Human 2FTGH, 293T, NIH 3T3, 3T3-v-Src, U3A, and U6A cell lines were maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% Cosnic cail serum (HyClone) and 1% penicillin-streptomycin (Gibco-BRL) as described previously (49). U266 myeloma cells (generous gift from Rich Jove) were grown in RPMI with 10% heat-inactivated fetal bovine serum. Mumps virus (Enders strain; ATCC VR-106) was cultivated and passaged in Vero cells. Cells and viruses.

**Plasmids, transfection, and reporter gene assays.** A cDNA copy of the mumps virus V gene was amplified by PCR from reverse-transcribed genomic RNA isolated from Vero cells infected with the Enders strain of mumps virus using primers that add restriction endonuclease recognition sequences for direct cloning into a mammalian expression plasmid downstream of an in-frame N-terminal Flag or hemagglutinin (HA) epitope tag. Several independently transcribed and spliced cDNAs were sequenced, and all were found to differ from the GenBank database sequence entry at three positions in the sequence: position 214, AGC(Ser) instead of AGA(Arg); position 382, GCG(Ala) instead of ACG(Thr); and position 433, ACC(Thr) instead of ATC(Ile). For luciferase assays in 293T cells, 60-mm dishes were transfected by the calcium phosphate method with 2 µg of reporter gene (for IFN-α responses, 5× ISRE-luciferase; for IFN-γ and IFN-β responses, 4× M678IE-luciferase), 6 µg of V plasmid, and 0.5 µg of CMV-LacZ. For luciferase assays in other cell lines, cells were transfected with Superfect reagent (Qiagen) or Lipofectamine reagent (Gibco) according to the manufacturer’s method with a cytomegalovirus-lacZ (CMV-LacZ) plasmid as a control for transfection efficiency, a reporter gene, and either empty vector or cDNA expression plasmids as indicated. After 24 h, the transfection medium was replaced with fresh medium or medium supplemented with cytokines: 1,000 U of recombinant human IFN-α per ml (Hoffman-La Roche) or 1,000 U of recombinant murine IFN-α per ml for NIH 3T3 cells (PBL Biomedical), or IL-6 (400 ng/ml) plus soluble IL-6 receptor (500 ng/ml) as described (25). Cells were harvested 6 to 12 h later in luciferase assay lysis buffer, and luciferase activity was measured according to the manufacturer’s protocol (Promega). For luciferase activity were normalized to β-galactosidase activity. In all cases, data represent the average values of triplicate samples normalized to cotransfected β-galactosidase activity, expressed as a percentage of the control stimulated sample value.

**Indirect immunofluorescence.** For immunofluorescence, cells were grown on Permanox chamber slides (Nalgene Nunc) and transfected and stained exactly as described previously (52) but with Flag antibody (Sigma; 10 µg/ml) STAT3 antibody (Santa Cruz C-20; 3 µg/ml) and TOTO 3 nuclear stain (Molecular Probes, 48 nM). For infections, cells were infected on chamber slides with 2 PFU of mumps virus per cell and stained with mumps virus NP antibody (Chemicon International; 10 µg/ml). Images were obtained with a Leica TCSSP confocal microscope, and representative fields illustrating transfected and untransfected cells in the same field are illustrated, but note that V protein effects on STATs were observed with 100% penetration, even in cells with relatively low V levels.

**Cell extracts, immunoblotting, and immunoprecipitation.** For preparation of cell extracts, samples were washed once with ice-cold phosphate-buffered saline and subsequently lysed with whole-cell extract buffer (WCEB) as described (49, 59). For immunoblotting, proteins were separated and transferred to nitrocellulose, then probed with antibodies and visualized with chemiluminescence (NEN Life Sciences). For immunoprecipitation, lysates were prepared in WCEB and precleared with protein A-agarose. Antibody-protein complexes were purified with Protein A beads and washed with WCEB. After elution with sodium dodecyl sulfate (SDS), proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and processed for immunoblotting. Antibodies used included STAT1 (Santa Cruz C20), STAT2 (Santa Cruz C24), STAT3 (Santa Cruz C20), Flag (Sigma), ubiquitin (Santa Cruz P41), DDB1 (Abcam 9194), and Cullin 4A (Santa Cruz 8557).

**Affinity purification.** Affinity purification was carried out exactly as described (59). Lysates from four transfected 10-cm plates per sample (~50 µg of total protein per sample) were treated with 1 µg of DNase I per ml for 2 h, and 50 µg of ethidium bromide per ml before incubation with anti-Flag (M2)-agarose beads (200 µl per sample; Sigma) overnight at 4°C. Beads were washed with WCEB, and eluates were complexed with 100 µl of Flag peptide per ml. Eluates were denatured in SDS loading buffer, separated by SDS-PAGE, and processed for silver staining (BioRad Stain Plus) or immunoblotting.

**Annexin staining.** Apoptosis was measured by staining with fluorescein isothiocyanate-conjugated annexin V according to the manufacturer’s protocol (Oncogene Science). Cells were transfected by Lipofectamine (3T3-v-Src) or electroporation (U266) with 16 µg of control vector, SV5, or mumps virus V vector or 5 µg of RIP vector and 3 µg of CD14 expression plasmid (generous gifts from Adrian Ting, Mt. Sinai), At 48 h later, CD14-positive cells were enriched by magnetic bead selection (Miltenyi Biotech) and subjected to annexin V staining and flow cytometry on a FacScan (Becton Dickinson). RIP samples were harvested at 24 h posttransfection.

**FIG. 1.** Analysis of STAT interference by mumps virus. (A) Mumps virus V protein blocks IFN signal transduction. 293T cells were transfected with an ISRE-luciferase reporter gene (left panel) and either empty vector or mumps virus V protein expression vector, as indicated. Cells were treated with 1,000 U of IFN-α per ml or not treated for 6 h prior to transfection. A GAS-luciferase reporter gene (right panel) and treatment with 5 ng of IFN-γ per ml. All bars represent average values from triplicate samples, normalized to cotransfected CMV-LacZ ± standard deviation. (B) Mumps virus V protein targets both STAT1 and STAT3. 2TGH cells were transfected with empty vector or Flag-tagged mumps virus V protein expression plasmid and subjected to indirect immunofluorescence staining 24 h later. Cells were fixed, permeabilized, and stained sequentially for Flag and then STAT1, STAT2, or STAT3, and analyzed by confocal microscopy. Nuclei were visualized by staining with TOTO3. Arrows point to the location of V-expressing cells. (C) Mumps virus V interferes with cytokine responses. Similar to panel B, except that cells were treated with IFN-α, IFN-γ, or IL-6 for 30 min prior to fixation. (D) Mumps virus infection causes STAT degradation and relocalization. Cells were infected with mumps virus (2 PFU/cell) and 20 h later processed for indirect immunofluorescence as described above, except that an antibody specific for mumps virus nucleocapsid protein (NP) was used to detect infected cells.
RESULTS

Mumps virus V protein eliminates both STAT1 and STAT3. A cDNA copy of the mumps virus V gene was tested in assays for IFN signaling inhibition (Fig. 1A). Transient expression of mumps virus V protein was capable of interfering with alpha/beta and gamma IFN reporter gene assays, consistent with loss of STAT1 signaling (17–19, 34, 64). To test the effects of V protein expression on individual STAT proteins, the steady-state levels of STAT1, STAT2, and STAT3 were examined by indirect immunofluorescence. Epitope-tagged V protein was detected with tag-specific antibodies, and STAT1, STAT2, and STAT3 were detected in the same cells by double labeling. The STAT1 protein level was efficiently reduced in cells expressing mumps virus V, but no reduction in STAT2 level was observed (Fig. 1B). In addition to its ability to reduce STAT1 levels, the mumps virus V protein also dramatically reduced the level of STAT3. This novel STAT3-directed activity is a unique property of the mumps virus V protein that is not shared by SV5 or HPIV2 V proteins (unpublished observations).

To formally verify that the loss of STAT protein corresponds to an inhibition of STAT protein activation, similar assays were carried out with IFN-γ to activate STAT1, IFN-α to activate STAT2, and interleukin 6 to activate STAT3 (Fig. 1C). As expected from loss of STAT1 and STAT3, no cytokine-induced nuclear translocation was observed in cells expressing mumps virus V protein, but adjacent untransfected cells exhibited normal cytokine-dependent nuclear accumulation. Interestingly, the pattern of STAT2 redistribution in response to IFN-α was altered, exhibiting a partial nuclear translocation phenotype, consistent with earlier conclusions regarding STAT2 nuclear transfer in the absence of STAT1 (32).

To evaluate the possibility that expression of mumps virus V protein outside the context of a virus infection confers the unique STAT3-directed activity, immunofluorescence was used to evaluate STAT protein levels in mumps virus-infected cells. An antibody that recognizes the mumps virus nucleocapsid protein (NP) was used to identify infected cells (Fig. 1D). The NP staining was localized to discrete cytoplasmic bodies, similar to a pattern that has been observed for other paramyxoviruses (14). All cells that stained positive for NP also exhibited loss of STAT1 and STAT3. A significant difference in STAT2 subcellular distribution was also observed in mumps virus-infected cells. STAT2 was localized to punctate cytoplasmic bodies that in many instances colocalized with the NP stain. These results demonstrate that in addition to STAT1, mumps virus V protein has a second host degradation target, STAT3. Mumps virus infection can also alter the subcellular localization of latent STAT2 protein.

Mumps virus V inhibits cytokine and oncogene signaling. STAT3 activation and transcription factor activity have been well studied for cytokine signaling systems similar to IL-6 (27). To test the consequences of V protein-induced STAT3 degradation in a biological context, STAT3-dependent transcription assays were carried out. Treatment with IL-6 potently induced reporter gene expression from a STAT3-responsive SIE/GAS-luciferase construct, but expression of mumps virus V completely prevented reporter gene induction by IL-6 (Fig. 2A). No inhibitory effect on IL-6 signaling was observed with con-
control SV5 V or HPIV2 V expression, but a notable and reproducible 30 to 50% increase in IL-6 reporter gene activity was observed with SV5 V (Fig. 2A).

To determine the ability of mumps virus V protein to block STAT3 signaling induced by an intracellular stimulus, v-Src, an oncogenic tyrosine kinase, was used to activate STAT3. Expression of v-Src potently induced the reporter gene, but this induction was specifically abolished by mumps virus V protein (Fig. 2B). Together, these results demonstrate that the reduction of the STAT3 protein level induced by mumps virus V protein inhibits both extracellular and intracellular STAT3-activating pathways.

**Distinct requirements for targeting STAT1 and STAT3.** Several systems, including IL-6, that rely on STAT3 signals can also activate STAT1, and the activated STAT factors recognize the same DNA sequence element, making STAT1 and STAT3 activities indistinguishable in reporter gene assays. To clearly define the mumps virus V protein IL-6 inhibition as the result of STAT3 and not STAT1 interference, assays were carried out in a STAT1-deficient cell line (U6A cells [39], Fig. 2C). In the absence of STAT1, IL-6 produced a potent activation of luciferase activity that was completely prevented by mumps virus V expression, demonstrating that STAT3 and not STAT1 is the mumps virus V target responsible for IL-6 evasion. This is distinct from HPIV2-induced STAT2 targeting, which requires STAT1 in an interdependent process (49). These data suggest a unique mechanism is used by mumps virus for STAT3 interference that is STAT1-independent.

Analogous to HPIV2, SV5 only degrades STAT1 in the presence of cellular STAT2 (49). To investigate a role for STAT2 in mumps virus V protein targeting, assays were carried out in STAT2-deficient cells (U6A [36]). IFN-γ-STAT1 signaling was robust in the STAT2-deficient U6A cells, and significant interference by either SV5 or mumps virus V protein was only observed when STAT2 was provided by cotransfection (Fig. 2D). This finding demonstrates that, like SV5 V, mumps virus V protein requires STAT2 in order to antagonize STAT1 signaling. IL-6-STAT3 signaling, which was robust in U6A cells, was unaffected by SV5 V either in the presence or in the absence of expressed STAT2 (Fig. 2E). In contrast, expression of mumps virus V protein potently prevented IL-6-STAT3 signaling in both the absence and presence of STAT2 (Fig. 2E). These data indicate conspicuously different STAT2 requirements in mumps virus-dependent STAT1 and STAT3 degradation.

For SV5, the critical role of STAT2 in STAT1 degradation is underscored by the discovery that differences between human and murine STAT2 orthologues provide a barrier to SV5-induced STAT1 degradation in the mouse system (50). To test the mumps virus V protein for a species-restricted STAT2 requirement, mouse NIH 3T3 fibroblast cell lines were used for transcription interference assays. While SV5 required human STAT2, mumps virus V was able to antagonize IFN-γ signaling in the murine system independently of human STAT2 expression (Fig. 2A). These results indicate that while STAT1 targeting by mumps virus V absolutely requires STAT2, mumps virus V activity is not restricted by murine and human STAT2 differences.

The ability of mumps virus V to antagonize murine STAT3-dependent signaling was also tested with v-Src as the inducer in either NIH 3T3 cells or a derivative line with stable expression of human STAT2 (Fig. 3B). Mumps virus V protein disrupted v-Src signaling in the mouse cells irrespective of human STAT2 expression. To test the activity of mumps virus V protein on a well-characterized STAT3-responsive gene, the activity of the cyclin D1 gene promoter was assessed in v-Src-transformed NIH 3T3 cell lines (Fig. 3C). Cyclin D1 transcriptional induction was inhibited by expression of mumps virus V protein but not SV5 V, consistent with STAT3 targeting by mumps virus V. Together, the differences in STAT2 dependence indicate that the mumps virus V protein utilizes distinct targeting mechanisms for STAT1 and STAT3 degradation.
Mumps virus V assembles STAT targeting complexes. STAT protein targeting by SV5 and HPIV2 requires a multisubunit VDC ubiquitin-ligase complex that includes cellular components STAT1, STAT2, DDB1, and Cul4A (59). To determine if the mumps virus V protein assembles a similar STAT degradation complex, Flag-tagged mumps virus V and SV5 V were subjected to affinity chromatography. Analysis of the affinity-purified material by SDS-PAGE and silver staining revealed a number of V-interacting protein species that ranged between 40 and 300 kDa and copurified with either mumps virus V or SV5 V protein but not a Flag-tagged green fluorescent protein control (Fig. 4A). The cellular proteins associated with the two viral proteins showed patterns that were overall very similar and likely represent a core degradation complex or complexes, but with virus-specific differences, providing a basis for differential STAT targeting specificity. Significant mumps virus V-specific polypeptides were apparent at ~85 and ~60 kDa, but less abundant mumps virus-specific bands were observed at ~300, 200, and 140 kDa.

Immunoblotting with specific antisera (Fig. 4B) revealed that both V protein affinity preparations contained STAT1 and STAT2, consistent with the genetic data described above. The STAT1 immunoblot revealed a laddering pattern for both SV5 and mumps virus V proteins, consistent with V-mediated STAT1 modification by polyubiquitylation. In addition, both viruses copurified with cellular DDB1 and Cul4A, two proteins with demonstrated roles in SV5 V-dependent STAT1 targeting (2, 59). Immunoblotting with STAT3 antiserum revealed that only the mumps virus V protein affinity preparation contained copurified STAT3. Importantly, none of these partner proteins was detected in the green fluorescent protein control.

To test the ability of the V proteins to induce specific STAT protein polyubiquitylation, transfection conditions that produced V protein levels suboptimal for complete degradation were used to capture the unstable polyubiquitylated STAT intermediates. Immunoprecipitation of the target STAT protein followed by immunoblot with ubiquitin-specific antisera revealed that both SV5 and mumps virus V protein induced polyubiquitylation of STAT1 (Fig. 4C, top panels). In contrast, only mumps virus V protein induced polyubiquitylation of STAT3 (Fig. 4C, bottom panels). These results are in agreement with the concept that the mumps virus V protein induces the formation of a STAT1- and STAT2-containing VDC ubiquitin-ligase complex or complexes that resemble the SV5 V-dependent degradation complex, but mumps virus V protein forms additional superimposed associations with STAT3 through a related but mechanistically distinct multiprotein ubiquitin ligase.

Oncolytic activity of mumps virus V protein. The constitutively activated STAT3 found in many human cancers often functions in a survival role for tumor maintenance (11), and inhibition of STAT3 has been demonstrated to induce apoptosis in tumor cells (23, 44). Malignant transformation of cultured murine fibroblasts by v-Src requires functional STAT3 signaling (6). Similarly, growth and survival of human myeloma tumor cells depend on IL-6-mediated STAT3 signaling (30). The human U266 myeloma cell line possesses an autocrine IL-6 self-stimulatory loop that produces constitutively acti-
TABLE 1. Mumps virus V protein induces apoptosis in cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transfection*</th>
<th>% Annexin-positive</th>
</tr>
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<tbody>
<tr>
<td>U266</td>
<td>Vector</td>
<td>11.1</td>
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<tr>
<td></td>
<td>SV5 V</td>
<td>15.8</td>
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<td></td>
<td>Mumps virus V</td>
<td>34.9</td>
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<tr>
<td></td>
<td>RIP</td>
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<tr>
<td>3T3/v-Src</td>
<td>Vector</td>
<td>29.7</td>
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<tr>
<td></td>
<td>SV5V</td>
<td>25.2</td>
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<tr>
<td></td>
<td>Mumps virus V</td>
<td>57.4</td>
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* Cells were cotransfected with expression vector for CD14.

** Percentage of 10^6 CD14-positive cells that stained with annexin V, measured by flow cytometry.

vated STAT3. In these cancer cells, disruption of STAT3 signaling induces spontaneous apoptosis (10).

To test for oncolytic activity, U266 and 3T3/v-Src cells were transfected with plasmids encoding V proteins along with an expression vector encoding CD14, a monocyte cell surface protein used as a marker for selecting transfected cells. As a positive control, parallel U266 samples were transfected with an expression vector for RIP, a proapoptotic death domain protein (56, 58). Enriched CD14-positive cell fractions were isolated with magnetic beads, and equal numbers of cells were stained with annexin V (Table 1). Slight increases in the percentage of annexin-positive apoptotic cells were observed after expression of control vector or SV5 V protein (11.1% and 15.8%, respectively, for U266; 29.7% and 25.2%, respectively, for 3T3/v-Src), but expression of mumps virus V protein substantially increased the number of annexin-positive cells (34.9% for U266; 57.4% for 3T3/v-Src). The apoptosis induced by mumps virus V protein in U266 cells was similar to that induced by the positive control, RIP (40.6%). Expression of mumps virus V also correlated with a reduced soft agar plating efficiency for the 3T3/v-Src cells (not shown). The induced apoptosis correlates with STAT3 degradation and v-Src or IL-6 suppression and suggests a therapeutic potential for the mumps virus V protein.

**DISCUSSION**

The results demonstrate that mumps virus targets and destroys both STAT1 and STAT3 proteins through the actions of its V protein. The loss of STAT1 inactivates both IFN-γ and IFN-α/β signal transduction, an effective means to disengage both innate and adaptive antiviral responses in host cells. The mechanism of STAT1 degradation by mumps virus is apparently very similar to that described for SV5, as the ability to target STAT1 requires the presence of STAT2 in the host cell (49, 50, 59). However, unlike SV5, which exhibits a species-specific requirement for human STAT2 to enable STAT1 degradation (50), the mumps virus V protein efficiently prevents IFN signaling in mouse cells without exogenous human STAT2. Importantly, the phenotypes of STAT1 and STAT3 degradation were also observed in mumps virus-infected cells, where an additional virus-induced effect on STAT2 was also noted. During infection, a portion of STAT2 was found to accumulate in punctate cytoplasmic bodies that colocalize with aggregates of the viral nucleocapsid protein.

Despite the high amino acid sequence conservation between the SV5 and mumps virus V proteins (~43% identity), the observed STAT3 targeting was a property unique to mumps virus and resulted in a cellular state refractory to IL-6 and v-Src, two well-known STAT3 activators. The mumps virus V STAT3 interference was completely independent of STAT2, in contrast to its own STAT1 targeting ability. This finding indicates a mechanistic distinction between the STAT1 and STAT3 degradation reactions and suggests that mumps virus V protein can assemble distinct targeting complexes for polyubiquitylation of either STAT1 or STAT3.

Analysis of protein affinity fractions indicated that the degradation complexes that purified with SV5 and mumps virus V proteins were similar in composition, yet mumps virus V copurified distinct subunits. Specific components of the mumps virus V-dependent complex in common with the SV5 V-dependent degradation complex include STAT1 and STAT2, DDB1, and Cul4A, thereby defining the mumps virus targeting complex as a VDC-type assembly similar to the SV5 and HPIV2 E3 ubiquitin ligases (59). Additional mumps virus V-specific interacting polypeptide species were identified, and one of these was STAT3. Significantly, while both SV5 and mumps virus V proteins could induce the polyubiquitylation of STAT1, only mumps virus V protein could induce the polyubiquitylation (and degradation) of STAT3, illustrating the targeting specificity of the respective V proteins.

Interestingly, it has recently been reported that a tryptophan-containing peptide in the mumps virus V protein C-terminal domain can participate in multiple STAT protein interactions assayed in mixed detergent extracts in vitro (43). It is possible that this motif is important for native V-dependent degradation complex functions, but the lack of STAT binding specificity and absence of biological activity in vivo suggest that additional determinants are required for specific STAT binding and targeting in vivo within the context of a complete protein complex. This is congruous with reports that both N- and C-terminal SV5 V protein regions are required for V-dependent degradation complex assembly and IFN antagonism (2, 59).

Viruses target STAT1 or STAT2 to evade the IFN antiviral responses, but the reason for STAT3 antagonism is not immediately apparent from the analysis of innate antiviral immunity. While STAT3 is not generally considered a major component of the IFN-induced antiviral system, STAT3 activation by IFN signaling has been reported (9, 29, 51, 60, 62, 63), implying a potential antiviral role for STAT3 that has yet to be fully elucidated. Another benefit from STAT3 evasion is avoidance of adaptive immune responses that occur downstream of inflammatory cytokines, and mumps virus-induced STAT3 loss will also antagonize mitogenic growth factors, tyrosine kinases, G proteins, and other STAT3 inducers. In this report, STAT3 signaling inhibition was investigated with two well-characterized STAT3 activators, IL-6 and v-Src. However, destruction of STAT3 will provide a much broader spectrum of cytokine and growth factor suppression in vivo (37). STAT3 destruction allows the virus to effectively evade diverse cellular responses, a property that could provide several general or tissue-specific replication advantages and might play a role in the ability of mumps virus to invade and replicate in activated T lymphocytes (15, 16).

A wide range of cytokines, growth factors, and oncogenic
stimuli that induce STAT3 activation have been associated with many human illnesses, including arthritis, lupus, autoimmunity, dwarfism, cardiac hypertrophy, obesity, and kidney disease. The ability to irreversibly remove STAT3 from cells is a clear therapeutic application of our results. A prime target for such a therapy derives from 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 (8). In most cases, the inducer of STAT3 activity is unknown, but inhibition of STAT3 leads to tumor regression and apoptosis (11).

Oncolytic viruses have a high potential for use in cancer treatment (5, 42), and the use of live mumps virus as a cancer treatment was described 40 years ago (3; reviewed in reference 42). 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 (4, 42, 47). More recent investigation demonstrated the ability of mumps virus to initiate programmed cell death in renal cell carcinoma (41). As secreted proteins such as IL-6 and epidermal growth factor act as autocrine or paracrine growth factors for many cancer types and intracellular stimuli such as c-Src are associated with transformation, it is likely that destruction of STAT3 will have a broad range of anticancer effects (33, 40, 41). Rather than live mumps virus, a more contemporary approach might involve delivery of the mumps virus V protein via a stealth vector system, and similar approaches could be adapted for treatment of other diseases resulting from STAT3 hyperactivity. Accordingly, the mumps virus V protein represents an efficient STAT3-inhibitory agent from which targeted therapies may be derived.

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