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Apoptosis induction and interferon signaling but not IFN-β promoter induction by an SV5 P/V mutant are rescued by coinfection with wild-type SV5

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Abstract

Infection of human cells with the paramyxovirus simian virus 5 (SV5) results in minimal cytopathic effect, and host interferon (IFN) and apoptotic pathways are not activated. We have previously shown that an rSV5 containing six naturally occurring P/V gene substitutions (rSV5-P/V-CPI−) displays premature and elevated expression of viral RNA and protein. In addition, cells infected with rSV5-P/V-CPI− show induction of the IFN-β promoter as well as activation of IFN signaling and apoptotic pathways. In this article, we have tested the hypothesis that rSV5-WT can supply *trans*-acting factors that prevent host cell antiviral responses induced by rSV5-P/V-CPI−. During coinfection of human A549 cells, rSV5-WT blocked cell rounding, loss of cell volume, and DNA fragmentation induced by rSV5-P/V-CPI−, three later events in the apoptotic pathway, but was not able to block the loss of mitochondrial membrane potential ($\Delta\Psi_m$), an early event in the cell death process. As expected, IFN signaling was blocked during coinfections, and this was attributed to the loss of STAT1 induced by the rSV5-WT V protein. Surprisingly, simultaneous infection with rSV5-WT could not suppress the activation of the IFN-β promoter by rSV5-P/V-CPI− infection. However, the IFN-β promoter was not activated in cells that were first preinfected for 1 h with rSV5-WT and then subsequently infected with rSV5-P/V-CPI−. A model is proposed for activation of host responses to infection with the rSV5-P/V-CPI− mutant and the steps that are blocked by rSV5-WT.

Introduction

Host antiviral responses can be important determinants of pathogenesis, tropism, and activation of the adaptive immune response (Roulston et al., 1999; Barber, 2001). These responses include induction of cytokines and cell death. During virus infection, the action of alpha/beta-interferon (IFNα/β) is considered to be the most important arm of the innate immune response (reviewed in Biron and Sen, 2001; Stark et al., 1998). Many of the identified IFN-induced gene products have potent antiviral activity and can contribute to the inhibition of host and viral protein synthesis, induction of apoptosis, and clearance of viral infections. As such, viruses have evolved mechanisms that counteract the induction of IFN, the activation of the IFN signaling pathways, and activation of apoptosis (reviewed in Garcia-Sastre, 2001; Goodbourn et al., 2000; Barber, 2001; Roulston et al., 1999). Paramyxoviruses are no exception to this phenomenon and have been found to inhibit IFN synthesis and/or signaling as well as apoptosis in a variety of ways (Garcia-Sastre, 2001; Goodbourn et al., 2000; Young et al., 2000; Iseni et al., 2002).

Many of these mechanisms for counteracting host responses have been attributed to the paramyxovirus P/V (and sometimes C) gene (Didcock et al., 1999b; Garcin et al., 1999, 2000; Kawano et al., 2001; Kubota et al., 2001; Parisien et al., 2001; Takeuchi et al., 2001). Transcription of the paramyxovirus P/V gene differs from transcription of other viral genes, since the viral polymerase produces two mRNAs from one gene by a process termed RNA editing (Thomas et al., 1988). For SV5, accurate transcription of the

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P/V gene produces an mRNA that encodes the V protein, while the P mRNA contains two additional nontemplate G residues added cotranscriptionally by the viral polymerase at a precise location in the P/V transcript (Thomas et al., 1988). Thus, the V and P proteins share the same amino-terminal segment (the P/V region) but have different C-terminal regions. Previous results have shown that infection of human cells with SV5 results in the loss of STAT1 protein, an essential component of the type I IFN signaling pathway (Didcock et al., 1999a; Young et al., 2000). Transfection experiments and expression of the SV5 V protein from heterologous vectors have shown that V is sufficient to inhibit IFN signaling in human cells by targeting STAT1 for degradation by the proteasome (Didcock et al., 1999b).

Naturally occurring strains of SV5 have been isolated that differ in their ability to block IFN signaling (Chatziandreou et al., 2002). Canine parainfluenza virus-plus (CPI+) is an SV5 strain isolated from a dog with neurological dysfunction (Baumgartner et al., 1981). CPI− was subsequently isolated from a dog experimentally infected with CPI+ (Baumgartner et al., 1991). Recent work has shown that while infection with rSV5-WT induces STAT1 degradation and blocks type I IFN signaling, the CPI− strain is defective in these two virus-induced alterations to the host cell (Chatziandreou et al., 2002). Sequence analysis has shown six amino acid differences in the amino-terminal region of the P/V genes of CPI−, which does not induce STAT1 degradation, and the W3 (WT) strain of SV5, which induces loss of STAT1 (Chatziandreou et al., 2002; Southern et al., 1991).

We and others have demonstrated that rSV5-WT is a poor inducer of type I IFN, and that mutations in the P/V gene can create an rSV5 that induces IFN (Wansley and Parks, 2002; He et al., 2002). Recent evidence from transfection experiments suggests that in addition to blocking IFN signaling, the V protein is also capable of blocking IFN synthesis induced by dsRNA (Poole et al., 2002). The CPI− virus is also a poor inducer of IFN, and transfection experiments have shown that CPI− V is as potent as rSV5-WT V in blocking dsRNA-induced IFN synthesis (Poole et al., 2002).

In our previous work, we created a virus in which the N-terminal region of the P/V gene from rSV5-WT was replaced with the same region of the CPI− virus, introducing six amino acid changes into the genome of the virus (Wansley and Parks, 2002). As expected, the rSV5-P/V-CPI− virus did not induce the degradation of STAT1 or the subsequent block in ISRE signaling due to the P/V mutations. Unexpectedly, this virus displayed several additional phenotypes that differed from rSV5-WT. We found that infection with rSV5-P/V-CPI− resulted in premature and elevated levels of viral RNA and protein as compared to rSV5-WT. In addition, we demonstrated that while rSV5-WT is a poor inducer of the IFN-β promoter, infection with rSV5-P/V-CPI− results in activation of this promoter and secretion of antiviral cytokines (Wansley and Parks, 2002; Young and Parks, 2003). Finally, while wild-type SV5 is noncytopathic in most cell types (Choppin, 1964; He et al., 2001; Parks et al., 2002), infection with rSV5-P/V-CPI− was found to induce severe cytopathic effect with characteristics of apoptosis in multiple cell lines (Wansley and Parks, 2002). Thus, when expressed in the context of the WT rSV5 genome (Wansley and Parks, 2002), the CPI− V protein behaves differently than when expressed by transfection (Poole et al., 2002).

In the present study, we have tested the hypothesis that rSV5-WT can supply *trans*-acting factors that prevent induction of host cell antiviral responses by rSV5-P/V-CPI−. Previous work on noncytopathic (ncp) and cytopathic (cp) strains of hepatitis A virus (HAV) and bovine viral diarrhea virus (BVDV) have used the approach of coinfecting cells to determine if the ncp strain could block host cell responses induced by the cp strain (Baigent et al., 2002; Brack et al., 2002). Likewise, previous coinfection experiments have shown that rSV5-WT can block apoptosis induced by an rSV5 containing a deletion in the SH protein (rSV5-ΔSH) (He et al., 2001). In this article, we present a model for the steps in host cell responses to rSV5-P/V-CPI− infection and use coinfections to show that rSV5-WT can block three of the new phenotypes seen with rSV5-P/V-CPI−.

Results

The cytopathic effect of infection with rSV5-P/V-CPI− is greatly reduced by coinfection with rSV5-WT

SV5 infection of most cell types results in minimal cytopathic effect (CPE) (Choppin, 1964; He et al., 2001; Parks et al., 2002). By contrast, we have previously discovered that infection with rSV5-P/V-CPI− results in extensive CPE and infected cells die by a mechanism displaying characteristics of apoptosis (Wansley and Parks, 2002). To determine whether infection with rSV5-WT could block induction of cell death by rSV5-P/V-CPI−, A549 cells were infected with rSV5-WT or rSV5-P/V-CPI− at an m.o.i. of 5, or coinfected with both viruses as indicated in Fig. 1. At 48 h pi, a fluorescence-based TUNEL assay for DNA fragmentation was performed, and the number of TUNEL-positive cells was quantitated as a percentage of total cells in each field. As seen in Fig. 1, ~30–35% of the cells were TUNEL positive when infected with rSV5-P/V-CPI− alone at an m.o.i. of 5, consistent with our previous results (Wansley and Parks, 2002). Less than 1% of cells infected with rSV5-WT were TUNEL-positive (Fig. 1), similar to that seen with mock-infected cells (not shown). During coinfection at an equal m.o.i. of 5, the percentage of TUNEL-positive cells dropped to ~10% and continued to drop as the m.o.i. of rSV5-WT infection increased. Background levels of TUNEL staining were seen when cells were infected with rSV5-WT and rSV5-P/V-CPI− at m.o.i.s of 50 and 5, respectively. These data support the hypothesis that during

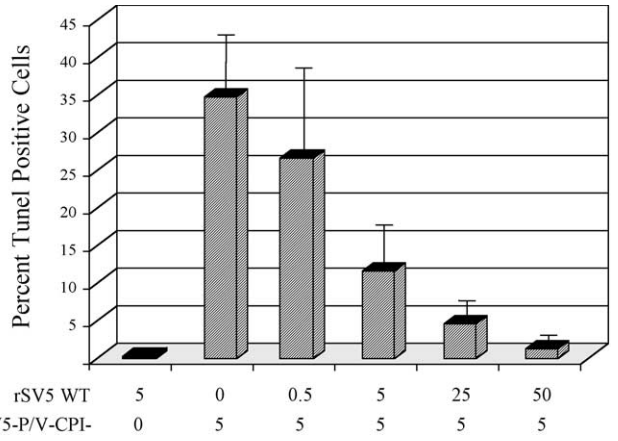

Fig. 1. TUNEL staining for cell death during coinfection. A549 cells were infected at an m.o.i. of 5 for each individual virus, or coinfected with rSV5-WT or rSV5-P/V-CPI− at the indicated m.o.i. At 48h pi, TUNEL staining was performed as described under Materials and methods. Random fields of cells were observed and the number of TUNEL-positive cells in each field was counted and calculated as the percentage of total cells in the field. Mock infected samples showed only ~1% TUNEL positive staining (data not shown). Data are the average of three independent experiments.

coinfection, rSV5-WT can block the cell death phenotype induced by infection with rSV5-P/V-CPI−.

The results in Fig. 1 could have been due to an ability of rSV5-WT to exclude infection by rSV5-P/V-CPI−. The rSV5-P/V-CPI− mutant had been constructed to contain a gene for the green fluorescence protein inserted between the HN and L genes, allowing it to be distinguished from rSV5-WT (He et al., 1997; Wansley and Parks, 2002). To determine whether rSV5-WT excluded rSV5-P/V-CPI− during coinfection, A549 cells were mock infected or infected with rSV5-WT or rSV5-P/V-CPI− alone at an m.o.i. of 50, or coinfected with both viruses at an m.o.i. of 25 for each virus, and then examined by fluorescence microscopy at 24 h pi. As shown in Fig. 2, the majority of cells infected with rSV5-P/V-CPI− alone or coinfected with both viruses were fluorescent above background levels seen during mock and rSV5-WT infection. These results indicate that infection with rSV5-WT did not prevent coinfection with rSV5-P/V-CPI−. In a converse experiment, we also determined that rSV5-P/V-CPI− did not prevent coinfection with a rSV5-WT expressing ovalbumin (rSV5-OVA) (Parks and Alexander-Miller, 2002). Cells coinfected with rSV5-OVA and rSV5-P/V-CPI− were found to express both OVA and GFP by immunofluorescence assays (Fig. 3A). It is noteworthy that the micrographs shown in Fig. 2 also demonstrate that during infection with rSV5-P/V-CPI− many of the cells had undergone morphological changes associated with cell death such as cell rounding (Fig. 2, arrows), while during coinfection with rSV5-WT and rSV5-P/V-CPI− there were fewer cells that were undergoing cell rounding. Together with Fig. 1, the data in Fig. 2 demonstrate that rSV5-WT can block elements of the cell death pathway induced by rSV5-P/V-CPI−, including cell rounding and DNA fragmentation.

The reduced cytopathic effect seen during coinfection with rSV5-P/V-CPI− and rSV5-WT could be explained by rSV5-WT reducing levels of gene expression from rSV5-P/V-CPI−. To test this hypothesis, A549 cells were mock infected or infected with rSV5-OVA or rSV5-P/V-CPI− individually, or coinfected with both viruses at an m.o.i. of 5. At 12 h pi, cells were pulse radiolabeled with ^{35}S -amino acids, and proteins were immunoprecipitated from cell extracts with antibodies to the OVA protein to detect protein synthesis from rSV5-OVA or with antibodies to GFP to detect protein synthesis from rSV5-P/V-CPI−. As seen in Fig. 3B, the rate of protein synthesis of rSV5-OVA was slightly reduced during coinfection as compared to infection with rSV5-OVA alone (compare lanes 4 and 2, respectively, left panel of figure). By contrast, the rate of protein synthesis of rSV5-P/V-CPI−-derived protein did not change during coinfection with rSV5-OVA (compare lanes 3 and 4). The data in Fig. 3 demonstrate that cells can be simultaneously infected by both rSV5-WT and rSV5-P/V-CPI−, and that the decreased cytopathic effect seen in coinfected cells (Figs. 1 and 2) cannot be accounted for by reduced levels of gene expression from rSV5-P/V-CPI−.

During coinfection, rSV5-WT cannot block the loss of mitochondrial membrane potential induced by rSV5-P/V-CPI−

DNA fragmentation detected by TUNEL staining is a relatively late event in apoptotic cell death, occurring after the activation of caspase-3 (Liu et al., 1997). To determine if infection with rSV5-WT blocked an earlier stage of cell death induced by rSV5-P/V-CPI−, we measured changes in mitochondrial membrane potential induced during SV5 infection. Mitochondrial membrane permeabilization (MMP) is an important early event in most apoptotic pathways, which serves to initiate downstream events and results in apoptotic death (Susin et al., 1996 and references therein). This permeabilization results in the disruption of the inner mitochondrial membrane potential ($\Delta\Psi_m$) and is thought to precede the fragmentation of DNA during apoptosis (Susin et al., 1996 and references therein). Changes in mitochondrial membrane potential during SV5 infection were assayed by flow cytometry using a cationic lipophilic fluorochrome, tetramethylrhodaminemethylester (TMRM), whose accumulation in the mitochondrial matrix is driven by the electrochemical gradient. Cells with a high $\Delta\Psi_m$ show bright fluorescence, while loss of $\Delta\Psi_m$ reduces fluorescence. In addition, the size of cells was assayed as a measure of cell shrinkage, a late step in apoptosis (Gomez-Angelats et al., 2000).

A549 cells were mock infected or infected alone with rSV5-WT or rSV5-P/V-CPI− at an m.o.i. of 50 or coinfected at an m.o.i. of 25 for each virus. At 1, 15, and 30 h pi, cells were harvested and analyzed by flow cytometry for

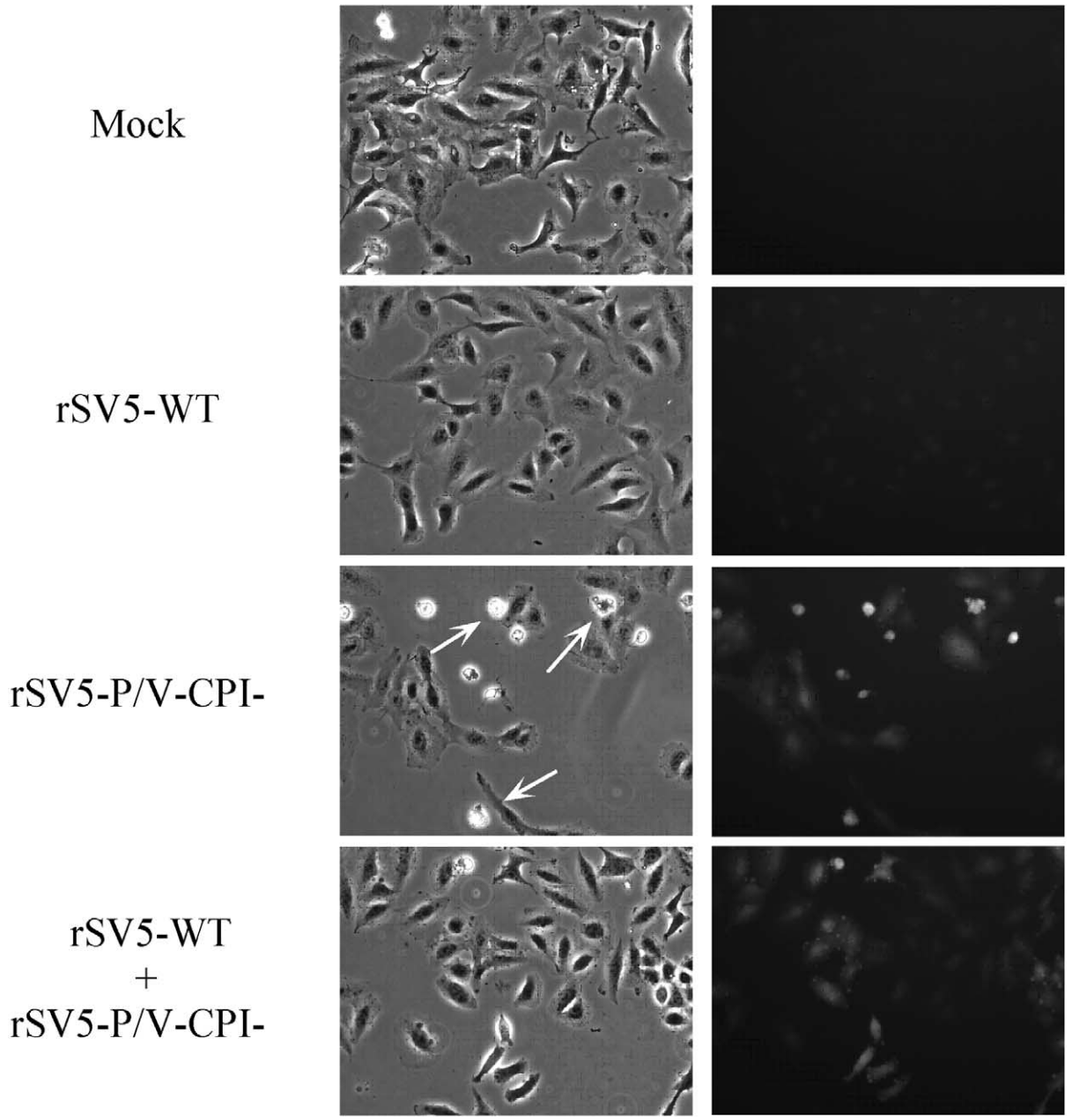

Fig. 2. Fluorescence microscopy of A549 cells coinfected with rSV5-WT and rSV5-P/V-CPI−. A549 cells were mock infected (top), infected with rSV5-WT (second row), or rSV5-P/V-CPI− (third row) at an m.o.i. of 50, or coinfected at an m.o.i. of 25 for each virus (bottom). At 48 h pi, cells were analyzed for expression of GFP by fluorescence microscopy. Phase contrast and GFP fluorescence for each sample are represented in the left and right columns, respectively. Arrows indicate rSV5-P/V-CPI− infected cells that have rounded up and show signs of later stages of apoptosis.

TMRM staining. A representative of three independent experiments is shown in Fig. 4, where the *y*-axis displays the level of TMRM staining as a measure of $\Delta\Psi_m$, while the *x*-axis displays forward scatter as a measure of the size of cells. Healthy cells are found in the upper right quadrant with high $\Delta\Psi_m$ and normal size. Cells that have dropped into the lower right quadrant have lost membrane potential but are still of normal size, so they have not yet entered the later stage of apoptosis (reviewed in Gomez-Angelats et al., 2000). Cells that are found in the lower left quadrant have lost membrane potential and have shrunk, identifying them as a population that are in the late stages of cell death or are already dead. As expected, ~98% of mock-infected cells were detected in the upper right quadrant at all time points examined (Fig. 4). During rSV5-WT infection, ~20% of cells showed a small decrease in $\Delta\Psi_m$ by 30 h pi (lower right quadrant). However, less than 1% of cells had additionally lost cell volume (lower left quadrant), consistent with the lack of TUNEL staining in rSV5-WT-infected cells (Fig. 1) (Wansley and Parks, 2002).

During infection with rSV5-P/V-CPI−, by 30 h pi a significant fraction of cells showed a large decrease in $\Delta\Psi_m$

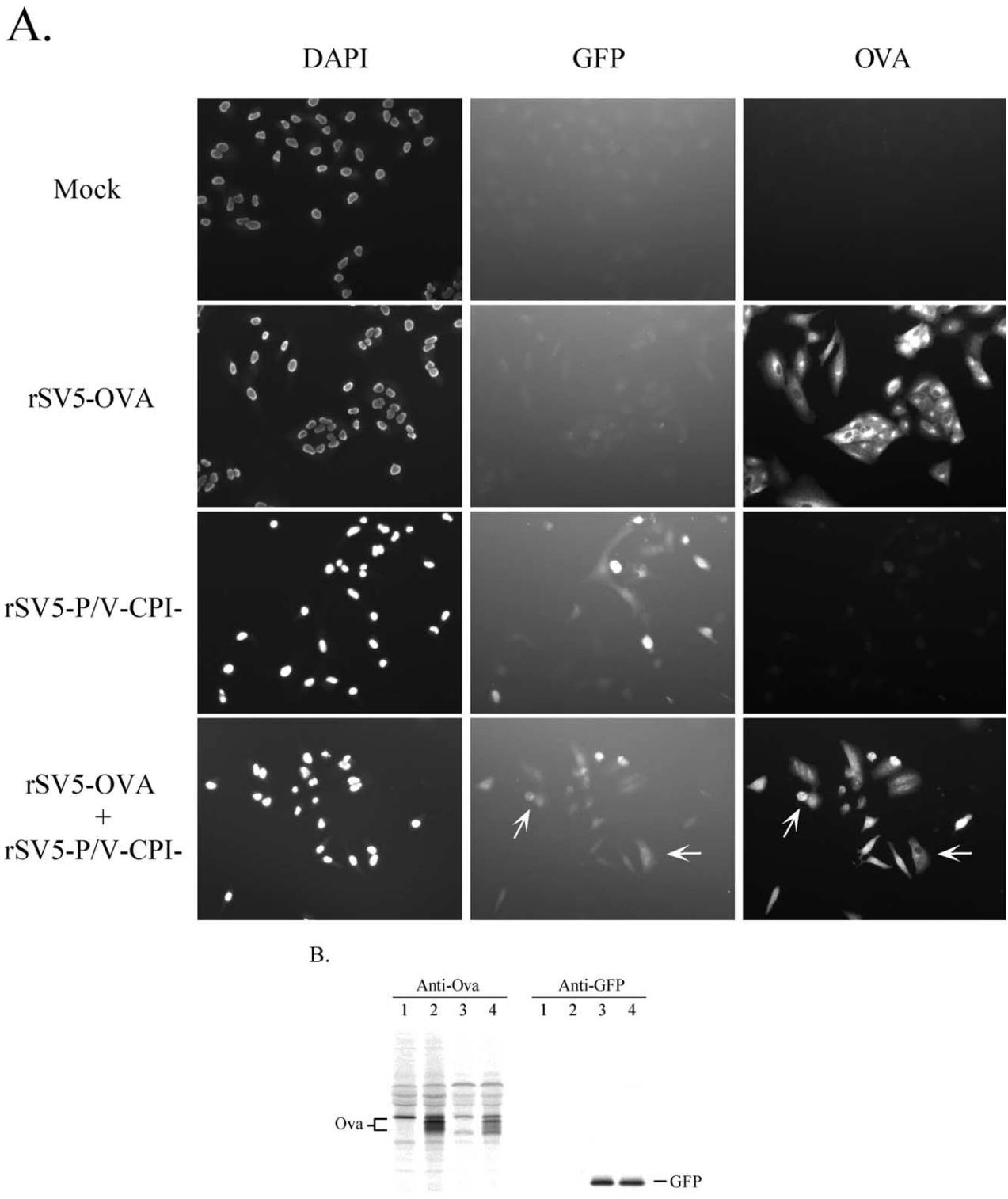

Fig. 3. Viral protein expression in cells coinfected with rSV5-OVA and rSV5-P/V-CPI−. (A) Immunofluorescence. A549 cells were mock infected (top), infected with rSV5-OVA (second row) or rSV5-P/V-CPI− (third row) at an m.o.i. of 5, or coinfected at an m.o.i. of 5 for each virus (bottom). At 48 h pi, cells were permeabilized and analyzed for expression of GFP (middle column) or ovalbumin (OVA; right column) by fluorescence microscopy and immunofluorescence, respectively. DAPI staining showed cells in each field (left column). (B) Rate of protein synthesis. A549 cells were mock infected (lane 1) or infected with rSV5-OVA (lane 2) or rSV5-P/V-CPI− (lane 3) at an m.o.i. of 5 for each virus, or coinfected with rSV5-OVA and rSV5-P/V-CPI− at an m.o.i. of 5 for each virus (lane 4). At 12 h pi, cells were radiolabeled for 15 min using Tran[^{35}S]-label and proteins were immunoprecipitated from cells extracts using antibodies to GFP or OVA before analysis by SDS–PAGE.

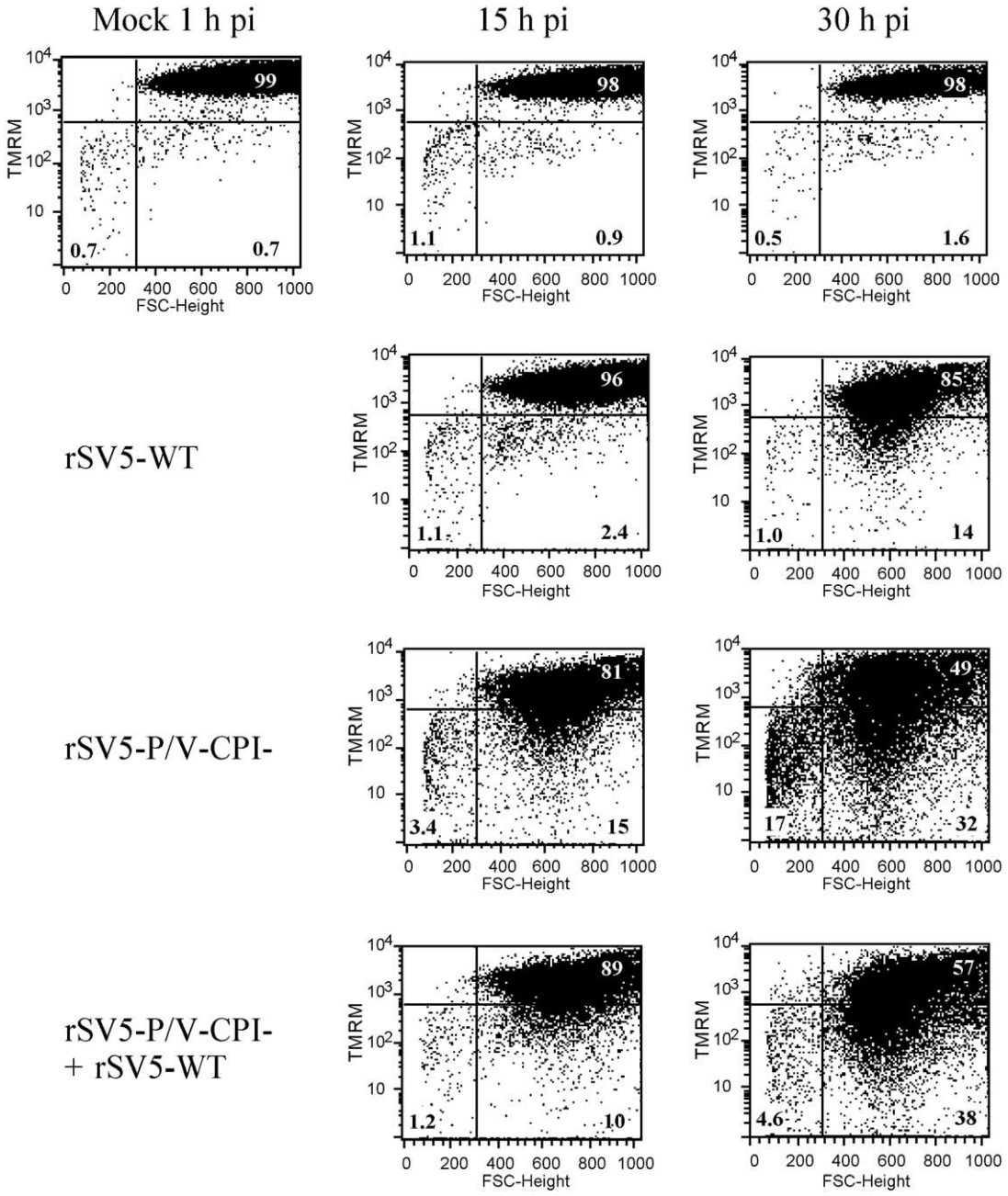

Fig. 4. Loss of mitochondrial membrane potential during coinfection. A549 cells were mock infected or infected with rSV5-WT or rSV5-P/V-CPI− at an m.o.i. of 50, or coinfected at an m.o.i. of 25 for each virus. At 15 and 30 h pi, cells were harvested and processed for flow cytometry using TMRM as a measure of $\Delta\Psi_m$ (*y*-axis) and forward scatter as a measure of cell size (*x*-axis). Data are representative of four independent experiments.

but not cell volume (32%, lower right quadrant), while 17% have lost membrane potential and cell volume (lower left quadrant), suggesting these cells had undergone cell death. Surprisingly, during coinfection with rSV5-WT and rSV5-P/V-CPI−, ~45% of the cells showed some loss of $\Delta\Psi_m$ by 30 h pi (combined lower right and left quadrants), suggesting that rSV5-WT was not able to block the loss of membrane potential induced by rSV5-P/V-CPI−. Importantly, however, only ~5% of cells that were coinfected with rSV5-WT and rSV5-P/V-CPI− had additionally lost cell volume, as compared to 17% of rSV5-P/V-CPI−-infected cells. The observation that infection with rSV5-WT reduces late-stage cell shrinkage is consistent with the data presented in Figs. 1 and 2 above showing that during coinfection, rSV5-WT can block cell rounding and DNA fragmentation induced by rSV5-P/V-CPI−. Taken together, these data indicate that while rSV5-WT can block steps associated with the later phase of apoptotic cell death (cell rounding, cell shrinkage, DNA fragmentation), it is not as effective at blocking the early initiation steps of cell death (loss

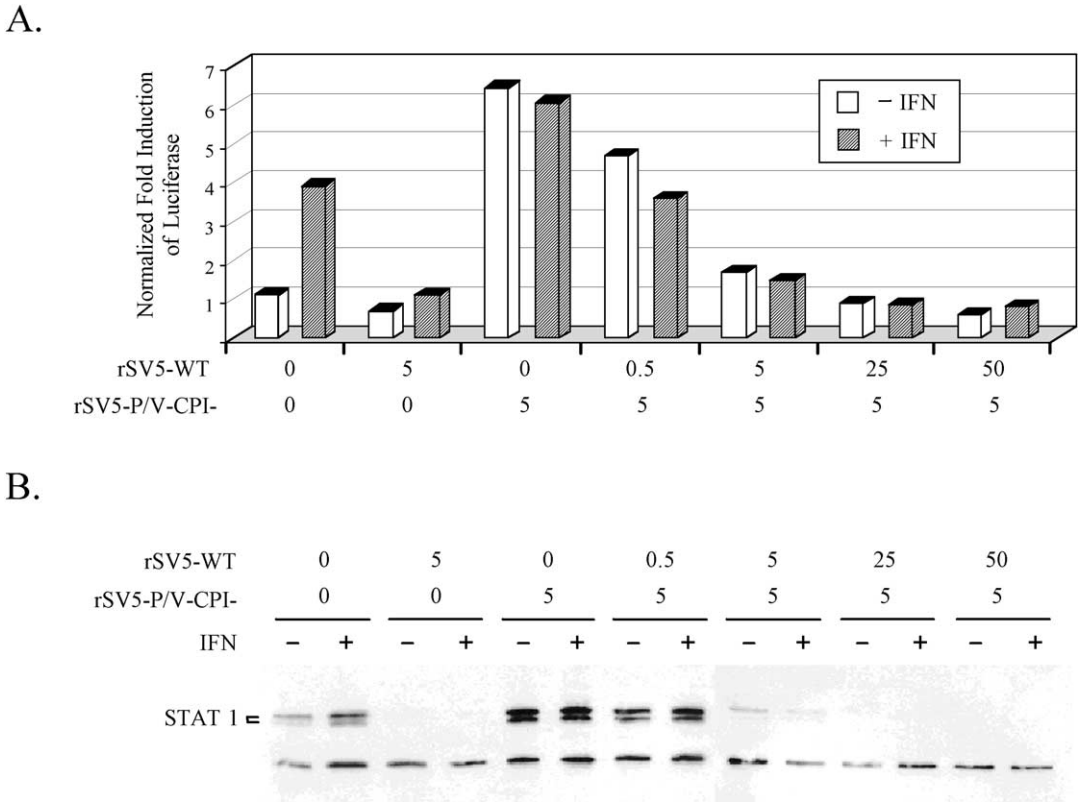

Fig. 5. IFN signaling and STAT1 degradation in cells coinfected with rSV5-V-CPI−. (A) Induction of ISRE. A549 cells were cotransfected with pSV-βgal and a plasmid containing the luciferase gene under control of an ISRE. Twenty-four hours later, cells were mock infected or infected individually or together with rSV5-WT and rSV5-P/V-CPI− at the indicated m.o.i.s. At 16 h pi, cells were incubated for 6 h with (hatched bars) or without (white bars) 2000 units of type I IFN. Normalized luciferase activity was calculated by dividing luciferase activity by β-gal activity and is expressed as a fold induction over that seen in mock-infected cells. Data are representative of three independent experiments. (B) STAT1 degradation during coinfection. A549 cells were infected as described in (A). After treatment with or without IFN for 6 h, cells were lysed and equivalent amounts of protein analyzed by Western blotting with antisera specific for the cellular STAT1 protein.

of mitochondrial membrane potential) induced by infection with rSV5-P/V-CPI−.

Infection with rSV5-WT can block ISRE signaling induced by rSV5-P/V-CPI− infection

Infection with wild-type SV5 has been shown to block the IFN-α/β signaling pathway by inducing the proteasomal degradation of STAT1 (Didcock et al., 1999b). In contrast, we have shown that infection with rSV5-P/V-CPI− does not induce degradation of STAT1 and results in an activation of the IFN signaling pathway as assayed by transfection of a plasmid containing the interferon-sensitive response element (ISRE) (Wansley and Parks, 2002). To determine if rSV5-WT could supply *trans*-acting factors that suppress IFN signaling induced by rSV5-P/V-CPI− infection, A549 cells were cotransfected with a plasmid containing the ISRE upstream of a luciferase reporter gene and the pSV-βgal plasmid to normalize for transfection efficiency. Twenty-four hours posttransfection, cells were infected with rSV5-WT or rSV5-P/V-CPI− alone or coinfected at various m.o.i.s as shown in Fig. 5A. Sixteen hours postinfection, cells were incubated with or without 2000 IU type I IFN to induce the ISRE-luciferase gene, and 6 h later cells were harvested and tested for induction of luciferase activity. As shown in Fig. 5A, infection with rSV5-WT did not induce the ISRE over levels observed in mock-infected cells, even with exogenous IFN added, consistent with previous results (Young et al., 2000). In contrast, infection with rSV5-P/V-CPI− induced ISRE activity with or without the addition of exogenous IFN (Wansley and Parks, 2002). When cells were coinfected with rSV5-WT and rSV5-P/V-CPI−, there was a progressive decrease in ISRE activity in cells infected with increasing amounts of rSV5-WT. Importantly, when equal amounts of each virus are added, the levels of ISRE induction were reduced to levels similar to that of rSV5-WT infection alone. This suppression of ISRE induction occurred in both the presence and the absence of IFN.

Cell lysates from the experiment in Fig. 5A were analyzed by Western blotting to determine the levels of STAT1 in A549 cells coinfected with rSV5-WT and rSV5-P/V-CPI. As seen in Fig. 5B, STAT1 was degraded in cells infected with rSV5-WT, and upregulated in rSV5-P/V-CPI−-infected cells. This increase in STAT1 expression is thought

to be because of the induction of IFN by this virus (Wansley and Parks, 2002). As the m.o.i. of rSV5-WT was increased during coinfection with rSV5-P/V-CPI−, there was a dose-dependent loss of STAT1 with levels of STAT1 similar to that seen with rSV5-WT alone when equal m.o.i.s were used. Taken together, these data indicate that during coinfection, the rSV5-WT phenotype of STAT1 degradation is dominant over that of rSV5-P/V-CPI−, and this loss of STAT1 prevents activation of ISRE.

Coinfection with rSV5-WT does not block activation of the IFN-β promoter by rSV5-P/V-CPI− infection

The original hypothesis to be tested was that rSV5-WT could supply *trans*-acting factors that suppress IFN and cell death induced by rSV5-P/V-CPI−. To determine whether rSV5-WT could block the induction of IFN synthesis by rSV5-P/V-CPI−, A549 cells were cotransfected with a plasmid containing the IFN-β promoter (pβlux), and the pSV-βgal plasmid for normalizing transfection efficiencies. Twenty-four hours posttransfection, cells were coinfected such that the m.o.i. of rSV5-P/V-CPI− infection was kept constant at 5 and increasing amounts of rSV5-WT were coinfected with rSV5-P/V-CPI−. Sixteen hours postinfection, cells were lysed and luciferase activity was measured. As expected, there was no detectable increase in luciferase activity over mock infection when cells were infected with rSV5-WT alone. As seen in Fig. 6, infection with rSV5-P/V-CPI− alone results in ~6.2-fold induction of the pβlux plasmid over mock-infected cells, as previously seen (Wansley and Parks, 2002). Surprisingly, this level of induction by rSV5-P/V-CPI− infection was not affected by increasing amounts of rSV5-WT added during coinfection.

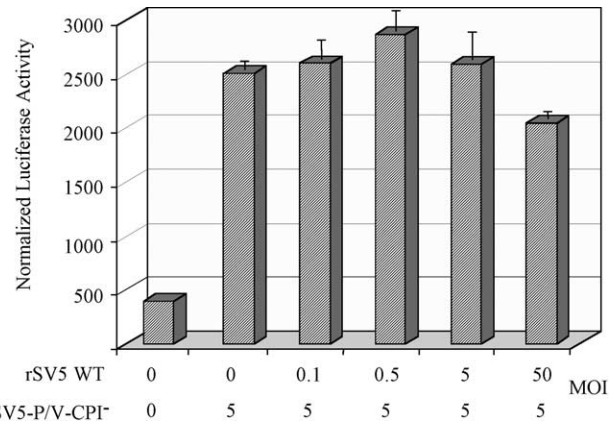

Fig. 6. Induction of pβlux plasmid during coinfection. A549 cells were cotransfected with a plasmid containing a luciferase gene under control of the IFN-β promoter and a pSV-βgal plasmid to normalize for transfection efficiency. Twenty-four hours posttransfection, cells were mock infected or coinfected at a constant m.o.i. of 5 with rSV5-P/V-CPI−, while rSV5-WT was added in increasing amounts. Sixteen hours pi, cells were harvested and normalized luciferase activity was calculated. Data are presented as the average of three experiments.

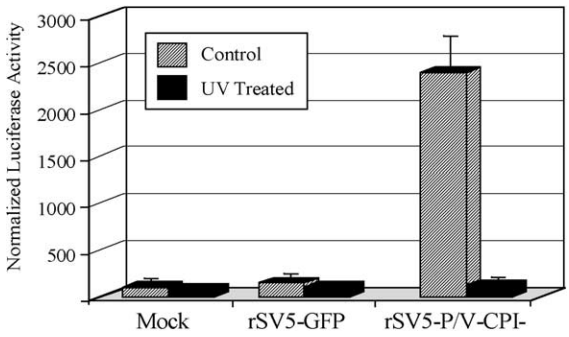

Fig. 7. UV treatment reduces the ability of rSV5-P/V-CPI− to induce the IFN-β promoter. A549 cells were transfected as described in the legend to Fig. 6. Twenty-four hours posttransfection, cells were mock infected or infected with rSV5-GFP and rSV5-P/V-CPI− at an m.o.i. of 50 (striped bars), or with virus that had been inactivated by UV treatment (solid bars). Sixteen hours pi, cells were harvested and normalized luciferase activity was measured. Data are presented as the average of three experiments.

The induction of the IFN-β promoter was dependent on gene expression from rSV5-P/V-CPI−. This was shown by UV inactivation of rSV5-P/V-CPI− (Fig. 7). A549 cells were cotransfected with the pSV-βgal and pβlux plasmids and then infected with ultraviolet (UV)-treated virus and tested for induction of the IFN-β promoter by luciferase assay. As expected, there was no luciferase activity observed in control or UV-treated mock- or rSV5-GFP-infected cells (Fig. 7). Infection with rSV5-P/V-CPI− resulted in induction of the IFN-β promoter, but when the virus was inactivated before being added to cells, induction of luciferase activity was abolished. These results indicate that while the activation of the IFN-β promoter by infection with rSV5-P/V-CPI− is dependent on viral gene expression, it cannot be blocked by coinfection with rSV5-WT.

Prior infection with rSV5-WT prevents activation of the IFN-β promoter by infection with rSV5-P/V-CPI−

All of the data presented in this article thus far suggest that during coinfection the phenotypes displayed during rSV5-WT infection are dominant over the phenotypes seen with rSV5-P/V-CPI− infection, with the exception of induction of the IFN-β promoter. One possible explanation for the result seen with the IFN-β promoter is that since infection with rSV5-P/V-CPI− results in earlier and higher than wild-type levels of protein and RNA expression (Wansley and Parks, 2002), activation of the IFN-β promoter is dominant in coinfections with rSV5-WT because rSV5-P/V-CPI− proteins are made earlier and in more abundance. In this hypothesis, an inducer of IFN (such as dsRNA) would be made early during coinfection, before the virus can establish mechanisms to counteract the host antiviral response. To test this hypothesis, we determined if prior infection with rSV5-WT would prevent the activation of the IFN-β promoter by infection with rSV5-P/V-CPI−. A timeline for the experimental design is shown in Fig. 8.

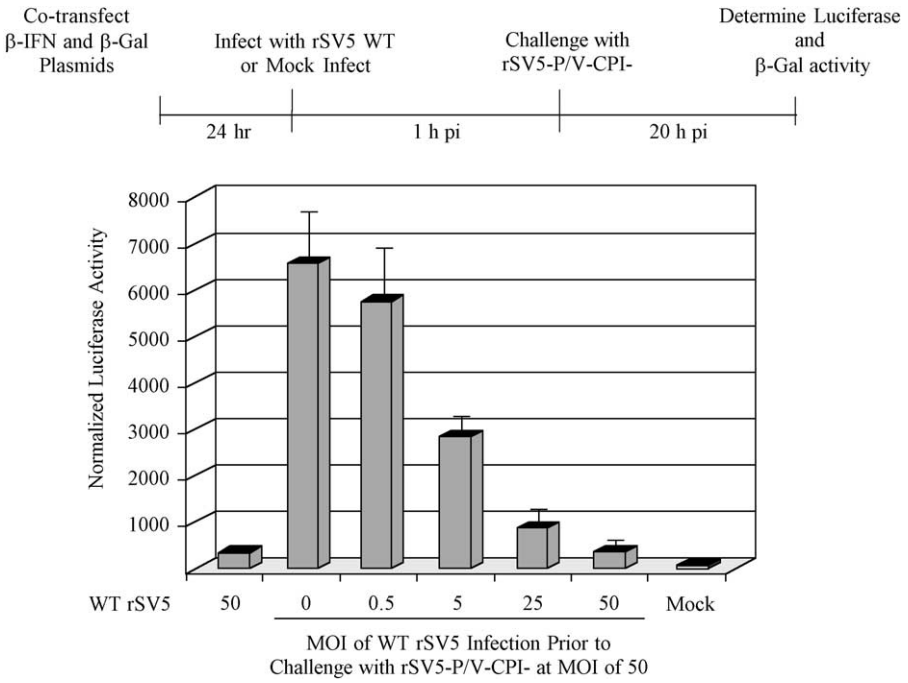

Fig. 8. Prior infection with rSV5-WT prevents activation of the IFN-β promoter by infection with rSV5-P/V-CPI−. A549 cells were cotransfected with the plasmids as described in the legend to Fig. 6, and 24 h later were mock infected, or infected with rSV5-WT at increasing m.o.i. as indicated. One hour pi, cells were then mock infected or infected with rSV5-P/V-CPI−. at an m.o.i. of 50. Sixteen hour pi, cells were harvested and luciferase activity was measured. Normalized luciferase activity was calculated as described in Fig. 5. Data are presented as the average of three experiments.

A549 cells were cotransfected with the pβlux and pSV-βgal plasmids, and 24 h later, infected with rSV5-WT at increasing m.o.i.s from 0 to 50. One hour later, the same cells were infected with rSV5-P/V-CPI− at an m.o.i. of 50, and luciferase activity was measured at 21 h after the initial infection with wild-type virus. Fig. 8 demonstrates that as the m.o.i. of prior infection with rSV5-WT increased, the level of IFN-β promoter activity induced by rSV5-P/V-CPI− decreased until it reaches approximately that of rSV5-WT alone when equal amounts of each virus are added. Prior infection with rSV5-WT did not exclude subsequent infection with rSV5-P/V-CPI− (data not shown). These data are consistent with the hypothesis that the timing of infection may play a role in the induction of IFN and that given only a 1-h head start, rSV5-WT can effectively prevent the activation of the IFN-β promoter seen during infection with by rSV5-P/V-CPI−.

Discussion

In a previous study, we discovered that by introducing six naturally occurring substitutions in the P/V gene of SV5 a number of phenotypes of the viral infectious cycle were affected including the control of RNA expression, activation of IFN synthesis and signaling pathways, and a dramatic increase in cytopathic effect (Wansley and Parks, 2002). Fig. 9 illustrates a model for the induction of host responses by infection with rSV5-P/V-CPI−. In this model, infection with rSV5-P/V-CPI− results in production of a viral inducer that leads to IFN-β synthesis, IFN-signaling, and expression of host antiviral genes (Fig. 9, left side).

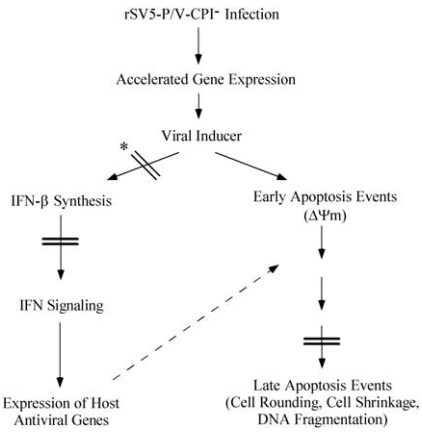

Fig. 9. Model for the steps in IFN and apoptotic pathways that are blocked by rSV5-WT during rSV5-P/V-CPI− infection. Depicted in the diagram are the events that occur during rSV5-P/V-CPI− infection to activate IFN and apoptotic pathways. Steps where rSV5-WT infection has been shown to block this induction by rSV5-P/V-CPI− are represented by (=), while steps where prior infection with rSV5-WT blocks induction by rSV5-P/V-CPI− infection are represented by an asterisk. Dotted line indicates a possible pathway whereby IFN induced gene products activate apoptotic pathways.

Additionally, production of an inducer (which may be the same or a different inducer than that seen with IFN) activates apoptotic pathways, resulting in cell death (Fig. 9, right side). Based on the previous finding that rSV5-WT has active mechanisms to prevent IFN synthesis (He et al., 2002; Poole et al., 2002) as well as IFN signaling (Didcock et al., 1999a,b), we have tested the hypothesis that the new phenotypes seen in rSV5-P/V-CPI− infections could be corrected by coinfection with rSV5-WT. Our results indicate that rSV5-WT possesses active mechanisms to prevent three phenotypes shown in our model for rSV5-P/V-CPI− infection: induction of IFN synthesis, induction of IFN signaling, and a late step in the apoptotic pathway.

In our previous study, we reported that while rSV5-WT is noncytopathic for most cell types, infection with rSV5-P/V-CPI− resulted in rampant cell death by a pathway with characteristics of apoptosis (Wansley and Parks, 2002). These data suggested that rSV5-WT either does not synthesize an inducer of cell death to levels seen with rSV5-P/V-CPI−, or rSV5-WT has mechanisms to actively block the induction of apoptosis, and these mechanisms are defective for rSV5-P/V-CPI−. We have found that during infection with rSV5-WT, the majority of the cells retained their normal mitochondrial membrane potential, with a slight loss in $\Delta\Psi_m$ in some infected cells. However, very few cells in the population with a slight loss in $\Delta\Psi_m$ also showed late stages of apoptosis (e.g., TUNEL staining, cell rounding, cell shrinkage), indicating that rSV5-WT may have a mechanism to block the induction of cell death at a later stage. This proposal was supported by results from coinfection experiments, where the induction of DNA fragmentation and cell rounding but not loss of $\Delta\Psi_m$ by rSV5-P/V-CPI− was blocked by coinfection with rSV5-WT. Our results indicate that rSV5-WT has active mechanisms to block the induction of cell death pathways located downstream of the loss of mitochondrial membrane potential, but before cell rounding and the fragmentation of DNA. In addition to being defective in blocking the late step in apoptosis, we propose that rSV5-P/V-CPI− makes more of the inducer of cell death, as evidenced by the finding of more rSV5-P/V-CPI−-infected cells showing a significant loss of $\Delta\Psi_m$.

Blocking the IFN signaling pathway is a common theme seen with a number of paramyxoviruses, although there are several different mechanisms used to accomplish the same goal. For example, human parainfluenza virus type 2 (HPIV2) blocks this pathway by inducing the degradation of STAT2 (Parisien et al., 2001), while Sendai virus (SeV) can accomplish this by preventing the phosphorylation of STAT1 (Takeuchi et al., 2001) or by changing the stability of STAT1 (Garcin et al., 2003). While infection with rSV5-WT results in a block in IFN signaling, we have previously shown that infection with rSV5-P/V-CPI− induced ISRE signaling in the presence or absence of exogenous IFN (Young et al., 2000; Wansley and Parks, 2002). In this study we show that during coinfection the wild-type phenotype of blocking the induction of ISRE is dominant as expected, since SV5 has a known active mechanism to disrupt the IFN signaling pathway by V-mediated degradation of STAT1 (Didcock et al., 1999b). These data support previous work using transfections of plasmids containing the V protein of SV5 or CPI− and shows that the ability to target STAT1 degradation seen with plasmid-derived V can be reproduced when expressed from the rSV5-WT genome.

α/βIFNs can induce apoptosis by activating the FADD/caspase 8 pathway (Balachandran, et al., 2000). In addition, previous results have shown that STAT1-null cell lines are resistant to apoptosis induced by tumor necrosis factor-α (Kumar et al., 1997), suggesting that STAT1 can function in the induction of apoptosis, possibly by activating ISRE elements upstream of antiviral genes required for apoptosis. Thus, SV5 actively blocks IFN signaling by targeting STAT1 for degradation and this could indirectly result in a block in activation of apoptosis. During rSV5-P/V-CPI− infection, α/βIFNs are synthesized (Wansley and Parks, 2002), and it is possible that this then activates cell death pathways since STAT1 is not degraded by rSV5-P/V-CPI− infection. A similar model has been proposed during coinfection with ncp and cp strains of HAV. An ncp strain of HAV can interfere with the ability of a cp strain to induce cell death, and it is proposed that this is because the ncp strain of HAV can inhibit the activation of IFN-induced apoptosis (Brack et al., 2002). Together, our model (Fig. 9) proposes that SV5 blocks cell death pathways by either direct (e.g., by affecting activation of caspases; right side of Fig. 9) or indirect methods (e.g., by blocking STAT1 induction of antiviral genes) and does so at a stage after the loss of $\Delta\Psi_m$, but before the execution step involving DNA fragmentation. Future work will focus on the mechanism of the induction of cell death by rSV5-P/V-CPI− and how rSV5-WT blocks activation of apoptosis.

rSV5-WT is a very poor inducer of IFN-β synthesis (Didcock et al., 1999a; Poole et al., 2002; He et al., 2002; Wansley and Parks, 2002). It is possible that the lack of IFN production by infection with rSV5-WT could occur because a viral protein acts to disable the host cell machinery that senses viral infection. For example, the human papillomavirus E6 protein binds IRF-3 (Ronco et al., 1998), while the influenza virus NS1 protein has been found to block the phosphorylation of IRF-3 and thereby prevent translocation to the nucleus (Talon et al., 2000). Previous data with SV5 has shown that the V protein prevents dsRNA-induced translocation of IRF-3 to the nucleus (He et al., 2002). In the case of rSV5-P/V-CPI− infection, the activation of the IFN-β promoter could be occurring because rSV5-P/V-CPI− has lost the ability to disable the host sensing machinery, possibly because the CPI− V protein in rSV5-P/V-CPI− is defective in blocking IRF-3 activation. However, activation of IFN responses is likely to be more complicated in the case of rSV5-P/V-CPI− infections, since the previous transfection experiments have shown that the

wild-type SV5 V protein and CPI− V protein are equally potent at blocking activation of the IFN-β promoter by dsRNA (Poole et al., 2002). Therefore, the activation of the IFN-β promoter during rSV5-P/V-CPI− infection cannot be explained simply by the inability of the CPI− V protein to block induction of IFN-β by dsRNA.

While dsRNA produced as a viral byproduct during infection is often a potent inducer of IFN (Biron and Sen, 2001; Goodbourn et al., 2000), it is possible that dsRNA is not primarily responsible for activating IFN pathways during rSV5-P/V-CPI− infection. In this regard, it has been found that the N gene product of measles virus can activate IRF-3 to induce IFN synthesis (tenOever et al., 2002). Thus, it is possible that the NP protein of SV5 acts as an inducer of IFN similar to that found with measles virus, and that the interactions of NP with P or V (Randall and Bermingham, 1996) normally act to block the NP-induced IFN response in cells infected with rSV5-WT. The hypothesis that dsRNA is not the inducer of IFN during rSV5-P/V-CPI− infection could account for the discrepancy seen with blocking activation of the IFN-β promoter with CPI− V expressed alone by transfection, and activation of the IFN-β promoter when CPI− V is expressed in the context of other viral components as seen during infection with rSV5-P/V-CPI−. In light of this discrepancy, it is important to emphasize that the recombinant rSV5-P/V-CPI− virus does not share all of the properties seen with the native CPI− virus, and it cannot be assumed that the transfer of the CPI− P/V mutations has transferred all of the CPI− phenotypes to WT rSV5.

The original CPI− virus which was the source of the P/V gene in rSV5-P/V-CPI− is a poor inducer of IFN-β (Poole et al., 2002). In contrast, our results have shown that rSV5-P/V-CPI− (which has the same CPI− P/V gene but on a different genetic background) was a strong inducer of IFN-β, resulting in the induction of similar levels of IFN as another known inducer of IFN, human parainfluenza virus type 2 (5000–10,000 U/ml as compared to 5000–20,000 U/ml, respectively, Wansley and Parks, 2002). By making the amino acid changes in the P/V gene with rSV5-P/V-CPI−, we may have abolished the ability of P or V to interfere with NP induction of IFN. In this case, the NP gene of CPI− would be prevented from inducing IFN when interacting with homologous CPI− P and V proteins. Likewise, rSV5-WT NP could be prevented from inducing IFN when interacting with homologous SV5 P and V proteins. However, when rSV5-WT NP interacts with the heterologous P/V gene products of CPI−, it could behave quite differently.

If the CPI− V protein can still function to block dsRNA-induced IFN pathways, what then is the mechanism by which rSV5-P/V-CPI− induces IFN? The most striking result from our studies is that rSV5-WT can block activation of the IFN-β promoter by rSV5-P/V-CPI− only after prior infection, but not during coinfection with both viruses. Similar results have been found with HAV, where it has been shown that preinfection with an ncp strain can reduce the replication of a cp strain (Brack et al., 2002). It was hypothesized that the ncp strain controls viral replication to ensure that dsRNA does not accumulate to levels high enough to overcome the block in IFN pathways. In this case, there is a balance between allowing enough gene expression necessary to inhibit the antiviral response early on, but not allowing viral byproducts to accumulate to levels where this inhibition could be overcome. We propose that a similar mechanism is in place with rSV5-WT, where gene expression early after infection is controlled to avoid inducing the antiviral response before inhibitory mechanisms are in place. By contrast, rSV5-P/V-CPI− has lost the early control mechanisms that prevent activation of IFN pathways. Alternatively, it is possible that there is a limiting host factor that is required for replication of rSV5-WT and rSV5-P/V-CPI−, and that during the prior infection with rSV5-WT, this host factor is titrated away and is no longer available to support the replication of rSV5-P/V-CPI−, resulting in decreased viral gene expression from rSV5-P/V-CPI−.

The SeV V protein is thought to interact with NP to inhibit RNA replication by blocking NP encapsidation of the genome (Horikami et al., 1992). Since the SV5 V protein binds to soluble but not polymeric NP (Randall and Bermingham, 1996), it is possible that the CPI−P/V substitutions decrease the ability of V to bind wild-type NP and effectively inhibit replication during rSV5-P/V-CPI− infection. Alternatively, the P/V mutations could increase the activity of the P protein, resulting in accelerated RNA synthesis due to a more active polymerase. In both of these cases, wild-type SV5 regulates RNA synthesis early during infection so as not to cross the threshold that activates the IFN response, while rSV5-P/V-CPI− has lost control of this regulation of RNA synthesis, and the IFN synthesis pathway is activated. It is important to note that the P and V proteins of SV5 serve multiple functions in the viral life cycle (Lamb and Kolakofsky, 2001), and it is not known whether the mutations in rSV5-P/V-CPI− affect the function of the P or the V protein. Therefore, we cannot attribute the induction of IFN to either the P or the V proteins of rSV5-P/V-CPI−. Work is in progress to determine which of these proteins are responsible for each phenotype seen with rSV5-P/V-CPI− infection.

In summary, we propose a model whereby rSV5-WT components can act *in trans* to correct the induction of apoptosis, IFN signaling, and the IFN-β promoter that is seen during infection with rSV5-P/V-CPI−. In this model, the P or V protein of rSV5-WT acts *in trans* to restrict an inducer of IFN (e.g., the synthesis of dsRNA or the activity of NP) to levels that will not trigger the induction of IFN. As an additional level of control, rSV5-WT can actively block the induction of IFN synthesis by inhibiting the translocation of IRF-3 to the nucleus (He et al., 2002) and IFN signaling by degrading STAT1 (Didcock et al., 1999a), blocking host cell factors involved in the antiviral response.

Either the same or additional viral inducers activate apoptotic pathways during rSV5-P/V-CPI− but not rSV5-WT infection. The degradation of STAT1 and block in IFN signaling and/or synthesis may contribute to the lack of cytopathic effect seen during rSV5-WT infection. Our data are consistent with the hypothesis that rSV5-WT also possesses an active mechanism to block the induction of cell death at a step after the loss of mitochondrial membrane potential, but before the fragmentation of DNA. Work is in progress to further elucidate the mechanisms by which rSV5-WT prevents the activation of host antiviral responses, as well as what functions are defective in the case of the rSV5-P/V-CPI− mutant.

Materials and methods

Cells, viruses, and UV inactivation

Monolayer cultures of cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). W3A strain of SV5 was grown in MDBK cells. rSV5-P/V-CPI− was recovered from cDNA and grown in Vero cells as described previously (Wansley and Parks, 2002). rSV5-OVA was recovered from cDNA and grown as described previously (Parks and Alexander-Miller, 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 UV lamp at a distance of 6.5 cm, and the resulting media was added to A549 cells and subsequently treated as infected samples. This procedure eliminated all infectivity as determined by plaque assays.

Transfection of reporter plasmids and IFN protection assays

Induction of the IFN-β promoter was assayed using pβlux, a plasmid containing the upstream 38−470 bases of the murine IFN-β regulatory region, including all four positive regulatory domains (PRDI to IV) linked to a luciferase reporter gene (Noah et al., 1999). 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. Transfection-infection experiments were performed in six-well dishes of A549 cells as described previously using FUGENE 6 (Wansley and Parks, 2002). Normalized luciferase activity was calculated as luciferase activity divided by β-galactosidase activity. Expression from the pSV-βgal plasmid was not responsive to IFN stimulation.

A pISRE-luc plasmid containing five copies of the ISG54 ISRE element upstream of a TATA box and luciferase reporter gene (Parisien et al., 2001) was used to measure induction of ISRE transcription. Transfection-infection experiments using A549 cells were carried out as described previously (Wansley and Parks, 2002). Sixteen hours pi, cell media was supplemented for 6 h with or without 2000 units human universal type I IFN (αA/D, PBL Biomedical Laboratories), as described (Young et al., 2000). Cells were harvested in reporter lysis buffer and normalized luciferase activity was calculated.

Western blotting, isotopic labeling of polypeptides, and immunoprecipitation analysis

Six-well dishes of A549 cells were infected with WT rSV5-GFP or rSV5-P/V-CPI$^-$ as described in each figure legend. Cell lysates were treated as described (Wansley and Parks, 2002) and analyzed by Western blotting with rabbit antisera to the cellular STAT1 protein (clone 554, Santa Cruz Biotechnology) followed by HRP-conjugated secondary antibodies and ECL.

To detect radiolabeled proteins, A549 cells that were mock infected, infected individually, or coinfected with rSV5-GFP or rSV5-P/V-CPI− were radiolabeled for 15 min at 12 h pi using 200 μCi/ml Tran[^{35}S]-label. Cells were lysed in 1% SDS and proteins immunoprecipitated from cell extracts using polyclonal antibodies to GFP (Invitrogen) or ovalbumin (OVA) (Parks and Alexander-Miller, 2002) before analysis by SDS–PAGE as described (Wansley and Parks, 2002).

Fluorescence microscopy assays

Microscopy experiments were performed as previously described on a Nikon Eclipse fluorescence microscope using a 20× lens (Wansley and Parks, 2002). For apoptosis assays, 3.5-cm dishes of A549 cells were mock infected or infected with rSV5 viruses and fixed with a 3:1 mix of ethanol:acetic acid for 10 min at RT. TUNEL staining was performed as described by the In Situ Death Detection Kit (Roche Molecular Biochemicals). For immunofluorescence assays, six-well dishes of A549 cells were mock infected or infected with rSV5-OVA (Parks and Alexander-Miller, 2002) or rSV5-P/V-CPI− as described in the figure legends. Cells were fixed and permeabilized with saponin before incubating with rabbit antisera to ovalbumin followed by rhodamine-conjugated goat anti-rabbit secondary antibodies and observed by fluorescence microscopy. Subconfluent monolayers were used to collect data on a per cell basis and to avoid crowding of cells.

Flow cytometry analysis

Six-well dishes of cells were mock infected or infected with rSV5-GFP or rSV5-P/V-CPI− as described in each figure legend. At each time p.i., both media and cells were harvested and cells were treated as described previously (Wansley and Parks, 2002). The GFP fluorescence of individual cells was measured using a FACScalibur flow cytometer (Becton Dickinson Biosciences). Loss of mitochon-

drial membrane potential ($\Delta\Psi_m$) was analyzed by TMRM staining as described (Castedo et al., 2002).

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