Variants of the Paramyxovirus Simian virus 5 with accelerated or delayed viral gene expression activate proinflammatory cytokine synthesis

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Abstract

Our previous results have shown that the parainfluenza virus SV5 is a poor inducer of proinflammatory cytokines interleukin-8 (IL-8) and macrophage chemoattractant protein 1 (MCP-1). By contrast, an engineered P/V mutant rSV5-P/V-CPI− and a naturally-occurring variant WF-PIV (Wake Forest–Parainfluenza Virus) are both potent activators of IL-8 and MCP-1. In the present study, we addressed the question of why rSV5-WT is such a poor inducer of host cytokine responses relative to the two SV5 variants, and we used the CC chemokine RANTES as a measure of host responses. Time course experiments showed high-level secretion of IL-6 and RANTES following infections of human A549 lung epithelial cells with the P/V-CPI− mutant and WF-PIV. By contrast, SV5-WT induced very low cytokine responses, with the notable exception of moderate induction of RANTES. The mechanism of RANTES induction by the two SV5 variants shared common properties, since RANTES secretion from infected cells had similar kinetics, depended on virus replication, correlated with increased RANTES mRNA levels and promoter activation, and was reduced by inhibitors of the p38 MAPK, ERK, and PI3K pathways. Despite the similar mechanisms of RANTES induction, the two SV5 variants differed dramatically in their growth and gene expression kinetics. By comparison to the P/V mutant rSV5-P/V-CPI− which has accelerated viral gene expression, WF-PIV infection showed a prolonged delay in viral replication, and infected cells did not show high-level viral RNA and protein expression until ∼12–24 hpi. Sequence analysis revealed that the N, P, V, and M genes from WF-PIV differed by 3, 8, 5, and 10 amino acids compared to rSV5-WT, respectively. Chimeric viruses harboring the WF-PIV P/V or M genes in the context of the other rSV5 genes had growth properties similar to rSV5-WT but had a RANTES-inducing phenotype similar to that of the bone fide WF-PIV virus. Our data indicate a role for both the P/V and the M gene products as determinants of RANTES induction in response to SV5 infection.

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Introduction

The synthesis of proinflammatory cytokines is a critical arm of the innate immune response to virus infection that can contribute to the outcome of an infection, virus tropism, and the potency of the adaptive immune response (Biron and Sen, 2001). In the respiratory tract, virus-infected epithelial cells are a major source of a large number of cytokines, including the type I interferons α and β, chemokines of the CC family such as macrophage chemoattractant protein-1 (MCP-1) and RANTES (regulated upon activation, normally T cell expressed and presumably secreted), and chemokines of the CXC family such as interleukin-8 (IL-8). Virus-induced cytokines can act on infected cells through the activation of anti-viral pathways within the infected cell or in neighboring cells. In addition, released cytokines can recruit immune cells that infiltrate the site of infection, thereby aiding in viral clearance as well as the generation of adaptive immune responses (reviewed in Biron and Sen, 2001). For example, the chemokine RANTES is a potent chemoattractant for T cells, monocytes, eosinophils, and basophils (Bacon and Schall, 1996) and is thought to play a key role in inflammation observed during some viral infections (Kujime et al., 2002; Nakamichi et al., 2005; Noe et al., 1999; Pazdrak et al., 2002).

Many members of the Paramyxovirus family of nonsegmented negative strand RNA viruses have been shown to be potent inducers of cytokine synthesis, including respiratory syncytial virus (RSV), Sendai virus, human parainfluenza virus type 2 (HPIV-2), HPIV-3, and Newcastle disease virus (e.g.,
from the IFN-P/V gene (rSV5-P/V-CPI2003). A recombinant SV5 with engineered substitutions in the inflammatory cytokines such as IL-8 and MCP-1 (Young and Parks, previously described as inducers of high levels of proinflammatory cytokines induced by rSV5-WT, two SV5 variants have been chosen as a positive control virus that has been shown to be a potent inducer of proinflammatory cytokines. At 22 hpi of A549 cells, rSV5-WT and rSV5-GFP infections resulted in very little induction of these genes by virus infection. Many Paramyxoviruses induce type I IFN but suppress the response to IFN through accessory proteins (e.g., Andrejeva et al., 2002; Didcock et al., 1999a, 1999b; Goodbourn et al., 2000; Young et al., 2000). In contrast, Simian virus 5 (SV5) evades the IFN response through multiple mechanisms. For example, SV5 counteracts the cellular IFN signaling pathway by inducing degradation of the transcription factor STAT1 (Young et al., 2000). The SV5 V protein, which is expressed from the bicistronic viral P/V gene, has been shown to be responsible for targeting STAT1 for degradation (Didcock et al., 1999b). Remarkably, the SV5 V protein has also been shown to play a role in inhibiting the induction phase of the IFN response (He et al., 2002; Poole et al., 2002). SV5-infected human cells show little transcription from the IFN-β promoter, and translocation of IRF-3 from the cytoplasm to the nucleus is inhibited following infection with WT SV5 but not with an rSV5 encoding a V protein that is truncated (He et al., 2002).

We have previously shown that in addition to limiting IFN activation, rSV5-WT is also a poor inducer of the host cell proinflammatory cytokines IL-8 and MCP-1, two cytokines that are typically associated with Paramyxovirus infections of respiratory epithelial cells (Young and Parks, 2003; Zhang et al., 2001). By contrast to SV5-WT, two SV5 variants have been previously described as inducers of high levels of proinflammatory cytokines such as IL-8 and MCP-1 (Young and Parks, 2003). A recombinant SV5 with engineered substitutions in the P/V gene (rSV5-P/V-CPI–) directs premature and elevated viral gene expression (Wansley and Parks, 2002) and induces high levels of IL-8 secretion (Young and Parks, 2003). In addition, a naturally occurring SV5 variant (WF-PIV; Wake Forest isolate of parainfluenza virus) was previously identified as an activator of proinflammatory cytokine secretion. While the ability of SV5 to limit IFN synthesis and signaling has been studied extensively, the mechanisms and viral factors that contribute to the low level of SV5 induction of proinflammatory cytokines have not been examined.

In the work described here, we hypothesized that the naturally occurring WF-PIV isolate and the engineered P/V mutant induced high levels of proinflammatory cytokines by a shared mechanism in which accelerated and elevated gene expression results in a high level of gene products that then induce RANTES secretion. Consistent with this hypothesis, RANTES induction by the two SV5 variants shared common properties: RANTES secretion from infected cells had similar kinetics, depended on virus replication, correlated with increased RANTES mRNA levels and promoter activation, and was reduced by inhibitors of the p38 MAPK, ERK, and PI3K pathways. However, our results indicate that the two SV5 variants do not share similar growth kinetics or express their genes to similar levels. Overall, our data indicate that RANTES induction by the SV5 variants cannot simply be explained by overexpression of viral gene products. Instead, our results are consistent with a model in which RANTES is induced by common gene products synthesized by the rapidly growing P/V– mutant and the slow growing WF-PIV variant. The WF-PIV RANTES-inducing phenotype could be transferred to rSV5-WT by exchange of genes encoding either the P/V or M protein. Our data demonstrate a role for both the P/V and the M gene products as determinants of RANTES induction in response to SV5 infection.

**Results**

Gene microarray studies were carried out using Affymetrix gene chip analysis to identify proinflammatory cytokines that were differentially induced by rSV5-WT and the variants rSV5-P/V– and WF-PIV. The related Paramyxovirus HPIV2 was chosen as a positive control virus that has been shown to be a potent inducer of proinflammatory cytokines. At 22 hpi of A549 cells, rSV5-WT and rSV5-GFP infections resulted in very little increase in most proinflammatory cytokine mRNAs (not shown), consistent with our previous analysis of IL-8 and MCP-1 induction (Young and Parks, 2003). A notable exception to this general property of SV5 infections was found in the case of RANTES, as described in detail below. The poor induction of host cell cytokine genes by rSV5-WT contrasted sharply with the high activation of these genes by HPIV2, as well as the two SV5 variants WF-PIV and rSV5-P/V– (not shown). Cells infected with these three viruses showed elevated mRNA levels for IL-6, IL-8, MCP-1, and MIP-3 alpha, but TNF-alpha RNA levels were not induced above background mock-infected samples (not shown). As with rSV5-WT and rSV5-GFP, RANTES was induced to the highest levels by infections with the SV5 variants and HPIV2.

To support results from the microarray analysis, a time course of virus-induced IL-6 and RANTES secretion was carried out. A549 cells were mock infected or infected with rSV5-WT, rSV5-P/V–, WF-PIV, or HPIV2, and at various times pi, extracellular media were analyzed by ELISA for the presence of IL-6 or RANTES. In the following experiments, data for rSV5-WT are shown as an example of the response to WT virus, since we have previously shown that rSV5-WT and rSV5-GFP (the proper control for rSV5-P/V–) are both very poor cytokine inducers (Young and Parks, 2003). As shown in Fig. 1, HPIV2 infection resulted in a rapid high-level secretion of both IL-6 and RANTES that was detected by 4–8 hpi. A549 cells infected with the two SV5 variants WF-PIV and rSV5-P/V– secreted final levels of these cytokines that were similar to that induced by HPIV2, but the kinetics of IL-6 and RANTES secretion were slower and detectable levels...
appeared between ~12 and 24 hpi. Type I interferon was also induced from WF-PIV-infected A549 cells with the same kinetics as IL-6 and RANTES (not shown). Thus, two IRF-3-dependent host genes (RANTES and interferon-beta) are induced by the two SV5 variants. For rSV5-WT, IL-6 levels were very similar to that seen for mock-infected cells (Fig. 1A), consistent with the hypothesis that WT virus is a poor inducer of proinflammatory cytokines. However, RANTES was an exception to this general property. This is evident in Fig. 1B, where cells infected with rSV5-WT showed levels of RANTES that were higher than mock-infected cells, but cytokine secretion appeared only at late times pi and to levels lower than that seen for the SV5 variants WF-PIV and rSV5-P/V-CPI−.

Virus-induced RANTES secretion requires virus replication and correlates with increased RANTES promoter activation

The properties of SV5-induced RANTES secretion were examined to determine if rSV5-WT activated RANTES through a mechanism that was distinct or shared with the variants WF-PIV and rSV5-P/V-CPI−. RANTES induction by WT and variant SV5 was dependent on virus replication. This is evident in Fig. 2A, where infection of A549 cells with increasing amounts of rSV5-WT, WF-PIV, rSV5-P/V-CPI−, or HPIV2 resulted in the secretion of increased amounts of RANTES by 24 hpi. SV5-induced RANTES secretion was also dependent on infection with live virus, since RANTES was not induced following infection with UV-treated virus (Fig. 2B).

Cytokine secretion can be induced through increased cytokine mRNA or by regulation of protein synthesis or mRNA stability. To distinguish between these possibilities, virus-induced RANTES mRNA accumulation was assayed by real-time PCR. As shown in Fig. 2C, HPIV2 infection induced early high levels of RANTES mRNA detectable by 8 hpi. Infection with the SV5 variants rSV5-P/V-CPI− and WF-PIV induced lower levels of RANTES mRNA at early times pi, but by 24 hpi cells infected with the P/V mutant had the highest levels of RANTES mRNA. Lower levels of RANTES mRNA accumulated at late times after infection with rSV5-WT.

A transfection/infecion assay was carried out to determine if the increased accumulation of virus-induced RANTES mRNA correlated with increased RANTES promoter activation. A549 cells were transfected with a plasmid encoding luciferase under control of the minimal RANTES promoter (Casola et al., 2001). Cells were then mock infected or infected at high MOI with rSV5-WT, P/V-CPI−, WF-PIV, or HPIV2. As shown in Fig. 2D, the P/V-CPI− mutant and WF-PIV induced the highest RANTES promoter activation, while rSV5-WT-infected cells had much lower levels of luciferase activity. Promoter activation by virus infection was much lower when cells were transfected with a plasmid containing luciferase under control of a RANTES promoter from which the critical ISRE regulatory element had been deleted (Fig. 2D). This result is similar to the previously described requirement for the ISRE site in RSV-induced RANTES secretion from A549 cells (Casola et al., 2001). Taken together, these data indicate that infection with rSV5-WT, the two SV5 variants P/V-CPI− and WF-PIV, and HPIV2, results in RANTES promoter activation and a corresponding increase in RANTES mRNA accumulation.

Previous work with RSV, influenza virus, and rabies virus has shown a role for MAPK activation in the control of RANTES secretion (Guillot et al., 2005; Kujime et al., 2002; Nakamichi et al., 2005; Pazdrak et al., 2002). To determine if RANTES secretion induced by rSV5-WT, rSV5-P/V-CPI−, or WF-PIV was dependent on p38 MAPK activation, A549 cells were infected in the presence of increasing amounts of the p38 inhibitor SB202190 (Fig. 3A). Addition of 50 μM inhibitor resulted in RANTES secretion that was significantly decreased relative to the control samples for rSV5-WT, rSV5-P/V-CPI−, and WF-PIV infections. A similar requirement for ERK signaling in virus-induced RANTES secretion was found following infection of A549 cells with WT and variant SV5, since infection in the presence of the MEK1/MEK2 inhibitor U0126 resulted in significant decreases in RANTES secretion for each virus infection at a concentration of 50 μM (Fig. 3B).

RANTES secretion from influenza virus-infected bronchial epithelial cells has been shown to also be dependent on phosphatidylinositol-3-kinase (PI3K) activity (Guillot et al., 2005). As shown in Fig. 3C, addition of increasing concentrations of the PI3K inhibitor LY294002 to virus-infected A549 cells resulted in a significant dose-dependent decrease in RANTES secretion. These data are similar to results on the mechanism of influenza-induced RANTES secretion from
bronchial epithelial cells (Guillot et al., 2005) and are consistent with a role for p38-, ERK- and PI3K-dependent pathways in RANTES secretion induced by rSV5-WT, rSV5-P/V-CPI− and WF-PIV. Interestingly, HPIV2-induced RANTES secretion was not significantly diminished by increasing amounts of the p38, ERK, and PI3K inhibitors (Figs. 3A–C). Taken together, these data indicate that the two SV5 variants induce RANTES secretion through common mechanisms and pathways which may be distinct from that induced by HPIV2 infection.

Delayed viral gene expression in cells infected with WF-PIV

The above results indicate that the two SV5 variants rSV5-P/V-CPI− and WF-PIV induce A549 cells to secrete RANTES through a similar mechanism and with very similar kinetics. rSV5-P/V-CPI− is an engineered mutant containing six naturally occurring substitutions in the P/V gene and has been shown to have accelerated kinetics of virus growth and gene expression relative to rSV5-WT (Wansley and Parks, 2002; Wansley et al., 2003). WF-PIV is a naturally occurring SV5 variant whose growth and gene expression properties have not been reported. Based on the similar mechanisms of RANTES induction by rSV5-P/V-CPI− and WF-PIV, we hypothesized that WF-PIV would also display accelerated kinetics of growth and gene expression relative to rSV5-WT.

Single-step growth kinetics of rSV5-WT and WF-PIV revealed that this hypothesis was incorrect. In contrast to the rapidly growing rSV5-WT, two independently plaque-purified stocks of WF-PIV failed to produce significant levels of progeny virus until after 24 hpi (Fig. 4A). In Western blot analysis, cells infected with rSV5-WT showed the accumulation of detectable viral N and P by 8 hpi, with high levels of these proteins plateauing by ∼24 hpi (Fig. 4B). By contrast, WF-PIV N and P were not detected at high levels until ∼24 hpi. By this time point, WF-PIV N and P had accumulated to similar levels to that seen in rSV5-WT-infected cells at ∼8–12 hpi. Delayed protein accumulation was seen for all WF-PIV gene products (not shown). Cells infected with WF-PIV also showed a delay in reaching a high rate of viral protein synthesis. This is evident in Fig. 4C, where pulse-labeling of infected A549 cells with 35S-amino acids showed a maximum rate of N protein synthesis for rSV5-WT between 8 and 12 hpi, and this was followed by a decreased rate of synthesis at later times pi, as reported previously (Young et al., 2001). By contrast, WF-PIV-infected
cells did not show a high rate of N protein synthesis until 24 hpi. Likewise, the rate of synthesis of P and V was delayed for WF-PIV compared to rSV5-WT (Fig. 4D). Similar results were obtained in Vero cells (data not shown), indicating that the delay in abundant viral gene expression was not due to antiviral effects of IFN.

The above delayed viral gene expression was not due to a low number of cells infected by WF-PIV. This is evident in Fig. 4E where nearly all cells in the population were infected with WF-PIV at a MOI of 10 as assessed by indirect immunofluorescence. Consistent with data in Figs. 4B and C, indirect immunofluorescence also revealed lower levels of N protein in WF-PIV-infected cells relative to WT rSV5-infected cells.

The levels of Paramyxovirus proteins are thought to be largely determined by levels of viral transcription (Lamb and Kolakofsky, 2001). Real-time PCR assays were carried out to determine if the relative delay in abundant WF-PIV viral protein expression was also seen at the level of viral mRNA accumulation. Total RNA was harvested from cells at various times pi with rSV5-WT or WF-PIV and used in a first strand synthesis reaction using oligo-dT primers. The resulting cDNAs were analyzed by real-time PCR for the presence of N and M viral mRNAs. As shown in Fig. 5A, rSV5-WT-infected cells accumulated N and M mRNA in a time-dependent manner with mRNA levels peaking at 12 hpi. In contrast, WF-PIV-infected cells accumulated N and M mRNA with delayed kinetics in which mRNA accumulation peaked at 24 hpi, indicating that the WF-PIV delay in protein synthesis and accumulation correlated with viral mRNA accumulation. As shown in Fig. 5B, this delay in WF-PIV mRNA accumulation also occurred in interferon-deficient Vero cells and in BHK cells (not shown), a cell line that does not respond to type I IFN. These results indicate that the lag in WF-PIV gene expression cannot be attributed to a virus-induced IFN response.

To determine if the WF-PIV delay in gene expression was occurring at the stage of primary transcription, A549 cells were infected with rSV5-WT or WF-PIV in the presence or absence of the translation inhibitor cycloheximide, and mRNA accumulation was measured by RT-PCR. By inhibiting translation, the switch from primary transcription to replication is blocked, and only primary transcripts accumulate in the infected cells (Lamb and Kolakofsky, 2001). As shown in Fig. 5C, rSV5-WT-infected cells accumulated about 2-fold more N mRNA by 6 hpi in the absence of cycloheximide than did corresponding WF-PIV-infected cells. However, in the presence of cycloheximide, N primary transcripts accumulated to approximately equivalent levels between rSV5-WT and WF-PIV-infected cells. These data indicate that the delay in WF-PIV gene expression occurs at a step in virus replication that is downstream of primary transcription, such as genome replication or secondary transcription.

Role of the WF-PIV 3′ end genes in delayed viral gene expression and RANTES induction

The Paramyxovirus 3′ end genes are important determinants of both viral replication and the control of host antiviral responses (Lamb and Kolakofsky, 2001). We tested the hypothesis that the WF-PIV 3′ end genes encoding N, P/V, and M contributed to both the delay in WF-PIV gene expression and virus-induced RANTES expression. RT-PCR was used as described in Materials and methods to clone and sequence 4478 bases of WF-PIV genome sequences that spanned from immediately downstream of the N start site through the P/V gene and up to the M gene 3′ end region. Consensus nucleotide sequences for the N, P/V, and M genes were generated by analysis of cDNA clones derived from multiple RT-PCR experiments. Fig. 6 summarizes the amino acid differences between rSV5-WT and WF-PIV N, P, V, and M proteins.

While the WF-PIV N gene had 43 nucleotide changes compared to rSV5-WT, these translated to only 3 amino acid differences, all located in the C-terminal half of N (Fig. 6A).
Of these changes mapped to known domains that are conserved among Paramyxovirus N protein sequences (Lamb and Kolakofsky, 2001). The WF-PIV P/V gene had 20 nucleotide changes compared to rSV5-WT, resulting in 8 amino acid changes in the P open reading frame (Fig. 6A). Within the M gene, WF-PIV had 37 nucleotide differences compared to rSV5-WT, resulting in 10 predicted amino acid changes scattered throughout the M protein. The recently identified SV5 late domain virus budding motif identified by Schmitt et al. (2005) was maintained at positions 20-FPIV-23 of the WF-PIV M protein, suggesting that the delayed growth of WF-PIV could not be solely attributed to a defect in this step of virus assembly.

Differences in the V protein of the W3A strain of SV5 and WF-PIV were of particular interest, since this is a multifunctional protein involved in controlling viral gene expression (Horikami et al., 1996; Lin et al., 2005; Randall and Bermingham, 1996), as well as host antiviral responses (Poole et al., 2002; Sun et al., 2004; Wansley et al., 2003). As shown in the alignment in Fig. 6B, the C-terminal Cys-rich region of the WF-PIV V protein contained no predicted amino acid changes, while the shared P/V N-terminal region had 5 predicted amino acid changes relative to W3A SV5.

To determine the contribution of the 3′ end genes of WF-PIV to the delayed gene expression kinetics and the induction of RANTES, a recombinant SV5 chimeric virus was generated such that the coding and noncoding regions of the WF-PIV N, P/V, and M genes were substituted into an rSV5-GFP background to generate rSV5-NPM-WF. The resulting rSV5-WF chimera was assessed for its single-step growth kinetics and gene expression profile compared to the control WT rSV5-GFP. As shown in Fig. 7, single-step growth analysis of rSV5-NPM-WF showed that the chimeric virus had similar growth kinetics to that of rSV5-GFP, with slightly higher growth seen at early times pi. Similarly, microscopy and Western blot analyses (Figs. 7B and C, respectively) showed equivalent or slightly higher protein expression for cells infected with rSV5-NPM-WF relative to that of rSV5-GFP. Thus, the changes within the 3′ end 4478 bases of the WF-PIV genome cannot by themselves account for the delay in growth and gene expression seen in infections with the bone fide WF-PIV virus.

Extracellular media harvested from cells infected with the rSV5-NPM-WF chimera showed elevated RANTES secretion compared to cells infected with WT rSV5-GFP, and these levels were very similar to that seen with the bone fide WF-
PIV virus (Fig. 7D). Although the levels of RANTES secreted from cells infected with rSV5-NPM-WF were always less than that seen with WF-PIV-infected cells, these differences were not statistically significant. Thus, the rSV5-NPM-WF chimeric virus which contains the WF-PIV N, P/V, and M genes has growth and gene expression properties similar to rSV5-WT but has a RANTES-inducing phenotype similar to that of WF-PIV.

To test the individual contribution of the WF-PIV P/V and M genes in RANTES induction, two additional chimeric viruses were constructed such that the WF-PIV P/V gene alone (rSV5-P/V-WF) or the M gene alone (rSV5-M-WF) was inserted in the context of the remaining rSV5-GFP genes. Both chimeric viruses grew to titers that were as high or higher than that of the WT control rSV5-GFP in single-step growth assays (Fig. 8A).

Fig. 5. Accumulation of WF-PIV mRNA is delayed compared to rSV5-WT-infected cells. (A) Kinetics of mRNA accumulation. A549 cells were infected at an MOI of 10 with WF-PIV or rSV5-WT and at the indicated times pi, total RNA was harvested and analyzed by real-time PCR as described in Materials and methods for the presence of viral N and M mRNA. Data are the mean plus standard deviation from three independent experiments. (B) mRNA accumulation in IFN-negative Vero cells. Levels of N mRNA were determined as described in Materials and methods at 8 and 24 hpi of Vero cells with rSV5-WT and WF-PIV. Data are representative of three independent experiments. (C) Accumulation of mRNA during primary transcription. A549 cells were infected at an MOI of 10 in the presence or absence of 100 μg/ml cycloheximide. At the indicated times pi, total RNA was harvested and analyzed by real-time PCR for the presence of N viral mRNA sequence. Data are representative of three independent experiments.

Fig. 6. Sequence analysis of WF-PIV N, P/V, and M genes. (A) The amino acid differences between WF-PIV N, P, and M proteins relative to rSV5-WT. SV5-WT amino acids are indicated above the box and WF-PIV residues below the box. (B) Alignment of V protein amino acid differences between WT SV5 (W3A), WF-PIV and the SV5 isolate CPI minus. Amino acid changes relative to W3A sequence are shown beneath each position.
viruses secreted RANTES to levels that matched that seen with the rSV5-NPM-WF chimera which contains the WF-PIV N, P/V, and M genes (Fig. 8B) and slightly less that that seen for the bone fide WF-PIV parental virus (not shown). Together, these data indicate that the P/V and M genes of WF-PIV can individually contribute to RANTES induction, at least when expressed in the context of the other rSV5-WT genes.

**Discussion**

The activation of proinflammatory cytokine expression following virus infection is an important host cell response that can limit virus growth and promote adaptive immunity. The viral factors that determine whether a Paramyxovirus induces or limits host cell cytokine responses are not completely...
understood. The work described here was initiated by two previous findings: rSV5-WT is a poor inducer of IL-8 and MCP-1, and the SV5 variants rSV5-P/V-CPI− and WF-PIV induce high levels of IL-8 (Young and Parks, 2003). We have addressed the question of why rSV5-WT is such a poor inducer of host cytokine responses relative to the two SV5 variants. Based on the similar kinetics and mechanisms of RANTES induction by the two variants, we initially hypothesized that an accelerated expression of viral gene products by rSV5-P/V-CPI− and WF-PIV was the basis for induction of RANTES. As described below, our results on the mechanism of differential cytokine induction by WT and variant SV5 viruses support four conclusions: (1) wild-type SV5 is an overall poor inducer of most cytokine responses in A549 lung epithelial cells, with the exception of RANTES which is secreted from infected cells only at moderate levels and at late times pi, (2) the SV5 variants rSV5-P/V-P/CPI− and WF-PIV activate expression of a number of cytokines and share common features in the pathways and mechanisms of RANTES induction, (3) the kinetics of viral gene expression relative to rSV5-WT differ dramatically for the rSV5-P/V-CPI− mutant with accelerated gene expression and the naturally occurring WF-PIV variant with delayed gene expression, and (4) rSV5-WT can be converted into a more potent inducer of RANTES by introduction of WF-PIV sequences from either the P/V gene or the M gene, but these genes do not confer the WF-PIV phenotype of delayed viral gene expression. Together, our data suggest that regardless of expression levels, gene products expressed by the SV5 variants are either inherent inducers of RANTES or are defective suppressors of the RANTES response.

We have focused our mechanistic studies on RANTES, a well-characterized cytokine response to virus infections in A549 cells (Casola et al., 2001 and references therein). Virus-induced transcription from the RANTES promoter requires IRF-3 and is dependent on an ISRE in the promoter (Casola et al., 2001; Genin et al., 2000; Lin et al., 1999). Previous work on the cis-regulatory elements controlling RSV-induced RANTES promoter activity in A549 cells has shown that the ISRE does not bind a STAT complex, but instead, IRF-3 is the major transactivating factor that binds this element in response to infection (Casola et al., 2001). Similarly, Lin et al. have shown that the STAT complex interferon-stimulated gene factor 3 (ISGF3) did not play a role in the induction of RANTES in 293 cells infected with Sendai virus nor did IRF-1 or IRF-7 largely contribute to RANTES activation following Sendai virus infection (Lin et al., 1999).

Why are WF-PIV and the P/V-CPI− mutant potent inducers of proinflammatory cytokines whereas rSV5-WT generally limits cytokine production? We have shown here that the two variant viruses activate RANTES secretion through similar replication-dependent mechanisms and with similar kinetics; therefore, we proposed a hypothesis that the P/V-CPI− mutant and WF-PIV may share a common phenotype of accelerated viral gene expression (Wansley et al., 2005) and may activate a host cell cytokine response before a viral antagonist could effectively block host responses. Our results here demonstrate that contrary to our hypothesis, WF-PIV has a remarkable delay in high-level viral replication and gene expression relative to both rSV5-WT and the P/V-CPI− recombinant. The exchange of WF-PIV N, P/V, and M genes into the background of rSV5-WT produced a chimeric virus rSV5-NPM-WF that had growth and gene expression properties that were equal to or slightly higher than rSV5-WT and much higher than the bone fide WF-PIV. Thus, the WF-PIV N, P/V, and M genes are not solely responsible for the dramatic differences in replication kinetics between rSV5-WT and WF-PIV. The WF-PIV L gene and leader are alternative candidate genes that may contribute to delayed viral gene expression, and work is in progress to identify determinants of replication kinetics within these viral genes. However, the rSV5-NPM-WF chimeric virus as well as the P/V-WF and M-WF viruses showed reproducibly higher induction of RANTES compared to rSV5-WT, indicating that these viruses had similar replication kinetics but different cytokine inducing phenotypes.

The outcome of whether cytokine responses are activated by a virus infection can be viewed as a combination of the level or type of viral components that act as inducers and the ability of a virus to actively block the cellular response (Biron and Sen, 2001; Goodbourn et al., 2000). Our data on the two SV5 variants with similar mechanisms of RANTES induction but dissimilar gene expression and growth profiles (Wansley and Parks, 2002; Figs. 4, 5) suggest that in our model system, it is not the level of viral components that activate the host response but rather the type of viral component. We propose two nonexclusive models to explain why the naturally occurring WF-PIV, the engineered rSV5-P/V-CPI−, and the chimeric rSV5-NPM-WF viruses induce higher levels of host cell responses compared to rSV5-WT. In the first model, the SV5 variants induce cytokines either by producing a different inducer than WT SV5 or a common inducer is produced at levels that are higher than rSV5-WT. The 3′ end genes of other negative strand RNA viruses have been shown to be important activators of host response, specifically IRF-3 activation. For example, IRF-3 can be activated by transcription of the Measles virus N gene outside of the context of a viral infection (Noe et al., 1999) or by purified VSV ribonucleoprotein complexes (TenOever et al., 2004). The three of the four SV5 variants that have high RANTES induction (WF-PIV; rSV5-P/V-CPI−; NPM-WF; and P/V-WF) contain differences in the P/V gene compared to rSV5-WT. Substitutions in the P protein, a multifunctional component of the viral RNA-dependent RNA polymerase (Horikami et al., 1992), could make the polymerase complex more prone to synthesize dsRNA, a known activator of host cell responses leading to RANTES induction (Rudd et al., 2005). Interestingly, an additional chimera containing an exchange of the M gene alone (rSV5-M-WF) was equally potent at RANTES induction compared to the other chimeras, indicating that viral components expressed from the M gene can also act directly or indirectly as determinants of host cell cytokine responses to Paramyxovirus infection.

A second model for differential cytokine induction proposes that a common inducer is produced by the WT and...
variant SV5, but the variants are defective in blocking the activation of the host response. It has been previously shown that the V protein of WT SV5 plays a role in counteracting host IFN responses by targeting signal transducer and activator of transcription 1 (STAT1) for degradation, as well as by preventing the translocation of IRF-3 to the nucleus in response to dsRNA stimulation (Andrejeva et al., 2002; Didcock et al., 1999a, 1999b; He et al., 2002; Poole et al., 2002). These results not only provide evidence for V protein acting as an antagonist of the host IFN response, but they also suggest that V protein should be an important antagonist of RANTES induction since RANTES induction following viral infection is IRF-3-dependent (Casola et al., 2001; Lin et al., 1999). Three of the cytokine-inducing variants contain substitutions in the V protein compared to WT SV5, and it is possible that these altered V proteins are less effective at counteracting IRF-3 activation. Future work will define the role of P and V proteins in inducing and counteracting RANTES activation as well as other proinflammatory cytokine responses.

While rSV5-WT is a poor inducer of type I interferon (He et al., 2002; Poole et al., 2002; Wansley et al., 2003) as well as many other prototype proinflammatory cytokines, an unexpected finding was the moderate induction of the IRF-3-dependent gene encoding RANTES at late times pi (Figs. 1–3). Previous results have shown that the V protein of WT SV5 prevents the translocation of IRF-3 to the nucleus (He et al., 2002; Poole et al., 2002), contributing to the low level induction of IFN-β even at late times following WT virus infection. If IRF-3 translocation is blocked by the SV5 V protein, then why does rSV5-WT virus activate moderate expression of RANTES, an IRF-3-dependent gene? A simple explanation is that RANTES may be more sensitive to low levels of IRF-3 activation than other IRF-3-dependent genes. Alternatively, differences between the very low level induction of IFN-β and the moderate induction of RANTES by WT virus infection may reflect differences in the requirement of these genes for IRF-3 alone or in combination with other factors such as NF-κB and AP-1. Spann et al. described an early activation of IRF-3 following infection with both wild-type RSV (wt RSV) and RSV deletion mutants lacking the IFN antagonist proteins NS1 and NS2. However at later times pi, nuclear IRF-3 was still detected in cells infected with the deletion mutants but not in wt RSV-infected cells, resulting in dramatic differences in the duration of the host response to wt RSV and mutant virus infections (Spann et al., 2005). These findings suggest that early in infection, prior to accumulation of a viral inhibitor to sufficient levels, some viruses initially activate a host response but then subsequently downregulate the host response once viral inhibitor has accumulated to sufficient levels. However, this model is inconsistent with our finding of RANTES mRNA accumulation and secretion of RANTES protein only at late times following rSV5-WT infection (Figs. 1B, 2C).

An alternative model for rSV5-induction of RANTES is based on the proposal that the rate and level of growth and gene expression plays a role in the balance between limiting versus activating host cell responses. This is supported by previous work showing that cytokine induction was associated with a rapid virus growth profile for a pathogenic isolate of West Nile virus (WNV), and IRF-3-dependent genes were turned on at late times pi (Fredericksen et al., 2004). Thus, the appearance of RANTES at late times pi with rSV5-WT may reflect a high rate of virus growth that eventually overwhelms the V-imposed block on IRF-3-dependent gene transcription.

The three viruses rSV5-WT, rSV5-P/V-CPI−, and WF-PIV shared a common mechanism of RANTES induction in which the RANTES promoter is activated through a replication-dependent pathway that could be blocked by p38 MAPK, ERK, and PI3K inhibitors. These data are consistent with previous results that identified MAPK and/or PI3K pathways in RANTES secretion induced by infection with RSV, rabies virus, and influenza virus (Kujime et al., 2002; Nakamichi et al., 2005; Pazdrak et al., 2002). The requirement for p38 MAPK, ERK, and PI3K could reflect signal transduction pathways that upregulate RANTES transcription or that result in stabilization of RANTES mRNA as shown previously for IL-8, IL-6, and RANTES activation in HeLa cells (Casola et al., 2001; Holtmann et al., 1999; Winzen et al., 1999). Infection with rSV5-WT or the SV5 variants results in transcriptional activation of the RANTES promoter, but additional roles for the MAPK signaling cascades in SV5-induced stabilization of RANTES mRNA are currently being tested.

Determining the molecular basis for the activation of host responses following viral infection is required for a better understanding of factors that impact pathogenesis. In addition, as viruses are engineered for therapeutic use, it will be of great importance to determine the impact of attenuating mutations and gene substitutions on virus-induced innate immune responses.

Materials and methods

Cells, viruses, growth analysis, and plaque assays

Monolayer cultures of cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). W3A strain of SV5 and the Greer strain of HPIV2 were grown in MDBK and CV-1 cells, respectively. WF-PIV and the rSV5-NPM-WF chimera were grown in Vero cells. WF-PIV was plaque purified from media derived from persistently infected Vero cells. rSV5-GFP virus was recovered from cDNA which was the kind gift of Biao He and Robert Lamb (Northwestern University). Plaque-purified virus was grown under low MOI conditions. rSV5-GFP virus was recovered from cDNA which was the kind gift of Biao He and Robert Lamb (Northwestern University). Single-step growth assays were carried out as described previously (Wansley and Parks, 2002).

For inactivation of virus by UV treatment, viruses diluted in DMEM/10% BSA were held in 60-mm dishes for 20 min under a handheld germicidal ultraviolet (UV) lamp at a distance of 6.5 cm. This procedure eliminated all infectivity as determined by plaque assays. When MAPK and phosphatidyl
inositol 3-kinase (PI3K) inhibitors were used, cells were pre-treated with the indicated inhibitor concentrations (or DMSO solvent for control samples) for 1 h and were then infected in the presence of inhibitor.

Sequence analysis of WF-PIV 3′ end genes and construction of chimeric virus

For cloning of viral genes, nucleocapsid RNA was isolated from WF-PIV-infected Vero cell lysates by banding on cesium chloride gradients followed by Trizol extraction. Reverse transcriptase PCR (RT-PCR) reactions were carried out with SV5-specific primer pairs spanning overlapping regions of the 3′ end genes and resulting products were cloned into pCR4-TOPO vectors (Invitrogen). Multiple clones from multiple RT-PCR reactions were used to obtain consensus sequence data for the WF-PIV genomic RNA bases 19–4460. Plasmids encoding the consensus sequences were assembled from PCR products using available restriction sites.

Chimeric recombinant viruses harboring the WF-PIV N, P/V, and M genes alone (rSV5-P/V-WF and rSV5-M-WF) or in combination (rSV5-NM-WF) were constructed using standard molecular biology techniques (Parks et al., 2001; details available upon request) by insertion of an NdeI-Asp718 fragment spanning from the 5′ end of the N gene to the 3′ end genes and resulting products were cloned into pCR4-TOPO vectors (Invitrogen). Multiple clones from multiple RT-PCR reactions were used to obtain consensus sequence data for the WF-PIV genomic RNA bases 19–4460. Plasmids encoding the consensus sequences were assembled from PCR products using available restriction sites.

Western blotting and isotopic labeling of polypeptides

For Western blotting, the protein concentration of cell lysates was determined by BCA assay (Pierce Chemicals), and equivalent amounts of protein were analyzed with rabbit antisera to the SV5 N or P proteins (Parks et al., 2001) followed by HRP-conjugated secondary antibodies and ECL. Mock-infected or virus-infected A549 cells were radiolabeled for 1 h and were then infected or virus-infected A549 cells were radiolabeled for 1 h and were then infected with the indicated inhibitor concentrations (or DMSO solvent for control samples) for 1 h and were then infected in the presence of inhibitor.

Analysis of transcription products

To measure the accumulation of viral mRNA, total RNA was isolated from mock-infected or virus-infected A549 cells at the indicated times post-infection, total RNA was harvested and analyzed for viral mRNA levels as described above.

Reporter gene assays

Induction of the RANTES promoter was assayed using pGL2-220, a plasmid containing the luciferase reporter gene downstream of 220 bases from the human RANTES regulatory region, defined by Casola et al. (2001) as being the minimal RANTES promoter fragment that retains full RSV inducibility. The ISRE mutant form of this construct (pGL2-120) was modified to lack the ISRE of the RANTES promoter (Casola et al., 2001). Both plasmids were the kind gift of Dr. Alan Brasier (University of Texas Medical Branch). The pSV-βgal plasmid (Promega) consists of the β-galactosidase gene under the control of a constitutive SV40 promoter and was used to normalize for transfection efficiencies between samples. Six wells of A549 cells were co-transfected with 1 μg pSV-βgal and 3 μg pGL2-220 or pGL2-120 per well using the calcium phosphate transfection protocol. Twenty-four hours post-transfection, cells were mock infected or infected with live or UV-treated rSV5-WT, rSV5-P/V-CPI−, WF-PIV, or HPIV2. Cells were harvested in reporter lysis buffer (Promega) at indicated times post-infection, total RNA was harvested, and luciferase and β-galactosidase activities were determined. Normalized luciferase activity was calculated as luciferase activity divided by β-galactosidase activity.

Microscopy assays

Infected cells were washed with PBS and visualized directly for fluorescence derived from GFP expression. In immunofluorescence assays, cells were fixed with 2% paraformaldehyde (PFA) for 10 min in the dark and permeabilized with 0.5% saponin for 10 min. Cell monolayers were labeled with SV5 anti-N monoclonal antibody D (kind gift of R. Randall; University of St. Andrews) followed by a secondary AlexaFluor 488 goat anti-mouse IgG (Molecular Probes) and mounting media containing DAPI. Samples were analyzed with the Nikon Eclipse fluorescence microscope and a 20× lens. Images were captured using a QImaging digital camera and processed using QCapture software. Exposure times were manually set to be constant between samples.

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