STAT2 Acts as a Host Range Determinant for Species-Specific Paramyxovirus Interferon Antagonism and Simian Virus 5 Replication

Jean-Patrick Parisien, Joe F. Lau, and Curt M. Horvath*

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

Received 7 January 2002/Accepted 24 March 2002

The antiviral state induced by alpha/beta interferon (IFN- α/β) is a powerful selective pressure for virus evolution of evasive strategies. The paramyxoviruses simian virus 5 (SV5) and human parainfluenza virus 2 (HPIV2) overcome IFN- α/β responses through the actions of their V proteins, which induce proteasomal degradation of cellular IFN- α/β -activated signal transducers and activators of transcription STAT1 and STAT2. SV5 infection induces STAT1 degradation and IFN- α/β inhibition efficiently in human cells but not in mouse cells, effectively restricting SV5 host range. Here, the cellular basis for this species specificity is demonstrated to result from differences between human and murine STAT2. Expression in mouse cells of full-length or truncated human STAT2 cDNA is sufficient to permit antagonism of endogenous murine IFN- α/β signaling by SV5 and HPIV2 V proteins. Furthermore, virus-induced STAT protein degradation is observed in mouse cells only in the presence of ectopically expressed human STAT2. The results indicate that STAT2 acts as an intracellular determinant of paramyxovirus host range restriction, which contributes to the species specificity of virus replication, and that human STAT2 can confer a growth advantage for SV5 in the murine host.

Alpha/beta interferons (IFN- α/β) are the primary innate antiviral cytokines for higher eukaryotic organisms. Exposure of cells to IFN- α/β rapidly establishes an antiviral state that blocks cytopathic effects and inhibits virus replication by activating a number of cellular mechanisms that inhibit viral RNA and protein synthesis (reviewed in reference 9). Most of the antiviral responses are regulated transcriptionally by the actions of the IFN- α/β -activated transcription complex, ISGF3, which consists of a heterotrimer of STAT1, STAT2, and IRF9 proteins. In response to IFN- α/β , latent cytoplasmic STATs are activated by tyrosine phosphorylation and undergo SH2 domain-mediated oligomerization to form ISGF3, which translocates to the nucleus, where it binds to the IFN- α/β -stimulated response element (ISRE) in the promoters of IFN- α/β -stimulated genes and induces their transcription (reviewed in reference 11). The IFN- α/β -induced antiviral state can be extremely effective in eliminating virus replication and has therefore appeared frequently as a target of viral immune suppression activities. As the IFN- α/β antiviral system is evolutionarily conserved and functional in most cell types studied, mechanisms of IFN- α/β suppression by viruses are typically found to be universally effective, irrespective of species or cell lineage.

It has been demonstrated recently that the *Paramyxoviridae* family of enveloped negative-strand RNA viruses counteract IFN- α/β responses by antagonizing the intracellular signaling pathways downstream of the activated IFN- α/β receptor. This virus family encompasses a wide range of medically important species including measles, mumps, and human parainfluenza viruses. Blocking IFN- α/β signaling is a broadly effective strat-

egy for preventing establishment of the antiviral state. For Sendai virus, a prototype of the *Respirovirus* genus, virus-encoded C proteins are used to block IFN- α/β signaling (6–8) and do so effectively in both murine and primate systems (3). Members of the *Rubulavirus* genus simian virus 5 (SV5) and human parainfluenza virus type 2 (HPIV2) use the virus-encoded V protein to block IFN- α/β signaling (4). Curiously, SV5 antagonizes IFN- α/β in primate cells but not in mouse cells (3). This species-specific IFN- α/β suppression by SV5 has been linked to an unprecedented mode of IFN- α/β escape by these viruses that principally involves the proteolytic degradation of cellular STAT proteins responsible for inducing IFN- α/β -specific antiviral genes. Expression of SV5 V protein in permissive cells induces a loss of cellular IFN- α/β -responsive transcription factor STAT1 (4), while expression of HPIV2 V induces a loss of cellular IFN- α/β -responsive transcription factor STAT2 (21). Available evidence suggests that the V proteins accomplish this degradation via subjugation of the cellular ubiquitin/proteasome degradation pathways (4, 21), but an additional explanation for the effect of HPIV2 V protein on STAT2 levels has been postulated to involve a selective defect in STAT2 protein synthesis (20). All the cellular determinants of V protein-induced STAT degradation and IFN- α/β antagonism have not been identified, but the degradation of STATs is independent of IFN- α/β signaling and requires a V protein-dependent multisubunit complex that contains both STAT1 and STAT2 (22).

The cellular basis for the differential ability of murine and human cells to create an innate antiviral response to SV5 was not immediately apparent from comparing the two species' proteolytic targets, as sequence comparisons reveal murine and human STAT1 orthologues to be 92.4% identical at the amino acid level (25). In contrast, murine and human STAT2

* Corresponding author. Mailing address: Box 1630, East Building Room 12-20D, One Gustave L. Levy Pl., New York, NY 10029. Phone: (212) 659-9406. Fax: (212) 849-2525. E-mail: curt.horvath@mssm.edu.

proteins are more divergent, with a modest 68.6% amino acid identity overall (5, 23, 25). Based on our recent discovery that STAT2 is required as an accessory for STAT1 degradation by SV5 in human cell lines (22), we hypothesized that differences between human and murine STAT2 orthologues might be the key to the observed murine host range restriction of SV5 IFN- α/β antagonism and STAT1 degradation. Experimental results indicate that IFN- α/β responses in STAT1-deficient human cells complemented with murine STAT1 are efficiently suppressed by expression of SV5 V. Further, expression of human STAT2 or truncated human STAT2 in mouse fibroblasts enables SV5 V to effectively disrupt murine IFN- α/β signaling. Mouse fibroblasts engineered to stably express human STAT2 acquire the ability to support specific degradation of murine STAT1 by SV5. This acquired IFN- α/β antagonism allows SV5 to replicate to higher titers in the human STAT2-expressing mouse cells, conferring a selective advantage for SV5 growth across species barriers. These findings demonstrate a novel role for STAT2 as a species-specific host range factor and a key participant in proteolytic degradation of STATs by paramyxoviruses.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 cells (gift from Stuart Aaronson, Mt. Sinai) and STAT1-deficient U3A cells (16, 18) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Cosmic calf serum (HyClone). STAT2-expressing NIH 3T3 clones were engineered by transfection of NIH 3T3 cells with pEFHA-STAT2 (22) and a vector encoding puromycin resistance (pBABE-puro [17]) and were selected in 2 μ g of puromycin/ml, and clones were screened by STAT2 immunoblotting (C20; Santa Cruz Biotechnology).

SV5 (strain W3A, derived from a genetically defined recombinant virus system [10, 14]) and HPIV2 (Greer strain) were propagated and titered in simian CV1 cells. Plaque assays were performed on CV1 cells by using an overlay containing 0.5% agar with DMEM and 10 mM HEPES (pH 7.2). Cells were fixed at 4 to 6 days postinfection (p.i.) with 3.7% formaldehyde, and plaques were visualized after being stained with 0.1% crystal violet in 20% ethanol as described elsewhere (22).

IFN- α/β -responsive transcription assays. For luciferase assays, cells were transfected with Superfect reagent (Qiagen) according to the manufacturer's method with a LacZ plasmid as a control for transfection efficiency, a reporter gene, and either an empty vector or cDNA expression plasmids. The IFN- α/β -responsive reporter gene contained five copies of the ISG54 ISRE upstream of the TATA box and the firefly luciferase gene open reading frame. After 24 h, the transfection medium was replaced with fresh medium or medium supplemented with 1,000 U of recombinant human IFN- α (Hoffman LaRoche)/ml or 1,000 U of recombinant murine IFN- β (PBL Biomedical)/ml. 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). Values for luciferase activity were normalized to β -galactosidase activity. In all cases, average values of triplicate experiments are shown. Expression vectors for human STAT1, V proteins, human STAT2, and human STAT2 fragments have been described elsewhere (12, 21, 22). The murine STAT1 cDNA expression vector was provided by David Levy (New York University). The murine STAT2 cDNA expression vector was provided by Christian Schindler (Columbia University).

Infection, cell extraction, and immunoblotting. For examination of STAT degradation, cells were infected with SV5 or HPIV2 at a multiplicity of infection (MOI) of 10 PFU/cell or mock infected and prepared for analysis at 16 h p.i. Whole-cell detergent extracts were prepared as described previously (21). Equal amounts of total protein (20 μ g) were separated by sodium dodecyl sulfate-7% polyacrylamide gel electrophoresis (SDS-7% PAGE) and transferred to nitrocellulose filters. Immunoblotting was performed with antiserum specific for human STAT2 (C20; Santa Cruz Biotechnology), antiserum recognizing murine STAT1 α and - β (E23; Santa Cruz Biotechnology), and antiserum specific for murine STAT2 (provided by Christian Schindler, Columbia University) (23), and filters were processed for chemiluminescence detection. For detection of viral

FIG. 1. Murine STAT1-dependent IFN- α/β signaling in human cells is blocked by SV5 V protein. STAT1-deficient human U3A cells were transfected with ISRE-luciferase reporter gene and expression vectors for human STAT1, murine STAT1, or SV5 V as indicated. Cells were treated with 1,000 U of human IFN- α /ml, as indicated, for 12 h prior to lysis and processing for the luciferase assay.

nucleocapsid proteins, antiserum raised against HPIV2-infected cells that recognizes both HPIV2 and SV5 nucleocapsid proteins was used (Whittaker Biochemicals; gift from Anne Moscona, Mt. Sinai) (22).

For examination of SV5 growth, cells were washed with serum-free medium and inoculated with SV5 at the MOIs indicated in the legend for Fig. 4. After a 2-h adsorption period, cells were washed and medium was replaced with DMEM plus 2% serum and, in some cases, IFN- α/β . After being harvested, supernatants were clarified by centrifugation and filtration through a 0.2- μ m-pore-size filter and adjusted to 0.75% bovine serum albumin, and virus titer was determined by plaque assays on monolayers of CV1 cells. For longer-term infections, assays were based on previous studies of SV5 persistence (28, 29). Cells were infected, and the inoculum was replaced at 2 h p.i. with low-serum medium with or without supplemental IFN- α/β . Virus was allowed to replicate for 48 h, and then the medium was harvested and replaced with low-serum medium with or without IFN- α/β . A second harvest was made 48 h later, and the chronically infected cell monolayer was washed and trypsinized for passage to larger dishes to allow for unhindered cell division in serum-containing medium lacking IFN- α/β . The supernatant of these cultures was harvested 7 days later (11 days p.i.).

RESULTS

Murine STAT1 is susceptible to SV5 V protein in human cells. A simple explanation for the failure of SV5 to antagonize murine IFN- α/β responses is that the small number of sequence differences between mouse and human STAT1 proteins create an intrinsic defect in V protein-mediated recognition or destruction. To test the ability of the mouse STAT1 to function as a V protein target, STAT1-deficient human U3A cells were used as the host for an IFN- α/β response antagonism assay. Due to their STAT1 deficiency, U3A cells cannot induce transcription of a transfected ISRE-luciferase reporter gene in response to IFN- α/β stimulation, but complementation of the cells by transfection with a human STAT1 expression vector restores a robust IFN- α/β -stimulated luciferase response (Fig. 1). This response is completely eliminated by cotransfection with an SV5 V protein expression plasmid. When a murine STAT1 cDNA is transfected, similar complementation of IFN- α/β -stimulated reporter gene activity is observed. Coexpression of SV5 V with murine STAT1 completely suppressed the IFN- α/β response. These results clearly demonstrate that murine and human STAT1 proteins are equally susceptible to V-dependent IFN- α/β suppression, indicating that the two STAT1 orthologues are not intrinsically different with respect to SV5 sensitivity.

Human STAT2 overcomes barriers to IFN- α/β antagonism.

FIG. 2. Human STAT2 enables IFN- α/β suppression in mouse cells. (A) NIH 3T3 cells were transfected with an ISRE-luciferase reporter gene in the presence or absence of cotransfected human or mouse STAT1 or STAT2 and HPIV2 V or SV5 V as indicated. Cells were stimulated with 1,000 U of murine IFN- β /ml for 6 h prior to lysis. Data represent normalized luciferase values from triplicate samples, expressed as percentages of IFN- β -stimulated controls. (B) The human STAT2 fragment comprising amino acids 1 to 578 is sufficient to allow V protein IFN- α/β antagonism. The experiment was conducted as for panel A, and human STAT2 or STAT2 fragments were expressed as indicated. (Inset) STAT2 domain structure and relevant amino acid numbers (11). ND, N domain; DBD, DNA binding domain; LD, linker domain; SH2, src homology 2 domain; Y690, activating tyrosine; P, phosphorylation site; TAD, transcriptional-activation domain.

The successful antagonism by SV5 V of murine STAT1-dependent IFN- α/β responses in a human cell line suggests that mouse cells are inherently deficient in either a targeting or enzymatic component of the STAT degradation machinery. In conjunction with the observation that both STAT1 and STAT2 must be present in human cells to create a permissive environment for selective STAT1 degradation by SV5 (22), it seemed reasonable to hypothesize that sequence differences between human and murine STAT2 might account for the functional deficiency for SV5 IFN- α/β antagonism in mice. To test this hypothesis, we assessed human STAT2 for the ability to complement defective STAT degradation in an IFN- α/β -responsive murine system. NIH 3T3 fibroblasts respond well to stimulation with murine IFN- β : there is a robust stimulation of an ISRE-dependent luciferase reporter gene after 6 h of treatment with mouse IFN- β (Fig. 2A). This IFN- β -stimulated transcription is insensitive to expression of the SV5 V protein, consistent with earlier reports of species specificity (3, 28). To examine the generality of this effect for STAT-destroying paramyxoviruses, the HPIV2 V protein was also tested in the murine system. Expression of the HPIV2 V protein also failed to antagonize the murine IFN- α/β response in these cells, a result possibly related to the reported incomplete HPIV2 replication in mouse L929 cells (13). The ability of both viral V proteins to antagonize the murine IFN- α/β response changed dramatically upon expression of human STAT2 in the mouse cell transcription assay. Coexpression of either SV5 or HPIV2 V protein along with human STAT2 in the NIH 3T3 cells resulted in a potent inhibition of IFN- α/β signaling, completely

eliminating ISRE-dependent luciferase activity (Fig. 2A). Control experiments with cells coexpressing either human or mouse STAT1 did not inhibit the IFN- α/β -responsive luciferase activity, but mouse STAT2 overexpression resulted in a slight V-dependent reduction of reporter gene activity (Fig. 2A). These results suggest that the STAT2 locus may be an important host range determinant for SV5, as mouse STAT2 is inefficient at mediating IFN- α/β inhibition, while expression of human STAT2 in mouse cells enables efficient V protein-dependent IFN- α/β antagonism.

Previous work demonstrated that defective STAT degradation in STAT2-deficient human cells could be rescued by expression of an amino-terminal STAT2 fragment encompassing amino acids 1 to 578 (22). The ability of human STAT2 fragments to confer V protein susceptibility to the murine cells was also tested. Expression of human STAT2 amino acids 1 to 578 was sufficient to render the murine cells susceptible to IFN- α/β antagonism by both paramyxovirus V proteins, but the isolated DNA binding domain and linker domain (amino acids 316 to 578) did not (Fig. 2B), confirming the important role of the STAT2 N terminus in the paramyxovirus-induced STAT degradation system. This result demonstrates that the crucial sequence differences underlying the species specificity of SV5 IFN- α/β suppression map to the same region of STAT2 that is absolutely required for STAT1 or STAT2 degradation by paramyxoviruses in human cells. The STAT2 fragment comprising amino acids 1 to 578 lacks domains required for IFN- α/β -responsive ISGF3 activation, tyrosine phosphorylation, and oligomerization, indicating that IFN- α/β signaling itself is

FIG. 3. Expression of human STAT2 in mouse cells permits STAT degradation. NIH 3T3 cells (3T3) and a derivative NIH 3T3 line expressing human STAT2 (3T3/HuS2) were infected with 10 PFU of HPIV2 (H) or SV5 (S)/cell. Lysates prepared 16 h p.i. were separated by SDS-PAGE and processed for immunoblotting along with control uninfected cells (C). (A) Immunoblot with antiserum that recognizes HPIV2 and SV5 nucleocapsid proteins (NP^{SV5}). (B) Immunoblot with antiserum specific for murine STAT2 (MuSTAT2). (C) Immunoblot with antiserum specific for murine STAT1 (MuSTAT1) that recognizes both full-length murine STAT1 α and truncated STAT1 β derived from alternative mRNA splicing. (D) Immunoblot with antiserum specific for human STAT2 (HuSTAT2).

not required for acquired IFN- α/β antagonism and affirming the mechanistic conclusions drawn from the study of human STAT degradation (22).

Expression of human STAT2 permits STAT degradation.

The transfection assays demonstrate that expression of human STAT2 in mouse cells transfers the ability of SV5 V to antagonize IFN- α/β signaling and suggest that STAT2 plays a role in restriction of the SV5 host range. To determine if human STAT2 expression enables virus-induced STAT protein degradation, an NIH 3T3 clone with stable expression of human STAT2 was isolated and subjected to infection with SV5 and HPIV2 (Fig. 3). Mock-infected- and infected-cell lysates were separated by SDS-PAGE and processed for immunoblotting. Probing with antiserum that recognizes both virus nucleocapsid proteins revealed that SV5 could synthesize viral proteins in the NIH 3T3 cells but HPIV2 could not (Fig. 3A). Importantly, infected-cell lysates from human STAT2-expressing cells revealed that expression of human STAT2 did not alter the level of SV5 protein synthesis (Fig. 3A). Probing with antiserum specific for murine STAT2 demonstrates that its level remained constant regardless of virus infection (Fig. 3B). Consistent with the overexpression results (Fig. 2B), a minor

reduction of STAT1 was observed in SV5-infected NIH 3T3 cells after probing with STAT1-specific antiserum, but SV5 efficiently induced complete and specific degradation of STAT1 only in the cells expressing human STAT2 (Fig. 3C). The effect of SV5 infection was limited to STAT1, as the level of human STAT2 in the SV5-infected cells remained intact (Fig. 3D). Remarkably, this experiment reveals that, despite the attenuation of HPIV2, a partial loss of both murine STAT1 and human STAT2, but not of murine STAT2, is achieved by the HPIV2 inoculum when human STAT2 is present. The finding of HPIV2-induced STAT degradation in the absence of significant viral protein synthesis suggests that the virus-associated V protein enters the cell upon virus-cell membrane fusion and is active for inducing degradation of its target (Fig. 3D). This degradation is similar to results obtained with UV-inactivated SV5 (3, 28) and is consistent with the demonstration that V is a virus component present at approximately 350 molecules per virion (calculated for SV5 in reference 24). The HPIV2 inoculum also decreased the abundance of murine STAT1, again only in the cells expressing human STAT2 (Fig. 3C). This is in agreement with the promiscuous actions of HPIV2 observed in STAT2-complemented human STAT2-deficient U6A cells, where both STAT1 and STAT2 degradation was noted (22). No significant difference in the abundance of murine STAT2 was observed for HPIV2 infection. These results indicate that the efficient degradation of STAT proteins requires human STAT2 and that murine STAT2 is not targeted by either virus, even in a degradation-permissive cellular environment. Importantly, all degradation activities were observed only in cells expressing human STAT2, confirming that paramyxovirus-induced STAT degradation can utilize human STAT2 to proceed across species barriers.

Expression of human STAT2 permits efficient SV5 replication in mouse cells. IFN- α/β antagonism and STAT degradation by SV5 were found to only occur in mouse cells if human STAT2 is expressed. The results suggest that STAT2 may function as a host factor important for the restriction of SV5 replication. To directly examine replication of SV5 in the presence or absence of human STAT2-dependent IFN- α/β antagonism, NIH 3T3 and NIH 3T3/human STAT2 cell lines were subjected to SV5 infection in the presence or absence of exogenous IFN- α/β (Fig. 4). To verify that expression of human STAT2 in the NIH 3T3 cells did not alter the intrinsic ability to mount an antiviral response induced by IFN- α/β , both lines were subjected to a 6 h of IFN- α/β pretreatment followed by infection with SV5 at a high MOI. Regardless of human STAT2 expression, IFN- α/β pretreatment resulted in a dramatic decrease in virus production in a 48-h growth period (Fig. 4A). This result demonstrates that expression of human STAT2 has neither positive nor negative effects on the ability of the mouse cells to mount an antiviral response.

To test the ability of SV5 to inhibit the endogenous IFN- α/β response in the NIH 3T3 cell lines, lower-multiplicity infections were used. Cells were infected with a range of SV5 concentrations, and virus replication was evaluated 48 h later. In all cases, SV5 infectious titers were higher in the presence of human STAT2, indicating that human STAT2 confers a growth advantage for SV5 in mouse cells (Fig. 4B). SV5 infection has been demonstrated to induce IFN- β biosynthesis, but the level of induction is low compared with that for Sendai virus (3). In

FIG. 4. Expression of human STAT2 does not influence establishment of the murine IFN- α/β antiviral state but enhances SV5 growth in IFN- α/β -responsive murine cells. (A) Pretreatment of cells with IFN- α/β reduces efficiency of virus replication. Cell lines indicated were incubated with or without murine IFN- β (1,000 U/ml) for 6 h and then infected with 10 PFU of SV5/cell. Supernatants were harvested 48 h later and titrated by plaque assay with CV1 cells. (B) Expression of human STAT2 confers a replication advantage to SV5 in mouse cells. Cell lines were infected with SV5 at the indicated MOI, and supernatants were harvested 48 h later for virus titration. (C) Expression of human STAT2 greatly enhances SV5 replication in mouse cells in the presence of excess exogenous IFN- α/β . The experiment was conducted as for panel B but with 1,000 U of murine IFN- β /ml added at the time the SV5 inoculum was replaced, i.e., after 2 h of adsorption. (D) Replication advantage conferred by human STAT2 allows more-efficient SV5 growth during long-term infection. The methodology was similar to that described for panel B. Infected cells were supplemented with IFN- α/β as indicated (+IFN) from 2 h p.i. until day 4. Cells were passaged to larger plates at the time of harvest on day 4 and grown in the absence of IFN- α/β for the next 7 days, at the end of which time accumulated virus in the supernatant was titrated. Time line insets (all panels), experimental designs.

addition, the endogenous IFN- α/β produced in the NIH 3T3 cell experiments is unlikely to attain optimal antiviral concentrations under the *in vitro* culture conditions in light of the medium changes required for washing and infecting cells with SV5. To assure high levels of IFN- α/β in our assays as a better imitation of a native infection, where high IFN- α/β concentrations accumulate locally, additional experiments were conducted with supplemental IFN- α/β added to the medium. The growth of SV5 was tested in the presence of IFN- α/β added exogenously at 2 h p.i. (1,000 U/ml, final concentration), coincident with replacement of the virus inoculum. Under these conditions, slightly lower titers were produced by all infections, but SV5 replication was dramatically improved in the presence of human STAT2, giving rise to ~10-fold differences in SV5 recovery (Fig. 4C). This result indicates that the ability to block IFN- α/β signaling conferred by the presence of human STAT2 provides a clear advantage for SV5 replication in mouse cells.

SV5 growth in murine cells has been characterized as having an early phase, permissive for virus protein synthesis, followed by loss of virus replication by 3 to 4 days after infection. Experiments were conducted to evaluate longer-term effects of acquired IFN- α/β antagonism on SV5 replication using meth-

odology developed for the study of SV5 persistence (28, 29). Cells were infected, and the inoculum was replaced at 2 h p.i. with low-serum medium with or without supplemental IFN- α/β . Virus was allowed to replicate for 48 h, and then the medium was harvested and replaced with low-serum medium with or without IFN- α/β . A second harvest was made 48 h later, and the chronically infected cell monolayer was washed and trypsinized for passage to larger dishes to allow for unhindered cell division in serum-containing medium lacking IFN- α/β . The supernatant of these cultures was harvested 7 days later (11 days p.i.). No significant differences in cellular cytopathic effects or plating efficiency were noted for any of the samples (data not shown). During the first 2 days following infection, only a minor SV5 replication difference between the two cell lines in the absence of exogenously added IFN- α/β was observed. In the presence of exogenous IFN- α/β , SV5 titers were reduced overall, consistent with data in Fig. 4C, but the presence of human STAT2 allowed SV5 to replicate five times more efficiently than in control NIH 3T3 cells under the same conditions (Fig. 4D). During the next 2 days, SV5 replicated much more efficiently in the human STAT2-containing cell lines, resulting in 100-fold increases in virus titers, regardless of

IFN- α/β supplementation. Cells were passaged and grown for 7 more days without added IFN- α/β . While SV5 replication was observed in control NIH 3T3 cells ($\sim 10^3$ PFU/ml), far more efficient replication was maintained for the human STAT2-containing cells regardless of prior IFN- α/β exposure, giving rise to a consistently 10-fold increase in virus yield. These results indicate that the acquisition of IFN- α/β suppression by SV5 in mouse cells containing human STAT2 results in higher viral titers, allowing for more productive replication in both acute- and chronic-infection scenarios.

DISCUSSION

The ability of a virus to successfully replicate in a specific host is the product of dynamic evolutionary competition between host and pathogen. As cellular antiviral immune forces battle viral subversion mechanisms, a balance of molecular interactions that defines the overall ability of a virus to encounter, infect, and replicate in a specific cell type or host species emerges. Cell surface interactions are often regarded as fundamental molecular determinants of cell tropism and species specificity, as expression of virus receptors can be restricted to certain cell types and the receptors can differ structurally between species (1). Intracellular factors also provide a basis for successful replication depending on viral requirements for particular components of the host enzymatic machinery that might be restricted in availability (26). Identification of the mechanisms that regulate cell or species specificity for viruses might ultimately provide targets for pharmaceutical intervention or novel means for producing debilitated viruses for vaccines.

The inability of SV5 to block IFN- α/β antiviral responses in murine cells has severe consequences for virus replication, leading to a self-limiting infection with most cells surviving infection and clearing the virus (3). Results presented here indicate that the reason SV5 fails to antagonize IFN- α/β in the mouse system is not due to intrinsic features of the SV5 proteolytic target, STAT1, as IFN- α/β responses dependent on murine STAT1 are efficiently blocked by V protein expression in otherwise human cells. Instead, we find that differences between human and mouse STAT2 proteins provide the molecular basis for SV5 species specificity. STAT proteins are modular in structure, with N-terminal protein interaction domains including a lengthy coiled coil, a central DNA binding domain, and C-terminal SH2 and transcriptional activation domains (reviewed in reference 11) (Fig. 2B). Expression of human STAT2 or the amino-terminal 578 amino acids of STAT2 enabled SV5 to overcome IFN- α/β antiviral effects in a murine host cell, and sole expression of the viral V protein was sufficient to mediate this IFN- α/β antagonism. The fact that the truncated STAT2 also enabled IFN- α/β inhibition demonstrates that IFN- α/β signal transduction through human STAT2 is not a requirement.

The V protein of a related paramyxovirus, HPIV2, also required expression of human STAT2 to antagonize IFN- α/β signaling in the mouse cell system, suggesting similar cellular bases for the two paramyxovirus STAT degradation systems. However, while the HPIV2 V protein shares the intracellular mechanistic requirement for human STAT2 when introduced into mouse cells by transfection, HPIV2 infection fails in the murine cells for reasons other than V-dependent IFN- α/β an-

tagonism. Apparently, absence of an additional essential host cell factor hinders an early step in HPIV2 infection of NIH 3T3 cells, preventing viral protein synthesis.

The species-specific ability of SV5 to selectively antagonize IFN- α/β signaling has been well characterized from the virologic perspective. SV5 naturally infects human, simian, and canine species, but the virus is unable to mount a productive infection of murine cells (3, 28). It has been demonstrated that while SV5 can enter murine cells and initiate viral protein synthesis, it is rapidly cleared by the endogenous IFN- α/β system. Neutralizing antibodies to IFN- α/β or IFN- α/β receptor deficiency enables SV5 replication in mouse cells, indicating the importance of IFN- α/β antiviral responses for controlling SV5 infection (29). The inability of SV5 to block IFN- α/β signaling in murine cells leads to a self-limiting infection, with most cells surviving infection and clearing the virus, but a few cells remain productive in persistent infections (3). After serial passage of persistently infected cells, SV5 variants that eventually dominate the pool can be selected (29). One of these mouse-adapted variants, mci-1, has acquired mutations leading to a phenotype of hyperactive intercellular membrane fusion, allowing the virus to more efficiently spread between cell cultures. Interestingly, this fusogenic variant remains as sensitive to IFN- α/β antiviral effects as the wild-type parental strain but can spread laterally to adjacent cells more rapidly and replicate at times when IFN- α/β concentrations are low in the culture medium during serial cell passage (29). A second mouse-adapted SV5 isolate, mci-2, was found to have lost reactivity with a monoclonal antibody that recognizes the V protein (28). Sequence analysis determined that one of the mutations in this virus results in a single amino acid substitution in the IFN- α/β antagonist V protein. A recombinant SV5 virus harboring the V protein mutation (N₁₀₀D) was demonstrated to retain the ability to block IFN- α/β signaling in human cells and to acquire the ability to effectively, albeit with decreased efficiency, block IFN- α/β signaling and target STAT1 in mouse cells (28). As a consequence of acquired murine IFN- α/β antagonism, this recombinant virus is capable of prolonged protein synthesis in mouse cells compared to wild-type SV5 and consequently replicates to higher titers during extended chronic infection. This finding provides strong support for the conclusion that IFN- α/β antagonism alone might sufficiently account for the restricted SV5 host range and complements the results presented here.

The finding that human STAT2 can enable cross-species interference with the murine IFN- α/β signaling system provides a unique molecular basis for the species specificity of SV5 based on expression of an intracellular innate antiviral signaling factor. Interestingly, species-specific differences in STAT2 have also been implicated in mouse-human differences in adaptive immune responses (5). Differential activation of T-cell cytokine (interleukin-12) responses downstream of the type I IFN- α/β receptor that are inducible in human but not murine cells were linked to sequence differences between the human and murine STAT2 C termini. The murine STAT2 orthologue was recognized as being uncharacteristically divergent for STATs, due in large part to interruption of the C terminus coding region of the murine locus by 12 copies of a 24-nucleotide minisatellite (5, 23, 25). This sequence disruption eliminates two tyrosine residues from the mouse STAT2 C

terminus that participate in recruitment of STAT4 to the IFN- α/β receptor in the human system. In contrast, the results obtained in IFN- α/β response assays with expression of human STAT2 protein fragments indicate that the region of STAT2 that is critical for paramyxovirus-induced STAT degradation is in the amino-terminal portion, suggesting that this region of STAT2 contains important sequence differences that account for species-specific innate immune responses. Indeed, the N-terminal 315 amino acids of STAT1 proteins for the two species are over 90% identical, while the corresponding regions of STAT2 for mice and humans are only 65% identical (5, 23, 25). Thus, the STAT2 N terminus is sufficiently divergent to account for the observed differential participation in IFN- α/β antagonism, but contributions of the more conserved STAT regions between 315 and 578 are not excluded. Informatic analysis and BLAST database searching do not reveal any features of the STAT N terminus that might implicate known degradation systems. Previous work indicates that the same portion of STAT2 is required for paramyxovirus-induced STAT degradation in STAT2-deficient human cells (22). It is likely that the mechanism of STAT degradation involves additional cellular machinery that is common to both human and mouse cells, but the mouse STAT2 fails to participate completely in this degradation complex.

Our findings demonstrate that virus host tropism can be controlled by intracellular signaling proteins. Paramyxoviruses that cause highly pathogenic human diseases (including measles and mumps viruses), as well as recently emerged deadly paramyxoviruses (e.g., Nipah and Hendra viruses), also encode V proteins that may participate in the inhibition of IFN- α/β signaling (2, 15, 19, 27). As the immunobiology and pathogenesis of human paramyxoviruses are being investigated with murine models, our findings may indicate potential complications with the extrapolation of results obtained with such models to the treatment of human diseases.

ACKNOWLEDGMENTS

We are grateful to Christina Ulane, Tom Kraus, and Jason Rodriguez for contributing preliminary results and helpful comments and to Chris Schindler, David Levy, Anne Moscona, Griff Parks, Bob Lamb, George Stark, and Stuart Aaronson for providing reagents. Thanks also to Griff Parks and Bob Lamb for advice and critical insights regarding paramyxovirus biology.

This study was supported in part by the New York City Council Speaker's Fund for Biomedical Research and NIH grants AI48722 and AI50707 to C.M.H.

REFERENCES

- Baranowski, E., C. M. Ruiz-Jarabo, and E. Domingo. 2001. Evolution of cell recognition by viruses. *Science* **292**:1102–1105.
- Chua, K. B., W. J. Bellini, P. A. Rota, B. H. Harcourt, A. Tamin, S. K. Lam, T. G. Ksiazek, P. E. Rollin, S. R. Zaki, W. Shieh, C. S. Goldsmith, D. J. Gubler, J. T. Roehrig, B. Eaton, A. R. Gould, J. Olson, H. Field, P. Daniels, A. E. Ling, C. J. Peters, L. J. Anderson, and B. W. Mahy. 2000. Nipah virus: a recently emergent deadly paramyxovirus. *Science* **288**:1432–1435.
- Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall. 1999. Sendai virus and simian virus 5 block activation of interferon-responsive genes: importance for virus pathogenesis. *J. Virol.* **73**:3125–3133.
- Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall. 1999. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. *J. Virol.* **73**:9928–9933.
- Farrar, J. D., J. D. Smith, T. L. Murphy, S. Leung, G. R. Stark, and K. M. Murphy. 2000. Selective loss of type 1 interferon induced STAT4 activation caused by a minisatellite insertion in mouse Stat2. *Nat. Immunol.* **1**:65–69.
- Garcin, D., J. Curran, M. Itoh, and D. Kolakofsky. 2001. Longer and shorter forms of Sendai virus C proteins play different roles in modulating the cellular antiviral response. *J. Virol.* **75**:6800–6807.
- Garcin, D., J. Curran, and D. Kolakofsky. 2000. Sendai virus C proteins must interact directly with cellular components to interfere with interferon action. *J. Virol.* **74**:8823–8830.
- Garcin, D., P. Latorre, and D. Kolakofsky. 1999. Sendai virus C proteins counteract the interferon-mediated induction of an antiviral state. *J. Virol.* **73**:6559–6565.
- Goodbourn, S., L. Didcock, and R. E. Randall. 2000. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *J. Gen. Virol.* **81**:2341–2364.
- He, B., R. G. Paterson, C. D. Ward, and R. A. Lamb. 1997. Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. *Virology* **237**:249–260.
- Horvath, C. M. 2000. STAT proteins and transcriptional responses to extracellular signals. *Trends Biochem. Sci.* **25**:496–502.
- Horvath, C. M., Z. Wen, and J. E. Darnell, Jr. 1995. A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. *Genes Dev.* **9**:984–994.
- Ito, Y., M. Tsurudome, H. Bando, H. Komada, and M. Nishio. 1989. Incomplete replication of human parainfluenza virus type 2 in mouse L929 cells. *Arch. Virol.* **108**:137–144.
- Keller, M. A., S. K. Murphy, and G. D. Parks. 2001. RNA replication from the simian virus 5 antigenomic promoter requires three sequence-dependent elements separated by sequence-independent spacer regions. *J. Virol.* **75**:3993–3998.
- Kubota, T., N. Yokosawa, S. Yokota, and N. Fujii. 2001. C terminal CYS-RICH region of mumps virus structural V protein correlates with block of interferon alpha and gamma signal transduction pathway through decrease of STAT 1-alpha. *Biochem. Biophys. Res. Commun.* **283**:255–259.
- McKendry, R., J. John, D. Flavell, M. Muller, I. M. Kerr, and G. R. Stark. 1991. High-frequency mutagenesis of human cells and characterization of a mutant unresponsive to both alpha and gamma interferons. *Proc. Natl. Acad. Sci. USA* **88**:11455–11459.
- Morgenstern, J. P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18**:3587–3596.
- Muller, M., C. Laxton, J. Briscoe, C. Schindler, T. Improta, J. E. Darnell, Jr., G. R. Stark, and I. M. Kerr. 1993. Complementation of a mutant cell line: central role of the 91-kDa polypeptide of ISGF3 in the interferon- α and - γ signal transduction pathway. *EMBO J.* **12**:4221–4228.
- Naniche, D., A. Yeh, D. Eto, M. Manchester, R. M. Friedman, and M. B. Oldstone. 2000. Evasion of host defenses by measles virus: wild-type measles virus infection interferes with induction of alpha/beta interferon production. *J. Virol.* **74**:7478–7484.
- Nishio, M., M. Tsurudome, M. Ito, M. Kawano, H. Komada, and Y. Ito. 2001. High resistance of human parainfluenza type 2 virus protein-expressing cells to the antiviral and anti-cell proliferative activities of alpha/beta interferons: cysteine-rich V-specific domain is required for high resistance to the interferons. *J. Virol.* **75**:9165–9176.
- Parisien, J.-P., J. F. Lau, J. J. Rodriguez, B. M. Sullivan, A. Moscona, G. D. Parks, R. A. Lamb, and C. M. Horvath. 2001. The V protein of human parainfluenza virus 2 antagonizes type I interferon responses by destabilizing signal transducer and activator of transcription 2. *Virology* **283**:230–239.
- Parisien, J.-P., J. F. Lau, J. J. Rodriguez, C. M. Ulane, and C. M. Horvath. 2002. Selective STAT protein degradation induced by paramyxoviruses requires both STAT1 and STAT2, but is independent of alpha/beta interferon signal transduction. *J. Virol.* **76**:4190–4198.
- Park, C., M. J. Lecomte, and C. Schindler. 1999. Murine Stat2 is uncharacteristically divergent. *Nucleic Acids Res.* **27**:4191–4199.
- Paterson, R. G., G. P. Leser, M. A. Shaughnessy, and R. A. Lamb. 1995. The paramyxovirus SV5 V protein binds two atoms of zinc and is a structural component of virions. *Virology* **208**:121–131.
- Paulson, M., S. Pisharody, L. Pan, S. Guadagno, A. L. Mui, and D. E. Levy. 1999. Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. *J. Biol. Chem.* **274**:25343–25349.
- Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh. 2000. Viral subversion of the immune system. *Annu. Rev. Immunol.* **18**:861–926.
- Wang, L., B. H. Harcourt, M. Yu, A. Tamin, P. A. Rota, W. J. Bellini, and B. T. Eaton. 2001. Molecular biology of Hendra and Nipah viruses. *Microbes Infect.* **3**:279–287.
- Young, D. F., N. Chatziandreou, B. He, S. Goodbourn, R. A. Lamb, and R. E. Randall. 2001. Single amino acid substitution in the V protein of simian virus 5 differentiates its ability to block interferon signaling in human and murine cells. *J. Virol.* **75**:3363–3370.
- Young, D. F., L. Didcock, and R. E. Randall. 1997. Isolation of highly fusogenic variants of simian virus 5 from persistently infected cells that produce and respond to interferon. *J. Virol.* **71**:9333–9342.