Exchange of P/V genes between two non-cytopathic simian virus 5 variants results in a recombinant virus that kills cells through death pathways that are sensitive to caspase inhibitors

Patrick J. Dillon, Elizabeth K. Wansley,† Virginia A. Young, Martha A. Alexander-Miller and Griffith D. Parks

Department of Microbiology and Immunology, Wake Forest University, School of Medicine, Winston–Salem, NC 27157-1064, USA

The paramyxovirus Simian virus 5 (SV5) is largely non-cytopathic in human epithelial and fibroblast cells. WF-PIV has been described previously as a naturally occurring SV5 variant that encodes P and V proteins differing from the wild-type (WT) SV5 proteins in eight and five amino acid positions, respectively. In this study, it is shown that WF-PIV is like WT SV5 by being largely non-cytopathic in A549 lung epithelial cells. However, substitution of the WF-PIV P/V gene into the background of WT SV5 resulted in a hybrid virus (P/V-WF) that induced apoptotic cell death not seen with either of the parental viruses. The kinetics of HeLa cell killing and induction of apoptosis by the P/V-WF chimera differed from those of the previously described P/V-CPI– chimera by being slower and less extensive. HeLa cell killing by the P/V-WF chimera was effectively reduced by inhibitors of caspase-9, but not of caspase-8. These results demonstrate that an exchange of P/V genes from two non-cytopathic SV5 variants can produce apoptosis-inducing chimeras, and that the role of the SV5 P/V gene products in limiting apoptosis can be dependent on expression in the context of a native viral genome.

The balance between apoptotic host-cell responses to virus infection and viral countermeasures to block apoptosis can be important for determining host-cell tropism, virus dissemination and pathogenesis (Barber, 2001; Lyles, 2000; Roulston et al., 1999). A number of negative-strand RNA viruses have been shown to induce apoptosis (e.g. Esolen et al., 1995; Koyama et al., 2003; Ludwig et al., 2006; Zorn et al., 1994). Vesicular stomatitis virus induces cell death in BHK and HeLa cells through pathways that can include activation of caspase-9 and caspase-8 (Kopecky & Lyles, 2003; Kopecky et al., 2001). In the case of the paramyxovirus Sendai virus (SeV), infection of CV-1 and HepG2 cells with the Fushimi strain results in cell death through activation of caspases-3 and -8 and, to a lesser extent, caspase-9 (Bitzer et al., 1999). The SeV proteins that modulate apoptosis have not been defined completely, but the C proteins and trailer RNA can contribute to the efficiency of cell killing (Garcin et al., 1998; Iseni et al., 2002; Koyama et al., 2003). For respiratory syncytial virus (RSV), infection of human lung cells results in the upregulation of caspase-3, -6, -7, -8 and -10 mRNAs (Kotelkin et al., 2003). As RSV induces apoptosis at late stages of infection, it has been hypothesized that anti-apoptotic genes induced during infection act to delay apoptosis, although the pathways involved are not understood completely (Kotelkin et al., 2003).

The W3A strain of the paramyxovirus Simian virus 5 (SV5) has the unusual property of being largely non-cytopathic in most epithelial and fibroblast cell types, and this results in highly productive persistent infections with minimal cell death (Choppin, 1964; He et al., 2001; Lin et al., 2003; Parks et al., 2002; Wansley & Parks, 2002). The SV5 V protein has been implicated in playing a role in limiting activation of apoptosis. The V protein is expressed from the viral P/V gene through a process called RNA editing (Lamb & Kolakofsky, 1996). For SV5, accurate transcription of the P/V gene produces an mRNA that encodes the V protein, whilst the P mRNA contains two additional non-template G residues added co-transcriptionally by the viral polymerase at a precise location in the P/V transcript (Lamb & Kolakofsky, 1996). Thus, the V and P proteins share the same N-terminal segment (the P/V region), but have different C-terminal regions. Whilst the P protein functions as an essential subunit of the viral polymerase, the V protein is thought to play multiple roles in counteracting host antiviral responses (e.g. Andrejeva et al., 2004; Young et al., 2000, 2006). Recombinant SV5 (rSV5) mutants encoding a V protein with N- or C-terminal alterations are ineffective at
counteracting the activation of host-cell apoptotic pathways (He et al., 2002; Sun et al., 2004; Wansley & Parks, 2002; Wansley et al., 2003).

We have previously described an rSV5 that was engineered to encode six naturally occurring P/V gene substitutions derived from the CPI– variant of SV5 (Wansley & Parks, 2002; Fig. 1); these CPI– P/V gene substitutions are associated with defects in V-mediated targeting of STAT1 for degradation (Chatziandreou et al., 2002). The P/V-CPI– chimeric virus has strikingly different phenotypes from WT rSV5, including being highly cytopathic to human epithelial and fibroblast cell types (Wansley & Parks, 2002). Whilst the introduction of P/V gene substitutions from the naturally occurring CPI– variant converts WT rSV5 into a cytopathic virus, the apoptosis-inducing properties of the bona fide CPI– variant, which was the source of the P/V substitutions in the P/V-CPI– chimera, were not tested in previously published reports. Thus, the inherent contribution of the CPI– P/V gene products to limiting or inducing cytopathic effects in the context of the bona fide virus versus in the P/V-CPI– chimera is not known.

Wake Forest parainfluenza virus (WF-PIV) is a previously described, naturally occurring variant of SV5 (Young et al., 2006) that differs from WT rSV5 in all of the genes that have been sequenced to date [Young et al., 2006; shown schematically in Fig. 1(a)]. The WF-PIV P and V proteins differ from those of WT rSV5 at eight and five amino acid positions, respectively (Fig. 1b). Here, we show that the native WF-PIV is similar to WT rSV5, in that cytopathic effects are not induced significantly following infection of human A549 cells. However, substitution of the WF-PIV P/V gene into the background of WT rSV5 resulted in a recombinant hybrid virus (P/V-WF) that activated cell-death pathways not seen with either of the two parental viruses.

**Fig. 1.** The naturally occurring variant WF-PIV is non-cytopathic in A549 cells. (a) The 3’ end region is shown for the WT rSV5 engineered to express GFP (WT rSV5-GFP; shaded box), the P/V-CPI– chimeric virus harbouring six substitutions in the shared P and V region (dark box), the WF-PIV variant (open box) and the chimera P/V-WF harbouring the entire P/V gene from WF-PIV in the context of the remaining WT rSV5-GFP genes. (b) P/V amino acid differences that distinguish the two chimeras P/V-CPI– and P/V-WF are shown compared with WT rSV5. (c) A549 cells were mock-infected or infected at an m.o.i. of 10 with WF-PIV or WT rSV5. Cells were examined by microscopy at 24 and 72 h.p.i. (d) Cell viability was measured by using an MTT assay at 72 h.p.i. as described in the text. Results are the mean of four experiments with SD.
Fig. 1(a) illustrates schematically the viruses analysed in this study. The recombinant W3A strain of SV5 (hereafter called WT rSV5) and rSV5 encoding green fluorescent protein (GFP) between HN and L (He et al., 1997) were the kind gift of Robert Lamb and Biao He (Northwestern University, IL, USA). The chimeric P/V-CPI− virus contains substitutions of six amino acids from the shared N-terminal P/V region of the SV5 variant CPI− (Y26H, V32I, T33I, L50P, L102P and S157F; Fig. 1b) inserted into the corresponding region of WT rSV5-GFP [black box in Fig. 1(a)]. Importantly, WF-PIV has five differences from WT rSV5 in the shared N-terminal P/V region (Fig. 1b), three of which are common to CPI− (V32I, T33I and S157F) and two of which are unique to WF-PIV (I13V and P152L). There are also three amino acid differences in the P-specific region between WF-PIV and WT rSV5 (G174E, K184R and T293K; Fig. 1b).

As shown in Fig. 1(c), A549 cells infected with the naturally occurring WF-PIV showed minimal cytopathic effects at 72 h post-infection (p.i.). This result was confirmed by an MTT-based cell-proliferation assay (Promega), where there was a minimal change in viability of cells at 72 h p.i. with either WT rSV5 or WF-PIV (Fig. 1d) and no significant difference in levels of apoptotic markers between infected cell cultures (not shown). Thus, WT rSV5 and WF-PIV are two naturally occurring viruses that are poor inducers of cytopathic effects.

The differences between the P/V genes of WF-PIV and WT rSV5 (Fig. 1b) raised the hypothesis that introducing the P/V gene from the non-cytopathic WF-PIV variant into the WT rSV5-GFP genome would result in a cytopathic chimeric virus, similar to our previous finding with the chimeric P/V-CPI− virus (Wansley & Parks, 2002). To test this hypothesis, we analysed the cell-killing phenotype of a previously described chimeric rSV5 that contained the entire P/V region of WF-PIV inserted into the backbone of WT rSV5-GFP (Young et al., 2006).

The P/V-WF chimera induced a cytopathic effect not seen with either of the parental viruses, WF-PIV or WT rSV5-GFP (data not shown). To determine whether the new chimeric P/V-WF virus induced apoptotic markers, cellsurface annexin staining was determined by flow cytometry using phycoerythrin-conjugated Annexin V (BD Biosciences) as described previously (Arimilli et al., 2006). As shown in Fig. 2(a), there was a time-dependent increase in the percentage of HeLa cells with high levels of Annexin V staining following infection with either P/V-CPI− or P/V-WF. However, cells infected with the new P/V-WF chimera showed a slower rate of appearance of Annexin V-positive cells relative to cells infected with the P/V-CPI− chimera [see 48 h time point in Fig. 2(a)]. Very similar results were seen in the case of infected A549 cells (Fig. 2b).

An MTT assay was used to quantify differences in the kinetics of cell viability following infection with the P/V-WF and P/V-CPI− chimeras. As shown in the middle panel of Fig. 2(c), HeLa cells infected with P/V-CPI− showed a rapid and extensive loss of cell viability, down to <20 % of that of mock-infected cells by 48 h p.i. In contrast, cells infected with the new P/V-WF chimera showed a slower loss of viability, and values at 48 and 72 h p.i. were approximately 50–60 % of those of mock-infected control samples [right-hand panel, − samples, Fig. 2(c)].

To determine whether death induced by the P/V mutants could be inhibited by a pan-caspase inhibitor, HeLa cells that

Fig. 2. Induction of apoptosis by infection with the chimeric P/V mutant viruses and inhibition of cell killing by a pan-caspase inhibitor. HeLa (a) or A549 (b) cells were mock-infected or infected at an m.o.i. of 10 with the indicated viruses. At 24, 48 and 72 h p.i., samples were analysed for cell-surface Annexin V staining. Tumour necrosis factor alpha-treated cells served as a positive control. Results are representative of two independent experiments. (c) HeLa cells were infected with the indicated viruses at an m.o.i. of 10 and treated with or without 100 μM pan-caspase inhibitor. At the indicated times p.i., cell viability was measured by using an MTT assay. Results are the mean of four experiments with SD.
were mock-infected or infected with WT rSV5-GFP, P/V-CPI− or P/V-WF were incubated with 100 μM of the pan-caspase inhibitor Z-VAD-FMK (Promega). As shown in the cell-viability assay in Fig. 2(c), addition of pan-caspase inhibitor to cells infected with either of the P/V chimeras resulted in a partial restoration of viability. Together, these data indicate that exchange of P/V genes between the two non-cytopathic viruses WT rSV5 and WF-PIV results in a chimeric virus that induces caspase-dependent death pathways, but the kinetics and extent of cell killing by the P/V-WF chimera are lower than those seen with the P/V-CPI− chimera.

Addition of a caspase-8 inhibitor (Z-IETD) did not restore viability to A549 or HeLa cells infected with either P/V mutant virus (data not shown). However, addition of a caspase-9 inhibitor (Z-LEHD; Biosource) resulted in a statistically significant (*P* < 0.001; Student’s *t*-test) increase in cell viability following infections of HeLa cells with both chimeras (Fig. 3a). Cell viability was restored to a higher percentage of that of mock-infected control samples in the case of the P/V-WF chimera compared with the P/V-CPI− chimera, as addition of 50 μM inhibitor resulted in MTT values that were approximately 90 and 70% at 48 and 72 h p.i., respectively (Fig. 3a, 50 μM, P/V-WF panel). By contrast, addition of caspase-9 inhibitor had no significant effect on loss of viability in the case of infected A549 cells (Fig. 3b). Taken together, these data indicate that (i) there are differences in the rate and extent of cell killing by the two P/V chimeric viruses, and (ii) there is a reduction in HeLa cell killing by the P/V chimeras in the presence of a caspase-9 inhibitor.

The overall goal of the work described here was to test the hypothesis that naturally occurring P/V substitutions from a non-cytopathic SV5 variant into the WT rSV5 genome can result in a chimeric virus that is highly cytopathic. This hypothesis could not be tested by using the previously described P/V-CPI− chimera (Wansley & Parks, 2002), as the cytopathic and apoptosis-inducing properties of the parental bona fide CPI− variant were not compared. To test this hypothesis, we have taken advantage of our recent isolation of WF-PIV, a naturally occurring SV5 variant that is largely non-cytopathic in most cell lines that we have examined (Young *et al.*, 2006). The WF-PIV P/V gene differs from WT rSV5 in eight positions, only three of which are common to the CPI− P/V gene (V32I, T33I and S157F; Fig. 1b). Importantly, whilst WT rSV5 and the naturally occurring WF-PIV variant are both largely non-cytopathic in human A549 and HeLa cells, the P/V-WF chimera induced cell killing with kinetics that were slightly slower than those of the original P/V-CPI− chimera. The common cytopathic phenotype of the two chimeras could be explained by a defective P or V protein, due to the P/V substitutions that are common to WF-PIV and CPI−. However, this hypothesis cannot explain why these P/V gene substitutions are not determinants of apoptosis in the non-cytopathic bona fide viruses.

An alternative hypothesis is that the apoptosis-inducing phenotype of both the P/V-CPI− and the P/V-WF chimeras is due to the expression of P/V gene products in the context of a non-native background genome. We hypothesize that, in the native non-cytopathic parental viruses, key interactions between P/V gene products and other viral components act to suppress or prevent apoptosis. These interactions would be inappropriate or suboptimal in the case of the chimeric P/V viruses. Which interactions with P/V gene products could be important for limiting...
apoptosis? As both the SV5 V and P proteins have been shown to interact with NP (Precious et al., 1995; Randall & Bermingham, 1996), we propose that proper V–NP or P–NP interactions are important for limiting apoptosis induction. Sequence analysis has shown that the WF-P/IV NP differs from that of WT rSV5 in three C-terminal amino acids (Young et al., 2006), and it is possible that these differences reside in a domain that influences NP–P or NP–V interactions. The finding that P/V-CPI− infections had a faster and more extensive loss of cell viability than that seen with the P/V-WF chimera could reflect differences in key P/V residues that influence protein–protein interactions to different degrees.

HeLa cell killing by the two chimeras was reduced by addition of a caspase-9 inhibitor, but this was not seen in the case of A549 cells. These data indicate that, whilst the two P/V mutants share a common phenotype of inducing apoptosis, there are significant cell-type differences in the response to infection. This result is similar to that seen with SeV mutants that display differences in cell type-dependent killing and inhibition by chemical inhibitors (Wiegand et al., 2005). We have recently reported an extreme example of this cell type-dependent activation of apoptosis by variants of SV5, as the cell-killing phenotypes of WT rSV5 and the P/V-CPI− mutant that are shown here in human epithelial cells are largely reversed in the case of infection of primary human dendritic cells (Arimilli et al., 2006). Work is in progress to identify the virus-activated pathways that lead to cell type-specific killing by SV5 mutants.

Acknowledgements

This work was supported by NIH grant AI-42023. P. J. D. and E. K. W. were supported by NIH training grant AI-07401. V. A. Y. was supported as a Howard Hughes Medical Institute predoctoral fellow.

References


