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All Four Sendai Virus C Proteins Bind Stat1, but only the Larger Forms also Induce Its Mono-ubiquitination and Degradation

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Sendai virus infection strongly induces interferon (IFN) production and has recently been shown to interdict the subsequent IFN signaling through the Jak/Stat pathway. This anti-IFN activity of SeV is due to its "C" proteins, a nested set of four proteins (C′, C, Y1, Y2) that carry out a nested set of functions in countering the innate immune response. We previously reported that all four C proteins interact with Stat1 to prevent IFN signaling through the Jak/Stat pathway. Nevertheless, only the longer C proteins reduced Stat1 levels and prevented IFN from inducing an antiviral (VSV) state, or apoptosis, in IFN-competent murine cells. Here, we investigate the mechanism by which the various C proteins differentially affect the host antiviral defenses. All four C proteins were found to physically associate with Stat1 during cell culture infections, and *in vitro* in the absence of other viral gene products (as evidenced by co-immunoprecipitation). In addition, the inability of a null mutant (C^{F170S}) to bind Stat1 suggests that this interaction is physiologically relevant. We have also shown that the proteasomal inhibitor MG132 can prevent the C protein-induced dismantling of the antiviral (VSV) state in murine cells; thus, the turnover of Stat1 correlates with the C protein-mediated counteraction of the antiviral (VSV) state. The C protein-induced instability of Stat1 was accompanied by a clear increase in the level of mono-ubiquinated Stat1, an unexpected hallmark of protein degradation. Finally, we show that a rSeV with mutant C proteins but wild-type Y proteins (CΔ10–15, that does not counteract the endogenous antiviral (VSV) state of MEFs even though their C proteins bind Stat1 and prevent its activity) is also unable to decrease bulk Stat1 levels or to increase the level of ubiquinated Stat1.

INTRODUCTION

To establish infections *in vivo*, viruses must replicate in the face of powerful immune defenses, including those mediated by interferons (IFNs) that are involved in both innate and adaptive immune responses. Type I (IFNα/β) and type II (IFNγ) IFNs bind to independent cell surface receptors and activate distinct but related signal transduction pathways, culminating in the activation of an overlapping set of IFN-stimulated genes (ISGs). ISGs encode a variety of intracellular enzymes, including protein kinase R and 2′–5′ oligoadenylate synthetase, through which IFNs induce an antiviral state within cells (Stark *et al.,* 1998). IFNs also downregulate the cell cycle (Thomas *et al.,* 1998) and may induce a pro-apoptotic state in cells (Chin *et al.,* 1997) and directly and indirectly enhance the adaptive immune response in a variety of ways (Pamer and Cresswell, 1998). Given the importance of the IFN system as the first line of defense against virus infections, many viruses have evolved specific mechanisms to overcome some of the IFN-induced responses (reviewed in Goodbourn *et al.,* 2000). The speed and efficiency by which viruses circumvent the innate immune response is critical in determining their host range and pathogenicity (Ploegh, 1998). Sendai virus (SeV), a model Paramyxovirus and respiratory pathogen of lab mice, has long been known for its ability to induce IFN production. There is now ample evidence that most, if not all, paramyxoviruses encode genes that interdict IFN signaling through the Jak/Stat pathway and that this is sometimes linked with a reduction of Stat1 or Stat2 levels as well (Didcock *et al.,* 1999a,b;Garcin *et al.,* 1999, 2000, 2001; Gotoh *et al.,* 1999; Komatsu *et al.,* 2000; Young *et al.,* 2000; Kato *et al.,* 2001; Parisien *et al.,* 2001; Nishio *et al.,* 2001). For SeV (genus *Respirovirus*), this anti-IFN function is coded by the C gene (Garcin *et al.,* 1997; Gotoh *et al.,* 1999), whereas for SV5 and hPIV2 (genus *Rubulavirus*), this function is carried out by the V gene (Didcock *et al.,* 1999b; Parisien *et al.,* 2001; Nishio *et al.,* 2001).

Paramyxovirus C and V genes are found within the viral P genes that are remarkable for the complexity of their genetic organization and expression (Lamb and Kolakofsky, 2001). The P gene, named for the phosphoprotein that is an essential component of the P/L viral RNA polymerase (vRNAP), contains additional ORFs that overlap the beginning and the middle of the P protein ORF (the C and V ORFs, respectively) (Fig. 1). The overlapping C and V ORFs are accessed by a variety of unusual mechanisms of ribosomal choice (Curran *et al.,*

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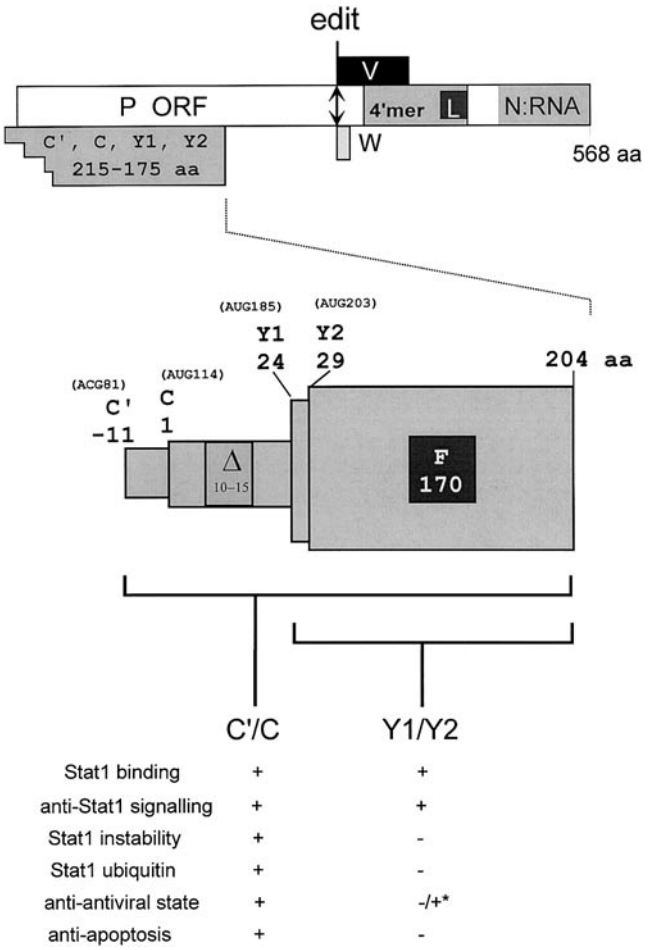

FIG. 1. Open reading frame organization and expression of the SeV P gene. The four ORFs expressed as protein (P, C, V, and W) are shown above as horizontal boxes, drawn roughly to scale. Several domains of the P protein, notably its tetramerization domain including the L-protein binding site, and that which binds to the N:RNA nucleocapsdid, are indicated. The double-headed arrow shows the site (codon 317) where G residues are added cotranscriptionally, to access the V and W ORFs. The crenellated beginning of the C ORF box above indicates the four independently initiated "C" proteins, which all terminate at codon 205. This feature is shown below in more detail and in an alternate fashion. For the C ORF, the AUG^{114}-initiated C protein is the point of reference for historical reasons; hence, the ACG^{81}-initiated C′ protein begins at codon −11. The numbers at the starts of the four C proteins below refer to codon position relative to AUG^{114}. The 18-nt deletion which eliminates codons 10–15 of C is highlighted. A summary of the various properties of the Y1/Y2 vs the longer C′/C proteins is given below. For explanation of +, −, −/+, and *, see Discussion.

1998) and mRNA editing (Thomas *et al.,* 1988; Vidal *et al.,* 1990; Hausmann *et al.,* 1999), respectively. For SeV, the overlapping ORFs are also referred to as "accessory" genes (Cadd *et al.,*1996), because in each case there is at least one virus within the subfamily *Paramyxovirinae* that does not express the V or C genes. For Sendai virus (also referred to as murine parainfluenza virus type 1 (mPIV1)), and human PIV1, a nested set of two longer "C" proteins [C'^{ACG81} and C^{AUG114}] and two shorter C proteins [$Y1^{AUG183}$ and $Y2^{AUG201}$] are expressed (Fig. 1). The two longer C proteins (and the P protein) are initiated by scanning ribosomes, whereas the two shorter C proteins are initiated by a ribosomal shunt (Latorre *et al.,* 1998).

The nested set of C proteins appears to carry out a nested set of functions in countering the innate immune response. The shorter Y proteins are almost as efficient as C′ and C in preventing IFN signaling to pISRE-luc, or the critical formation of pY701-Stat1 required for this IFN signaling (Garcin *et al.,* 2000, 2001; Kato *et al.,* 2001). The interdiction of IFN signaling through the Jak/Stat pathway is therefore one function that can be carried out by all of the C proteins. In contrast, several properties of this gene (its ability to reduce Stat1 levels, to prevent the infection from inducing an antiviral, vesicular stomatitis virus (VSV) state, or programmed cell death (PCD)), are associated with only the longer C proteins (Garcin *et al.,* 2001). These latter three properties may be linked. The IFN-induced antiviral (VSV) state and PCD are complex cellular responses that integrate signals from different pathways, and Stat1 is prominent in both cellular responses. Eliminating Stat1 could act to prevent PCD (Chin *et al.,* 1997; Kumar *et al.,* 1997; Schindler, 1998), and there may be elements of IFN action that require Stat1 independent of its phosphorylation status (Chatterjee-Kishore *et al.,* 2000). We have continued to investigate the mechanisms of action of the various C proteins. Here we report that all four C proteins bind Stat1, but that only the larger forms of C induce the ubiquitination of Stat1 and its degradation. Unexpectedly, only a single ubiquitin moiety appears to be added to Stat1.

RESULTS

C forms a complex with Stat1 both in infected cells and *in vitro*

As mentioned above, there is ample evidence that the SeV C proteins function to interdict IFN signaling, and they sometimes induce Stat1 instability as well. The simplest explanation for these results is that C and Stat1 directly interact with each other. However, a direct interaction between any paramyxovirus protein and Stat1 had not been reported when the experiments below were carried out. While this article was being prepared, Takeuchi *et al.* (2001) reported that a high molecular weight (HeLa cell) complex containing Stat1 further increases its M_{app} upon SeV infection, apparently due to the presence of the C proteins. They also reported that C and Stat1 could be co-immunoprecipitated from these complexes. The results below confirm and extend these studies, using SeV with mutant C genes.

To determine whether C and Stat1 were stably associated during SeV infection, an NIH 3T3-derived mouse embryo fibroblast (MEF) cell line (Yang *et al.,* 1995) and the IFN-competent murine BF cell line (Didcock *et al.,* 1999a) were infected with either wild-type SeV^{M}, SeV^{M}-C^{F170S}, or SeV^{Z}-C[Δ10–15]. The C^{F170S} mutation strongly

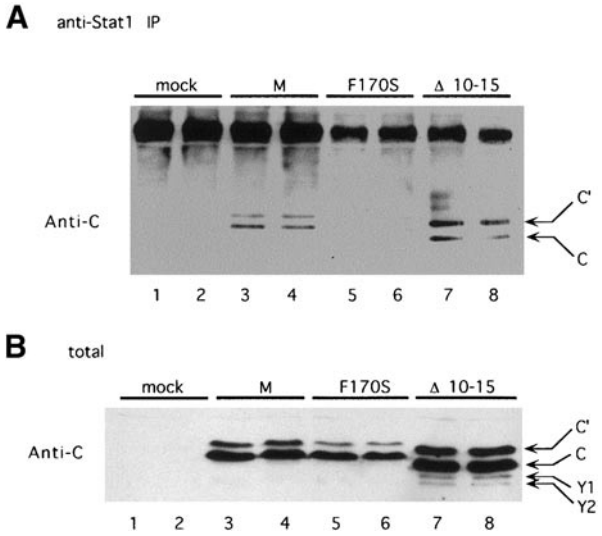

FIG. 2. SeV C protein/Stat1 complex formation during BF cell infection. Duplicate, parallel cultures of BF cells were infected with 20 PFU/cell of wild-type SeV^{M}, SeV^{M}-C^{F170S}, or SeV^{Z}-CΔ10–15, or mock infected. Whole-cell extracts were prepared at 24 hpi, and equivalent amounts of extract (normalized for total protein by Bradford, ca. 3×10^{6} cells) were immunoprecipitated with anti-Stat1 and then Western blotted with anti-C. Total extracts were also directly Western blotted with anti-C (B). The various C proteins are indicated on the right. The C proteins of the M and Z strains migrate slightly differently; the CΔ10–15 mutant also overexpresses C′ relative to SeV^{Z}-wt.

reduces all C gene activities, whereas the C[Δ10–15] mutation eliminates only some of these activities (Garcin *et al.,* 2001). Whole-cell extracts of infected cultures were prepared at 24 hpi, and either immunoprecipitated with anti-Stat1 followed by Western blotting with anti-C or vice-versa. In BF cells, which contain undetectable levels of Stat1 and where all three SeV replicate well (see relative C protein levels, Fig. 2B), the anti-Stat1 precipitate from the SeV^{M} [wt] and SeV^{Z}-C[Δ10–15] infections clearly contained C′ and C (Fig. 2A, lanes 3, 4, 7, and 8), whereas no C proteins could be detected in the SeV^{M}-C^{F170S} precipitate (Fig. 2A, lanes 5 and 6). The MEF cell line contains considerably more endogenous Stat1 than the BF cells, but the more highly debilitated viruses such as SeV^{M}-C^{F170S} do not grow in these cells. In MEFs, however, there was clear evidence of a C/Stat1 complex in the SeV^{M} and SeV^{Z}-C[Δ10–15] infections when either antisera was used for precipitation (Figs. 3A and 3B). There is thus good evidence that the SeV C protein(s) and Stat1 are stably associated during infection of murine cells (Figs. 2 and 3) and that F170 appears to be essential for this interaction (Fig. 2).

To determine whether C/Stat1 complex formation would occur *in vitro*, extracts of uninfected MEFs were combined with those of BSR T7 cells that had been transfected with plasmids expressing various C proteins. These included GFP-tagged C and C^{F170S} proteins, with the tag fused to either end of C^{AUG114} (GFP-C and C-GFP). The GFP tag not only adds a new antibody combining site(s) to C, it may also increase the half-life of the C proteins, as significantly higher levels of GFP-C than untagged C are attained in the transfection (not shown). The four GFP fusion proteins were first expressed in BF cells and examined for their ability to prevent IFN signaling to cotransfected pISRE-luc (Didcock *et al.,* 1999a). GFP-C was as active as untagged C (on a molar basis) in reducing luciferase levels, and approximately fivefold more active than C-GFP, whereas both C^{F170S} fusion proteins were inactive (data not shown). The four GFP fusion proteins (and GFP alone) were then expressed in BSR-T7 cells, and extracts containing comparable levels of each protein (Fig. 4B) were examined for their ability to form a complex with Stat1 by co-immunoprecipitation (Fig. 4). Increasing amounts of GFP-C extract added to a constant amount of MEF extract resulted in the recovery of increasing amounts of Stat1 after precipitation with anti-C (Fig. 4D). More than 20% of the input Stat1 was recovered at the highest amounts of GFP-C extract used. This GFP-C/Stat1 complex forms within 30 min at 20°C in the absence of added nucleotides and in the presence of

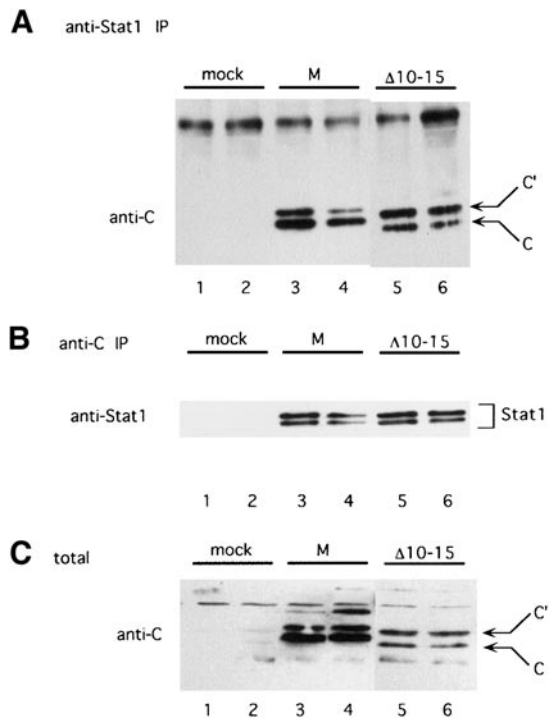

FIG. 3. SeV C protein/Stat1 complex formation during infection of MEF cells. Parallel cultures of MEF cells were infected with 20 PFU/cell of wild-type SeV^{M} or SeV^{Z}-CΔ10–15, or mock-infected (SeV^{M}-C^{F170S} does not grow in MEFs). Whole-cell extracts were prepared at 24 hpi, and equivalent amounts of extract (normalized for total protein, ca. 3×10^{6} cells) were immunoprecipitated with anti-Stat1, and then Western blotted with anti-C (A). Alternatively, equivalent amounts of extract were immunoprecipitated with anti-C, and then Western blotted with anti-Stat1 (B). Total extracts were also directly Western blotted with anti-C (C). The various C and Stat1 proteins are indicated on the right. The C proteins of the M and Z strains migrate slightly differently; the CΔ10–15 mutant also overexpresses C′ relative to SeV^{Z}-wt.

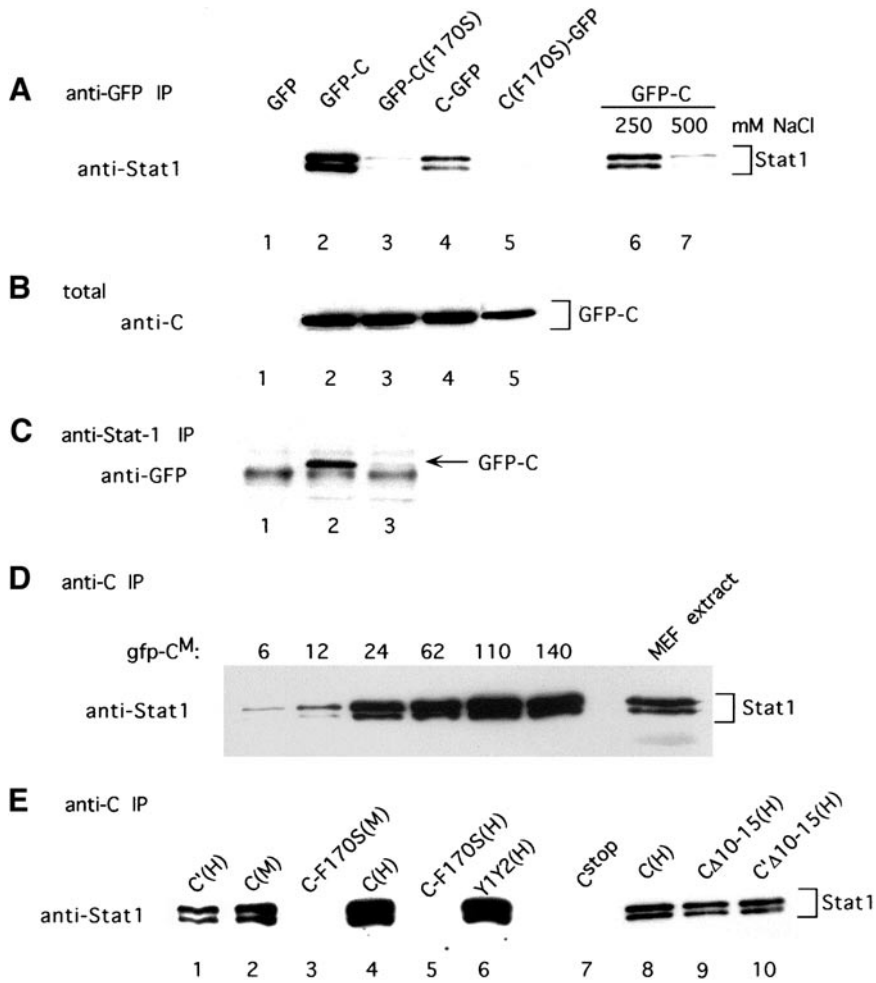

FIG. 4. SeV C protein/Stat1 complex formation *in vitro*. Equivalent amounts of total cell extracts (normalized for total protein) of uninfected MEF cultures and BSR T7 cultures transfected with various GFP-C expression constructs (or GFP alone as a negative control) were combined for 30 min at 20°C, and then (A) precipitated with anti-GFP and Western blotted with anti-Stat1; (B) directly Western blotted with anti-C; (C) precipitated with anti-Stat1 and Western blotted with anti-GFP. (D) Increasing amounts of GFP-C^M-transfected BSR T7 extract was combined with a constant amount of MEF extract (ca. 3×10^6 cells), precipitated with anti-C, and Western blotted with anti-Stat1. One-twentieth of the MEF extract was directly blotted for reference (right-most lane). (E) Extracts of BSR T7 cells transfected with various (non-GFP fusion) C protein expression plasmids (as indicated above) were combined with a constant amount of MEF extract, precipitated with anti-C, and Western blotted with anti-Stat1.

EDTA and thus does not appear to require energy. The complex is stable to washing three times in normal salt conditions (150 mM NaCl) and largely survives washing with 250 mM NaCl, but the complex is mostly lost upon washing with 500 mM NaCl (Fig. 4A).

When BSR T7 extracts from cells transfected with GFP alone, or either of the GFP-C^{F170S} constructs, were combined with the MEF extract, anti-GFP precipitated little or no Stat1 (Fig. 4A, lanes 1, 3, and 5). In contrast, when comparable extracts from cells transfected with either of the GFP/C^{wt} constructs were combined with the MEF extract, C/Stat1 complex formation was clearly evident, and C-GFP appeared to be approximately fivefold less active in this respect than GFP-C (lanes 2 and 4). The ability of the four GFP fusion proteins to form a complex with Stat1 *in vitro* thus correlates nicely with their ability to prevent IFN signaling to cotransfected pISRE-luc. Specific C/Stat1 complex formation was also detectable when anti-Stat1 was used for precipitation and GFP-C was present, but not when GFP alone or GFP-C^{F170S} was present (Fig. 4C). Untagged C′, C, CΔ10–15 or C′Δ10–15, and Y1/Y2 (from the H and M strain backgrounds), expressed in BSR T7 cells, but not C^{F170S}, also formed a complex with Stat1 *in vitro*, and this complex could be precipitated with either anti-Stat1 or anti-C (Fig. 4E and not shown).

In summary, there is evidence that C and Stat1 are physically associated in SeV infected murine cells and that this complex also forms *in vitro* in the absence SeV infection (i.e., does not require other viral gene products). The critical phe[170] required for all C protein activity, including preventing pY701-Stat1 formation and IFN signaling to pISRE-luc, is also required for this association. In contrast, this association does not require the highly

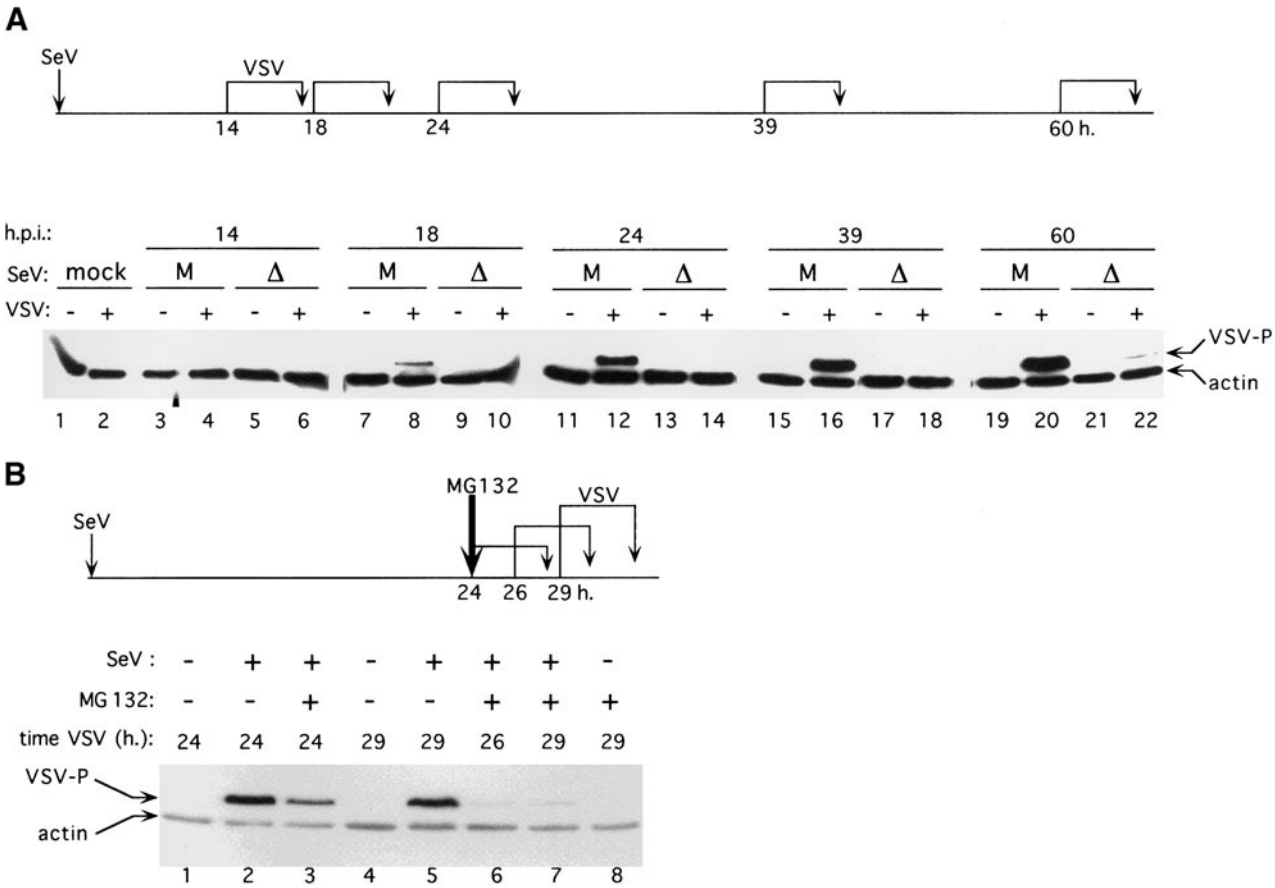

FIG. 5. Dismantling the endogenous antiviral (VSV) state of MEFs by SeV infection is an ongoing process that can be reversed by MG132 treatment. Parallel cultures of MEFs were infected with 20 PFU/cell of either SeV^M (M) or SeV^Z-CΔ10–15 (Δ), or mock infected. Some cultures were also superinfected with 50 PFU/cell of VSV at the various times post-SeV infection indicated. Cytoplasmic extracts were prepared 4 h post-VSV infection, and equivalent amounts were immunoblotted with anti-VSV P, and anti-actin as a loading control (A). Other cultures were infected with 20 PFU/cell of SeV^M and treated with 10 μM of MG132 at 24 hpi (see Materials and Methods). Some of the cultures were then superinfected with VSV at 24, 26, or 29 h post-SeV infection. Cytoplasmic extracts were prepared and examined as above (B).

basic residues [10]**lys**-leu-**arg**-gly-**arg**-**arg**[15] of C^{AUG114}. Figures 2, 3, and 4 thus provide physical and genetic evidence that the C proteins function via their direct interaction with Stat1.

Stat1 instability and the antiviral (VSV) state

If the C proteins function by physically interacting with Stat1 to inhibit its activity, thereby preventing IFN from inducing an antiviral (VSV) state, then why bother to induce Stat1 instability as well? Is this Stat1 instability physiologically relevant to the infection of murine cells or simply the consequence of some other cellular reaction to the infection unrelated to the IFN-induced antiviral (VSV) state? We previously reported that SeV-wt infection of MEFs led to the dismantling of the endogenous antiviral (VSV) state of this cell line and that this was associated with a reduction of Stat1 levels (Garcin *et al.,* 2000, 2001; see also Fig. 6). As the turnover of Stat1 is thought to occur via the proteasome (Kim and Maniatis, 1996; Didcock *et al.,* 1999b), we examined whether treatment of SeV-infected MEFs (now susceptible to VSV infection) with the proteasomal inhibitor MG132 would reestablish the antiviral (VSV) state. If so, this would indicate that the Stat1 instability was not an epiphenomenon, but associated with the viral program to counteract the IFN-mediated antiviral (VSV) state.

We first determined the kinetics with which SeV^M reverses this endogenous antiviral (VSV) state of MEFs and found that this reversal is a relatively slow process. As shown in Fig. 5, which examines VSV P protein levels 4 hpi, a minimum of 18 h of SeV^M infection is required before VSV replication becomes detectable (Fig. 5A, lane 8). The relative ability to VSV to replicate in these cells then continues to increase with time of SeV^M infection until 60 hpi (Fig. 5A, lanes 12, 16, and 20, note relative intensities of VSV P and actin bands). Neither SeV-CΔ10–15 infection (Fig. 5A) nor SeV-[C′/C-minus] infection (data not shown), in contrast, was able to reverse the endogenous antiviral (VSV) state of the MEFs (Fig. 5A, lanes 10, 14, 18, and 22) and thus serve as negative

controls. MEFs were therefore infected with SeV^M for 24 h, a time sufficient to permit a clear level of VSV replication. The cultures were then treated with MG132 or lactacystin and superinfected with VSV at various times following treatment (MG132 or lactacystin treatment by itself has no effect on VSV growth (not shown)). Both drugs produced similar effects and only that of MG132 is shown. MG132 treatment started coincident with the 4-h VSV superinfection clearly reduced the level of VSV replication (lane 3 vs lane 2, Fig. 5B), and a further 2 h of pretreatment was sufficient to almost completely reverse the effects of 24 h of SeV^M infection (lane 6). Three conclusions can be drawn from Fig. 5, as follows: (1) The reversal of the endogenous antiviral (VSV) state of MEFs by SeV infection requires proteasome activity (as defined by MG132 and lactacystin sensitivity); (2) residues ^{10}**lys**-leu-**arg**-gly-**arg**-**arg**^{15} of the C protein are essential for this virus function; (3) C-induced instability of Stat1 is not an epiphenomenon, but correlates with the viral program to counteract the antiviral (VSV) state of MEFs. However, we cannot conclude from this data that C-induced Stat1 instability is the causative agent responsible for this effect. It is entirely possible that Stat1 is not the only protein important in the innate immune response whose abundance (or activity) is altered by the SeV C proteins. Any or all of these putative proteins could also play a role.

Only the longer C proteins target Stat1 for ubiquitination and degradation

Most proteins are marked for proteasomal degradation by the attachment of ubiquitin(s) to lysine residues of the target. Once multiply ubiquitinated, the protein is quickly degraded to short peptides by the 26S proteasome. We therefore examined whether SeV^M or SeV^Z-CΔ10–15 infection of MEFs would differentially affect the level of ubiquitinated Stat1. Whole-cell extracts of MEFs infected for 28 h were precipitated with either antiubiquitin (Fig. 6A) or anti-C (Fig. 6B), and the levels (and electrophoretic mobilities) of Stat1 recovered were examined by immunoblotting (lanes 3, 4, 5, and 6, Figs. 6A and 6B) and compared to those in the unfractionated extract (lanes 1, 2, 7, and 8, 6A and 6B). As shown in Fig. 6A, uninfected MEFs contain a small but clearly detectable level of ubiquitinated Stat1 that only becomes visible following precipitation with anti-ub (lane 3), and which migrates in the gel slightly slower than the bulk of unmodified Stat1α (top band, lanes 1, 2, 7, and 8), consistent with the addition of a single 76-aa ubiquitin. (Although there is only a single ubiquitinated Stat1 band visible in Fig. 6A, a double band of Stat1α and Stat1β is visible in other experiments.) Treatment of the infected cultures with MG132 at 24 hpi clearly increases the level of ubiquitinated Stat1 following precipitation with anti-ub (lane 4), as expected. SeV^M infection also led to a clear

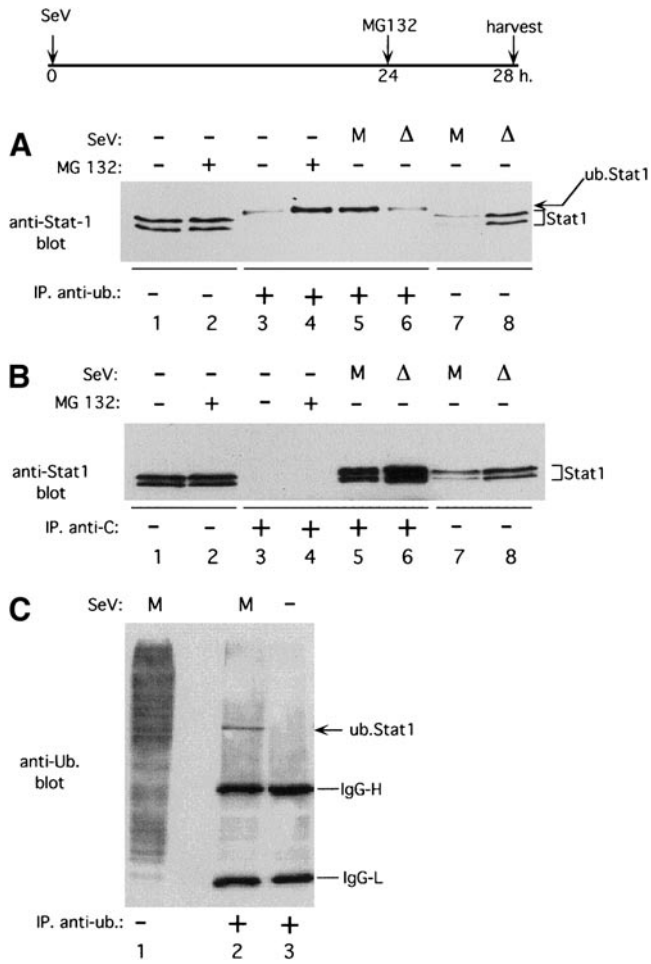

FIG. 6. SeV infection of MEFs induces Stat1 ubiquitination. Parallel cultures of MEFs were infected with 20 PFU/cell of either SeV^M (M) or SeV^Z-CΔ10–15 (Δ), or mock infected. MG132 (see Materials and Methods) was added to some cultures at 24 hpi as indicated. Total cell extracts were prepared at 28 hpi, and equivalent amounts were either precipitated with anti-ubiquitin and Western blotted with anti-Stat1 (lanes 3, 4, 5, and 6, A), or precipitated with anti-C and Western blotted with anti-Stat1 (lanes 3, 4, 5, and 6, B). Lanes 1, 2, 7, and 8 show the direct immunoblotting of one-tenth of the sample used for immunoprecipitation (lanes 3, 4, 5, and 6, respectively). (C) One-fortieth of the SeV^M-infected MEF cell extract was directly immunoblotted with anti-ubiquitin (lane 1), or one-fourth was first precipitated with anti-ubiquitin and then immunoblotted with anti-ubiquitin (lane 2). Uninfected MEF extracts were also examined (lane 3). The ubiquitinated form of Stat1 and the IgG chains are indicated on the right.

increase in the level of ubiquitinated Stat1 (lane 5 vs lane 3), concomitant with a decrease in the level of bulk Stat1 (lane 7 vs lane 1). SeV-C[Δ10–15] infection, in contrast, did not increase the level of ubiquitinated Stat1 (lane 6 vs lane 3), nor did it decrease the level of bulk Stat1 (lane 8 vs lane 1). The inability of SeV-C[Δ10–15] infection to induce Stat1 ubiquitination and turnover was not due to lack of C/Stat1 complex formation, as there was, in fact, more C/Stat1 complex present in SeV-C[Δ10–15] than in SeV^M infections (lanes 5 and 6, Fig. 6B), reflecting the bulk levels of Stat1 present (lanes 7 and 8).

The more slowly migrating form of Stat1 seen following precipitation with anti-ub (lanes 3, 4, 5, and 6, Fig. 6A) is absent in the bulk Stat1 (lanes 1, 2, 7, and 8) and thus appears to be a very minor component of bulk Stat1. To further determine whether this slower form is indeed ubiquitinated, MEF extracts were immunoblotted with anti-ub before and after precipitation with anti-ub. Anti-ub recognizes a large number of proteins of all sizes in MEF extracts prior to immunoprecipitation, reflecting the plethora of ubiquitinated proteins intracellularly (lane 1, Fig. 6C). These bands increase in intensity upon MG132 treatment, but are unaffected by SeV^{M} infection (data not shown). However, when the anti-ub precipitate of the SeV^{M}-infected extract is immunoblotted, a very clear band can be seen whose mobility (relative to colored molecular weight standards) is the same as ub-Stat1 in panel A (Lane 2, Fig. 6C). Moreover, this band is much reduced in uninfected extracts (lane 3, however, it can be seen in some cases). Thus, unexpectedly, the anti-ub antibody appears to selectively precipitate this slower form of Stat1 from cell extracts. The same results of Fig. 6A are obtained with a rabbit polyclonal antiubiquitin serum, and in 2C4 human cells as well as MEFs (data not shown). Thus, this slower form of Stat1 does appear to be ubiquitinated. Whether the slower form of Stat1 is also modified otherwise, e.g., by phosphorylation, remains to be determined. In summary, our results show that Stat1 ubiquitination and turnover during SeV infection, similar to counteracting the antiviral (VSV) state and PCD, appears to require the longer C proteins.

DISCUSSION

More than 50 viral genes have now been identified which act as immune modulators, and several act directly to subvert the innate immune response (Ploegh, 1998). In many cases, the evasive measures specified by the viral genomes remain to be linked directly to the virulence of the pathogen in question, for lack of a relevant animal model. SeV, however, is a well-studied respiratory pathogen of lab mice (Itoh *et al.,* 1990). The SeV C^{F170S} mutation, moreover, was uncovered as one of two mutations (along with L^{E2050A}) in one of the most virulent virus strains reported to date (SeV^{M}; LD_{50} of 40) that became avirulent (SeV^{MVC}; LD_{50} of >800,000) on adaptation for growth in monkey kidney cells (Itoh *et al.,* 1997). As far as we know, the C^{F170S} mutation may account for the entire 5 logs of difference in LD_{50} (Garcin *et al.,* 1997). The SeV C gene is thus clearly required for virulence in a relevant animal model, and the precise manner in which this gene functions may prove revealing.

This paper extends previous studies by providing evidence that the C proteins physically associate with Stat1 during infection (Figs. 2 and 3), and *in vitro* in the absence of other viral gene products (Fig. 4). In addition, the inability of the C^{F170S} mutant protein to interact with Stat1 suggests this interaction is physiologically relevant. The C protein also induces instability of Stat1in MEFs (Garcin *et al.,* 2000), and we have shown that this instability is accompanied by an increase in the level of ubiquitinated Stat1, an expected hallmark of protein degradation. Moreover, the proteasomal inhibitor MG132 prevents Stat1 turnover in these cells (Fig. 6). The SeV C protein induced instability of Stat1, similar to that induced by the SV5 V protein (Didcock *et al.,* 1999b), thus appears to be due to proteasomal degradation (for a caveat, see below). We have also found that MG132 and lactacystin prevent the C protein induced dismantling of the antiviral (VSV) state in MEFs (Fig. 5). The turnover of Stat1 thus correlates with C protein induced dismantling of the antiviral (VSV) state in these cells. In contrast to SeV-wt, SeV-CΔ10–15 is unable to decrease bulk Stat1 levels or increase the level of ubiquitinated Stat1, nor to counteract the endogenous antiviral (VSV) state of MEFs. Since only the longer C proteins of the CΔ10–15 gene contain this deletion, the shorter Y proteins are apparently unable to destabilize Stat1. rSeV-[C′/C-minus], which express only the Y proteins but at the same level as the longer C proteins in SeV-wt infections (Latorre *et al.,* 1998), also behaves similar to rSeV-CΔ10–15 in this respect (data not shown). One cannot exclude that the effects of MG132 and lactacystin may be due to cellular factors other than Stat1, whose levels are also affected by these drugs. Moreover, it is possible that Stat1 is not the only protein important in the innate immune response whose abundance (or activity) is altered by the SeV C proteins, as C and Stat1 can be found in very high molecular weight complexes (Takeuchi *et al.,* 2001). Any or all of these putative proteins could also play a role. Nevertheless, the degradation of Stat1 can account, at least in part, for the ability of the C gene to prevent the induction of the antiviral (VSV) state, as well as PCD.

HeLa cell lines expressing the various SeV C proteins have recently been reported, in which Y1 or Y2 expression alone was able to prevent IFN from inducing an antiviral (VSV) state (Kato *et al.,* 2001). Y proteins constitutively expressed at high levels (in the absence of IFN stimulation) are apparently sufficient to counteract subsequent IFN treatment. However, when the Y proteins are expressed during a natural SeV-CΔ10–15 infection of murine BF cells, they are unable to prevent IFN from inducing an effective antiviral (VSV) state, in contrast to SeV-wt (Garcin *et al.*, 2001). These are clearly two different tests of Y protein action, in two cell lines. High levels of Y are present long before IFN stimulation in the HeLa cell lines, whereas in the murine cell experiments IFN is added long before there are high levels of Y intracellularly due to viral genome amplification. Without a head start, the Y proteins do not appear to be as effective as the longer C proteins in counteracting IFN action. Consistent with this, viruses that can only express wild-type Y proteins such as CΔ10–15 or C′/C-minus are avirulent

in mice (Latorre *et al.,* 1998; Garcin *et al.,* 2001), even though they can be adapted to grow relatively well in some cell lines. rSeV-[C′/C-minus], moreover, expresses the Y proteins at the same level as the longer C proteins in SeV-wt infections (Latorre *et al.,* 1998). However, there is also evidence that the Y proteins expressed in virus infection are active to some degree, as SeV-CΔ10–15 (wild-type Y1/Y2) grows relatively well in MEFs, whereas SeV-C^{F170S} (null mutant) infections are highly restricted.

Since MG132 inhibits SeV-induced Stat1 turnover in MEFs, we expected this drug (and SeV infection) to increase multiply-ubiquitinated forms of Stat1, by which the 19S cap of the 26S proteasome identifies substrates for rapid degradation (Hershko and Ciechanover, 1998). However, we could only detect mono-ubiquitinated Stat1 in many experiments, and in 2C4 human cells as well as MEFs (data not shown). This may be relevant, as mono-ubiquitinated proteins are the preferred substrates for the vacuolar pathway of protein degradation (that is distinct from the proteasome) (Terrell *et al.,* 1998; Roth and Davis, 2000; Hatakeyama *et al.,* 2001), and the ubiquitin-ligase that modifies Stat1 has not been reported. While Kim and Maniatis (1996) showed poly-ubiquitin attachment to Stat1 in an overexpression system, the MG132-sensitive step they noted was later identified as probably that of receptor degradation (Haspel *et al.,* 1996). The current view is that Stat1 is normally inactivated by dephosphorylation and not proteolysis. The longer SeV C proteins and the rubulavirus V proteins may then act as connectors to a cellular ubiquitin ligase. Drugs such as MG132 and lactacystin can have very broad effects, and it is not inconceivable that they act indirectly to stabilize Stat1 that is nevertheless degraded by the vacuolar pathway. Alternatively, mono-ubiquitinated proteins may be degraded by the proteasome under certain conditions. Further experiments will be required to elucidate the SeV C protein-induced Stat1 degradation pathway and to determine how the N-terminal sequences of the longer C proteins induce Stat1 ubiquitination.

MATERIALS AND METHODS

Cells and viruses

Murine BF cells (cloned from a primary cell culture of a BALB/c mouse embryo) (Didcock *et al.,* 1999a), MEFs (Yang *et al.,* 1995), and BSR T7 cells (Buchholz *et al.,* 1999) were grown as monolayers in DMEM supplemented with 10% FBS. The generation of rSeV expressing alternate C (and P) proteins has been described elsewhere (Garcin *et al.,* 1995, 1997; Latorre *et al.*, 1998). All SeV stocks were grown in the allantoic cavity of 10-day-old embryonated chicken eggs. Virus present in the allantoic fluid was analyzed by SDS–PAGE and Coomassie blue staining after virus pelleting. Virus titers were determined by plaquing on LLC-MK2 cells. The VSV stock (Mudd-Summers) was grown in BHK cells. Virus released into the culture medium was clarified by centrifugation, and the titer was determined by plaque formation on LLC-MK2 cells.

Virus infections

SeV infections were performed at a multiplicity of infection (m.o.i.) of 20 in DMEM containing 10% fetal bovine serum. After an absorption period of 1–2 h, the inoculum was removed and replaced with fresh medium. For VSV infections, an m.o.i. of 50 was used. For proteasome inhibitor experiments, the medium contains 0.2% dimethyl sulfoxide (DMSO) supplemented or not with 10 μM MG132 (Sigma).

Co-immunoprecipitation

Cells were washed once with PBS and scraped into PBS, and whole-cell extracts were prepared with lysis buffer containing 0.5% NP-40, 50 mM Tris–Cl, pH 7.4, 150–250 mM NaCl, 10 mM EDTA, 3 μg/ml aprotinin, and 1 mM AEBSF. The mixture was sonicated for 5 s (Branson sonifier B12, position 3) and centrifuged at 13,000 *g* for 2 min. The supernatant was used for immunoprecipitation with a rabbit polyclonal antiserum to SeV C protein (1/300 dilution) (provided by Y. Nagai, Tokyo, Japan); a mouse monoclonal antibody to Stat1 C terminus (1/300 dilution) (Transduction Laboratories, S21120); a rabbit polyclonal antibody to GFP (1/300 dilution) (Clontech); and a mouse monoclonal antibody to ubiquitin (1/100 dilution) (P4D1, Santa-Cruz). For the *in vitro* binding assay, two-thirds of total MEF extracts (corresponding to 4×10^6 cells) were mixed with two-thirds of transfected BSR T7 cells (corresponding to 8×10^5cells) at 20°C for 30 min before immunoprecipitation.

Immunoblotting

Proteins were separated by SDS–PAGE and transferred to Immobilon-P membranes by semidry transfer. The primary antibodies used included the following: a rabbit polyclonal antiserum to VSV P protein (provided by J. Perrault and D. Summers); a rabbit polyclonal antiserum to SeV P protein isolated from an SDS gel (anti-P^{SDS}; L. Roux, Geneva, Switzerland); a rabbit polyclonal antiserum to SeV C protein; a mouse monoclonal antibody to Stat1 C terminus; a rabbit polyclonal antiserum to phospho-stat (Y701) (Upstate Biotechnology, 06-657); a rabbit polyclonal antiserum to actin (provided by G. Gabbiani, Geneva, Switzerland); a mouse monoclonal antibody to ubiquitin (P4D1, Santa-Cruz); and a rabbit polyclonal antibody to GFP. The secondary antibodies used were alkaline phosphatase conjugated goat antibodies specific for either rabbit or mouse immunoglobulin G (Bio-Rad). The immobilized proteins were detected by light-enhanced chemiluminescence (Bio-Rad).

Plasmids and transient transfections

Various SeV C genes were cloned into pTM1, which contains a T7 promoter and an EMCV IRES (B. Moss, National Institutes of Health). For transfections, 200,000 BSR T7 cells (that express T7 RNAP, Buchholz *et al.,* 1999) were plated in six-well plates 20 h before transfection with 0.5 μg DNA and 1.5 μl Fugene (Roche) according to the manufacturer's instructions. After 48 h, the cells were harvested and treated as described above.

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REFERENCES

Buchholz, U. J., Finke, S., and Conzelmann, K. K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J. Virol.* **73,** 251–259.

Cadd, T., Garcin, D., Tapparel, C., Itoh, M., Homma, M., Roux, L., Curran, J., and Kolakofsky, D. (1996). The Sendai paramyxovirus accessory C proteins inhibit viral genome amplification in a promoter-specific fashion. *J. Virol.* **70,** 5067–5074.

Chatterjee-Kishore, M., Wright, K. L., Ting, J. P., and Stark, G. R. (2000). How Stat1 mediates constitutive gene expression: A complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *EMBO J.* **19,** 4111–4122.

Chin, Y. E., Kitagawa, M., Kuida, K., Flavell, R. A., and Fu, X. Y. (1997). Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. *Mol. Cell. Biol.* **17,** 5328–5337.

Curran, J., Latorre, P., and Kolakofsky, D. (1998). Translational gymnastics on the Sendai virus P/C mRNA. *Semin. Virol.* **8,** 351–357.

Delenda, C., Hausmann, S., Garcin, D., and Kolakofsky, D. (1997). Normal cellular replication of Sendai without the *trans*-frame, nonstructural V protein. *Virology* **228,** 55–62.

Delenda, C., Taylor, G., Hausmann, S., Garcin, D., and Kolakofsky, D. (1998). Sendai viruses with altered P, V, and W protein expression. *Virology* **242,** 327–337.

Didcock, L., Young, D. F., Goodbourn, S., and Randall, R. E. (1999a). Sendai virus and simian virus 5 block activation of interferon-responsive genes: Importance for virus pathogenesis. *J. Virol.* **73,** 3125–3133.

Didcock, L., Young, D. F., Goodbourn, S., and Randall, R. E. (1999b). The V protein of simian virus 5 inhibits interferon signaling by targeting STAT1 for proteasome-mediated degradation. *J. Virol.* **73,** 9928–9933.

Garcin, D., Pelet, T., Calain, P., Roux, L., Curran, J., and Kolakofsky, D. (1995). A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: Generation of a novel copy-back nondefective interfering virus. *EMBO J.* **14,** 6087–6094.

Garcin, D., Itoh, M., and Kolakofsky, D. (1997). A point mutation in the Sendai virus accessory C proteins attenuates virulence for mice, but not virus growth in cell culture. *Virology* **238,** 424–431.

Garcin, D., Latorre, P., and Kolakofsky, D. (1999). Sendai virus C proteins counteract the interferon mediated induction of an antiviral state. *J. Virol.* **73,** 6559–6565.

Garcin, D., Curran, J., and Kolakofsky, D. (2000). Sendai virus C proteins must interact directly with cellular components to interfere with interferon action. *J. Virol.* **74,** 8823–8830.

Garcin, D., Curran, J., Itoh, M., and Kolakofsky, D. (2001). The longer and shorter forms of the SeV C proteins play different roles in modulating the cellular antiviral response. *J. Virol.* **75,** 6800–6807.

Goodbourn, S., Didcock, L., and Randall, R. E. (2000). Cell signaling, immune modulation, antiviral response and virus countermeasures. *J. Gen. Virol.* **81,** 2341–2364.

Gotoh, B., Takeuchi, K., Komatsu, T., Yokoo, J., Kimura, Y., Kurotani, A., Kato, A., and Nagai, Y. (1999). Knockout of the Sendai virus C gene eliminates the viral ability to prevent the interferon-alpha/beta-mediated responses. *FEBS Lett.* **459,** 205–210.

Haspel, R. L., Salditt-Georgieff, M., and Darnell, J. E., Jr. (1996). The rapid inactivation of nuclear tyrosine phosphorylated Stat1 depends upon a protein tyrosine phosphatase. *EMBO J.* **15,** 6262–6268.

Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K. I. (2001). U box proteins as a new family of ubiquitin-protein ligases. *J. Biol. Chem.* **276,** 33111–33120.

Hausmann, S., Garcin, D., Delenda, C., and Kolakofsky, D. (1999). The versatility of paramyxovirus RNA polymerase stuttering. *J. Virol.* **73,** 5568–5576.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* **67,** 425–479.

Itoh, M., Ming, T. D., Hayashi, T., Mochizuki, Y., and Homma, M. (1990). Pneumopathogenicity of a Sendai virus protease-activation mutant, TCs, which is sensitive to trypsin and chymotrypsin. *J. Virol.* **64,** 5660–5664.

Itoh, M., Isegawa, Y., Hotta, H., and Homma, M. (1997). Isolation of an avirulent mutant of Sendai virus with two amino acid mutations from a highly virulent field strain through adaptation to LLC-MK2 cells. *J. Gen. Virol.* **78,** 3207–3215.

Kato, A., Kiyotani, K., Sakai, Y. Yoshida, T., and Nagai, Y. (1997). The paramyxovirus Sendai virus V protein encodes a luxury function required for viral pathogenesis. *EMBO J.* **16,** 678–587.

Kato, A., Ohnishi, Y., Kohase, M., Saito, S., Tashiro, M., and Nagai, Y. (2001). Y2, the smallest of the Sendai virus C proteins, is fully capable of both counteracting the antiviral action of interferons and inhibiting viral RNA synthesis. *J. Virol.* **75,** 3802–3810.

Kim, T. K., and Maniatis, T. (1996). Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. *Science* **273,** 1717–1719.

Komatsu, T., Takeuchi, K., Yokoo, J., Tanaka, Y., and Gotoh, B. (2000). Sendai virus blocks alpha interferon signaling to signal transducers and activators of transcription. *J. Virol.* **74,** 2477–2480.

Kumar, A., Commane, M., Flickinger, T. W., Horvath, C. M., and Stark, G. R. (1997). Defective TNFα-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. *Science* **278,** 1630–1632.

Lamb, R. A., and Kolakofsky, D. (2001). Paramyxoviridae: The Viruses and Their Replication. *In* "Fields Virology" (D. M. Knipe, P. M. Howley, *et al.,* Eds.), 4th ed. Lippincott-Raven Publishers, Philadelphia, PA.

Latorre, P., Cadd, T., Itoh, M., Curran, J., and Kolakofsky, D. (1998). The various Sendai virus C proteins are not functionally equivalent and exert both positive and negative effects on viral RNA accumulation during the course of infection. *J. Virol.* **72,** 5984–5993.

Latorre, P., Kolakofsky, D., and Curran, J. (1998). Sendai virus Y proteins are initiated by a ribosomal shunt. *Mol. Cell. Biol.* **18,** 5021–5031.

Nishio, M., Tsurudome, M., Ito, M., Kawano, M., Komada, H., and Ito, Y. (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.

Pamer, E., and Cresswell, P. (1998). Mechanisms of MHC class I-restricted antigen processing. *Annu. Rev. Immunol.* **6,** 323–358.

Parisien, J. P., Lau, J. F., Rodriguez, J. J., Sullivan, B. M., Moscona, A., Parks, G. D., Lamb, R. A., and Horvath, C. M. (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.

Ploegh, H. L. (1998). Viral strategies of immune evasion. *Science* **280,** 248–253.

Roth, A. F., and Davis, N. G. (2000). Ubiquitination of the PEST-like endocytosis signal of the yeast a-factor receptor. *J. Biol. Chem.* **275,** 8143–8153.

Schindler, C. (1998). STATs as activators of apoptosis. *Trends Cell Biol.* **8,** 97–98.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998). How cells respond to interferons. *Annu. Rev. Biochem.* **67,** 227–264.

Takeuchi, K., Komatsu, T., Yokoo, J., Kato, A., Shioda, T., Nagai, Y., and Gotoh, B. (2001). Sendai virus C protein physically associates with Stat1. *Genes Cells* **6,** 545–557.

Terrell, J., Shih, S., Dunn, R., and Hicke, L. (1998). A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol. Cells* **2,** 193–202.

Thomas, S. M., Lamb, R. A., and Paterson, R. G. (1988). Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the parmyxovirus SV5. *Cell* **54,** 891–892.

Thomas, N. S., Pizzey, A. R., Tiwari, S., Williams, C. D., and Yang, J. (1998). p130, p107, and pRb are differentially regulated in proliferating cells and during cell cycle arrest by alpha-interferon. *J. Biol. Chem.* **273,** 23659–23667.

Vidal, S., Curran, J., and Kolakofsky, D. (1990). A stuttering model for paramyxovirus P mRNA editing. *EMBO J.* **9,** 2017–2022.

Yang, Y. L., Reis, L. F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A. Williams, B. R., Aguet, M., and Weissmann, C. (1995). Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J.* **14,** 6995–7006.

Young, D. F., Didcock, L., Goodbourn, S., and Randall, R. E. (2000). Paramyxoviridae use distinct virus-specific mechanisms to circumvent the interferon response. *Virology* **269,** 383–390.