Transformed cell-specific induction of apoptosis by porcine circovirus type 1 viral protein 3

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Several members of the family Circoviridae have been shown to encode proteins with apoptotic activity. For example, both porcine circovirus type 2 (PCV2) and chicken anemia virus (CAV) encode a third viral protein (VP3) that has been shown to be cytotoxic. Interestingly, in the case of the CAV protein (designated apoptin), apoptosis is specific to transformed cell types. Similarities in genome structure and organization suggest that PCV type 1 (PCV1) may also contain a third ORF, which codes for a protein with homologous activity. To investigate this, ORF prediction followed by gene expression analyses were conducted on a gene found to be homologous to CAV and PCV2 VP3. Our data presented herein elucidate a putative ORF3 that codes for a viral protein with functional similarity to that of apoptin and PCV2 VP3. Unlike its homologues, sequence analysis revealed a highly hydrophobic, extended C-terminal domain in PCV1 VP3, which harbours a strong nuclear export signal. Subcellular localization analysis demonstrated divergent PCV1 VP3 localization patterns compared with that of CAV VP3. Interestingly, cytotoxicity studies revealed evidence that apoptosis may be selective to transformed cell types, similar to apoptin; however, PCV1 VP3 induced a dramatic G1 cell cycle arrest as opposed to the G2/M arrest observed with apoptin. These results indicate that nuclear localization of PCV1 VP3 is necessary neither for induction of apoptosis nor for transformed cell selectivity, and suggest a mechanism of action distinct from that of apoptin.

INTRODUCTION

Viruses typically manipulate a range of cellular functions to facilitate production of progeny and subsequent cellular egress. Strategies for cellular exit commonly employ proteins capable of inducing apoptosis near the end of the viral life cycle. Many animal viruses have been found to encode pro/anti-apoptotic protein homologues, proteins that disrupt anti-apoptotic pathways, or proteins that stimulate pro-apoptotic proteins (Roulston et al., 1999; Teodoro & Branton, 1997). One such example is the adenovirus E4orf4 protein, which is known to aid in viral spread through the induction of apoptosis towards the end of the virus life cycle (Müller et al., 1992; Van Hoof & Goris, 2003).

Several members of the family Circoviridae have been shown to encode proteins with apoptotic activity. The circoviruses are characterized by their non-enveloped spherical structure and small, circular, single-stranded ambisense DNA (Crowther et al., 2003; Phenix et al., 2001). Porcine circovirus type 2 (PCV2), the aetiological agent of post-weaning multisystemic wasting syndrome, has been shown to induce apoptosis by an as yet uncharacterized mechanism. This virus is divided into two genotypes, a and b (Cheung et al., 2007), each of which produces a protein from the third ORF that has reported apoptotic capacity (Kiupel et al., 2005; Liu et al., 2005). Studies suggest that the PCV2b isoform is more pathogenic (Gagnon et al., 2007), and other isoforms are also being discovered with varying degrees of pathological severity (Pereda et al., 2011).

Similarly, the chicken anemia virus (CAV), the aetiological agent of chicken infectious anemia, causes large-scale apoptosis of cortical thymocytes and erythroblastoid cells in the bone marrow (Jeurissen et al., 1992). Pathogenesis of CAV is accomplished by the third viral protein (VP3), which has been shown to be a potent inducer of apoptosis (Noteborn et al., 1994). Interestingly, in the case of CAV VP3, the protein induces apoptosis only in transformed cells, leaving normal cells intact (Heilman et al., 2006). This activity occurs regardless of the status of the p53 tumour suppressor (Heilman et al., 2006) by a caspase 3/7-dependent pathway (Burek et al., 2006). More than half of human cancers are non-responsive to many chemotherapeutics owing to loss of p53 activity; therefore continued study of pathways to cell death such as this have obvious therapeutic value.

Very little is known about porcine circovirus type 1 (PCV1), a non-pathogenic cousin of PCV2 and CAV. The
genome and architecture of this virus are highly related to those of PCV2, with nearly identical genome size (~1760 nt) and placement of the first and second ORFs, which encode the viral replicase and capsid protein, respectively (Finsterbusch et al., 2009). It is possible that this virus also harbours a third ORF, which may produce a protein with similar apoptotic capacities. The study of apoptotic proteins in this virus family may be fruitful for the discovery of novel p53-independent pathways to apoptosis in cancer cells.

RESULTS

A third ORF exists within the PCV1 genome, which is approximately twice the length of PCV2a and PCV2b ORF3s

Both the PCV2a and PCV2b genomes contain a third ORF, which codes for a protein capable of inducing apoptosis similarly to CAV apoptin. This raises the possibility that PCV1 may also contain a gene product that has homologous activity. Fig. 1 shows the results of an ORF search on the PCV1 genome using GenBank accession number AY193712.1, which revealed a third ORF consisting of 621 nt. Comparison of the translated PCV1 ORF3 (putative VP3) with PCV2a and PCV2b (UniProt O56124 and B6V519, respectively), shown in Fig. 2, resulted in 60% sequence identity and 78% sequence similarity. The high degree of homology among aa 1–104 (nt 1–312) suggests that PCV1 VP3 may have structural and functional similarity. Furthermore, a nucleotide alignment revealed a difference at residue 315 resulting in a tyrosine codon in PCV1 where a stop codon exists in both PCV2 genotypes. After nt 315, the PCV1 ORF3 sequence codes for a highly hydrophobic C-terminal domain (aa 105–207), which nearly doubles the size of the gene compared with homologous sequences. By contrast, the sequence alignment with CAV apoptin (UniProt Q99152) overall did not yield high homology, although clustered regions of homologous residues were found concentrated around the apoptin nuclear export and import sequences (unpublished data). These sequences have been shown to be critical for apoptin function, which may suggest some functional overlap with PCV1 VP3. Additionally, it is possible that tertiary structure similarities may exist despite primary sequence discrepancies (Lesk & Chothia, 1980).

PCV1 ORF3 contains several putative signalling sequences and a strong nuclear export sequence in the C-terminal region

Apoptin, the best characterized VP3 of the family Circoviridae, has been shown to possess both nuclear import and export signals. These signal sequences have been linked to the ability of this protein to engage in cell-type specific apoptosis. Specifically, apoptin seems to be regulated by nuclear localization prior to induction of apoptosis; thus it is found in the nucleus of transformed cells, while in the cytoplasm of primary cells (Heilman et al., 2006). To examine PCV1 ORF3 for putative signal sequences, NetNES prediction software was first used to predict putative export signals. This database uses sequence information gathered from experimentally validated leucine-rich export sequences and scores potentially positive signals using in-house trained neural networks and a hidden Markov model (la Cour et al., 2004). Fig. 3(a) shows the results of this search, where an export sequence was predicted at residues 42–49, and also a strong probability of a nuclear export signal (NES) at residues 134–149. These regions also adhere to the accepted canonical NES consensus sequence (L-(2,3)-[LIVFM]-(2,3)-L-X-[LII]) (la Cour et al., 2003). Further examination of predicted NES sequences was performed by aligning the established NES sequence from CAV VP3 (residues 37–46) with regions predicted for PCV1 VP3 from NESbase (residues 42–49 and 134–142) using the CLUSTALW2 program (Goujon et al., 2010; Larkin et al., 2007). Fig. 3(b) shows a schematic of these two proteins (top), indicating the relative positions of each NES sequence, and an alignment of these NES regions (bottom). Sequences show a high level of homology, especially in conserved hydrophobic positions known to be essential for NES function (Kosugi et al., 2008). Interestingly, the alignment revealed an additional NES sequence overlapping a region just upstream of the predicted area for the C-terminal NES (residues 127–136). This sequence also strongly matches the established consensus sequence. Together, these data indicate a strong possibility of functional nuclear export in this C-terminal region of the PCV1 VP3. Next, nuclear localization signal (NLS) sequences were searched using two different prediction algorithms, cNLS Mapper (Kosugi et al., 2009) and NLStradamus (Nguyen Ba et al., 2009). Although no strong prediction of NLS sequences were found, a bipartite yeast NLS sequence was predicted in regions 30–59 (unpublished data).

PCV1 VP3 localizes to the cytoplasm in transformed cells

Differences in subcellular localization of apoptin between cell types has been linked to its ability to induce apoptosis selectively (Heilman et al., 2006). Several comparative localization studies have previously been performed between H1299 (non-small cell lung carcinoma cells) and primary foreskin fibroblasts (PFF), as these cells display representative differences in localization and killing capacity for apoptin. Although apoptin does not have close sequence homology with PCV1 VP3, clusters of sequence homology exist, primarily at known apoptin localization sequences. We hypothesized that PCV1 VP3 may behave in a similar manner in these cell types. To investigate this, N-terminal GFP fusion constructs were created for PCV1 VP3, PCV2a VP3, PCV2b VP3 and CAV apoptin, and these were separately transfected into H1299 cells. Twenty-four hours post-transfection, cells were fixed with paraformaldehyde and mounted onto microscope slides. Epifluorescence
imaging of the mounted cells revealed strong cytoplasmic localization for PCV1 VP3 compared with that of apoptin, as seen in Fig. 4(a). Additionally, PCV2a/b VP3 displayed a diffuse localization throughout the cell compared with the distinct localization patterns of PCV1 VP3 and CAV apoptin, Fig. 4(a). PCV1 VP3 images revealed punctate localization patterns, similar to CAV apoptin images, which have previously been shown to correlate with multimerization of the protein (Heilman et al., 2006). To explore what predicted sequences are responsible for this localization, a series of N-terminally GFP fused truncation mutants representing each of the putative signal sequences was prepared. These mutants were transfected into H1299 cells as described previously. Imaging confirmed the prediction that the C-terminal extension (aa 105–207) is directing strong cytoplasmic localization, as seen in Fig. 4(b). Additionally, truncation mutants of the forward section containing the two predicted NES sequences (aa 19–28 and 42–49) may also be biologically active. Although localization analysis of truncation mutant PCV1 VP3(1–63) showed no distinct cytoplasmic localization, PCV1 VP3(1–105) showed cytoplasmic preference for localization. This discrepancy in localization behaviour may be attributed to the shortness of the former mutant, which was unable to meet the conformational requirement for an active NES signal. Quantification of the confocal images compared with an EGFP control confirmed the visual
PCV1 VP3 selectively induces apoptosis in transformed cells

PCV1 VP3 may possess the ability to induce apoptosis in its homologues in PCV2 and CAV. Despite the result that subcellular localization does not differ between primary and transformed cells, cell-type specific cytotoxicity may yet exist. To investigate this behaviour, WT FLAG-tagged CAV apoptin and PCV1 VP3 constructs were created instead of GFP due to excitation wavelength similarity of GFP with the Promega Apo-One caspase 3/7 assay. H1299 cells were transfected in 96-well format. After 24 h, the Promega Apo-One Caspase 3/7 reagent was used to detect evidence of apoptosis. Fluorescence resulting from caspase-specific cleavage was quantified by fluorescence spectrophotometry. Apoptotic induction was calculated by subtracting background apoptosis from medium alone and normalizing against apoptin apoptosis levels. As shown in Fig. 6(a), apoptosis resulting from expression of apoptin and PCV1 VP3 were statistically significant (CI 95%, P value **<0.005 and ***<0.0005, respectively; n=10) when compared with control parental vector. These results also indicate that PCV1 VP3 induces apoptosis by a caspase 3-dependent pathway, as is the case for apoptin. To determine if PCV1 VP3 cytotoxicity is cell-type specific, PFF and H1299 cells (canonical cell types for apoptin differential activity) were transfected with FLAG-tagged CAV apoptin and PCV1 VP3. Interestingly, H1299 apoptosis induction was substantially higher than in PFF cells, as seen in Fig. 6(b) (PFF n=6; H1299 n=3). The difference in PFF signal from background was not statistically significant, indicating no detectable apoptotic activity in this cell type. These results indicate that not only does PCV1 VP3 induce apoptosis, but it does so in a cell-selective manner. These results combined with the localization information suggest that PCV1 VP3 may also possess the ability to induce apoptosis selectively depending on the transformation status of the cell, although in the absence of the differential subcellular localization observed with apoptin.

PCV1 VP3 causes cell cycle arrest in G1

The selective induction of apoptosis exhibited by PCV1 VP3 is analogous to that of CAV VP3. However, the cytoplasmic localization of PCV1 VP3 in transformed cells suggests that the mechanics of its action on cell selection and programmed cell death may be distinct. CAV VP3 has been shown to cause arrest of cells at the G2/M stage of the cell cycle, resulting from interaction with the anaphase-promoting complex/cyclosome (APC/C) (Teodoro et al., 2004). To address whether the PCV1 VP3 mechanism leading to cell death is similar, the cell cycle profile of H1299 cells expressing GFP PCV1 VP3 was analysed. H1299 cells were seeded in six-well format and transfected with either parental GFP vector or GFP PCV1 VP3. Cells were harvested by trypsinization at approximately 24, 48 and 60 h and were fixed and stained with propidium iodide. Flow cytometry was performed to collect ~30 000

**Fig. 3.** A NetNES prediction of VP3 sequences from the porcine circoviruses. (a) For each PCV type, a NES was weakly predicted in the forward region. NES prediction of PCV2 genotype ‘a’ resulted in a predicted sequence between residues 40 and 50. NES prediction of PCV2 genotype ‘b’ resulted in a predicted sequence in a similar location to PCV2a. PCV1 contained two NES predictions in the forward region near residues 20 and 50. Additionally, a strong NES was predicted in the extended C-terminal domain (probability threshold 0.5). (b) Above: mapping of NES predictions in the forward region near residues 20 and 50. NES prediction of PCV2 genotype ‘a’ resulted in a predicted sequence between residues 40 and 50. In the forward region. NES prediction of PCV2 genotype ‘b’ resulted in a predicted sequence in a similar location to PCV2a. PCV1 contained two NES predictions in the forward region near residues 20 and 50. Additionally, a strong NES was predicted in the extended C-terminal domain (probability threshold 0.5). (b) Above: mapping of predicted signalling sequences for PCV1 VP3, and published signalling sequences for CAV apoptin. Below: sequence alignment of PCV1 VP3 with CAV apoptin’s NES sequence, performed with the European Bioinformatics Institute (EBI) CLUSTALW2 program. Amino acids are colour-coded by biophysical properties similar to the colour codes used in EBI CLUSTAL w2.

The cytoplasmic localization of PCV1 VP3 is independent of cell type

Apoptin localization has been shown to differ between transformed and primary cell lines. Nuclear–cytoplasmic shuttling has been linked to the ability of apoptin to selectively induce apoptosis (Heilman et al., 2006). To determine if PCV1 VP3 behaves in a similar manner, the localization of GFP-tagged WT PCV1 VP3 was compared in PFF and H1299 cells. Confocal microscope imaging of the transfectants revealed identical localization patterns and virtually no difference in subcellular localization between cell types, as shown in Fig. 5(a). Quantification was performed confirming the visual interpretation, depicted in Fig. 5(b). This result suggests that either PCV1 VP3 has no ability to discriminate between cell types as apoptin does, or an alternative mechanism for cell-type discrimination may exist.
events for each sample and GFP-positive cells were gated and analysed for DNA content by applying Watson (pragmatic) cell cycle modelling to resultant profiles. Results of this analysis are shown in Fig. 7(a), with purple lines indicating model fit in each sample and shaded regions indicating integrations of cell cycle stage that correlate with DNA content. In strong contrast to CAV VP3, expression of PCV1 VP3 in these cells induces a dramatic G1 cell cycle accumulation, with G1/G2 ratios increasing from control levels of 5.74 to >9 after 48 h. Nearly 90% of the GFP-positive cell population is in G1 at 60 h, shown in Fig. 7(b). Apoptotic cells were not readily apparent as the GFP gated cells will typically exclude any subG1 population due to loss of GFP fluorescence by proteolytic activity. To assess the level of apoptosis, samples processed in parallel to those for cell cycle analysis were stained at 48 h with a cell-permeable red fluorescent caspase 3/7 inhibitor and quantified by fluorescence

**Fig. 4.** Fluorescence microscopy of N-terminal-tagged GFP fusion proteins demonstrates a non-nuclear localization. (a) Epifluorescence microscopy of GFP-PCV1 VP3 as compared with various GFP-VP3 fusion proteins from other circoviruses. Localizations are compared with GFP alone. (b) Epifluorescence microscopy of various truncation mutants of PCV1 VP3, indicating several putative localization sequences. (c) The nuclear/cytoplasmic fraction for each panel was calculated by green pixel intensity using ImageJ (*P<0.05, **P<0.005). Error bars indicate SD.
microscopy followed by image densitometry. The apoptotic index was plotted as the ratio of red/green pixel densities for GFP vector controls or GFP PCV1 VP3, as shown in Fig. 7(c). Results indicate a strong level of caspase 3/7-dependent apoptosis concurrent with observed G1 accumulation. Together, these results strongly suggest that the mechanism of PCV1 VP3-induced apoptosis is distinct from that of CAV VP3.

DISCUSSION

In this study, we have demonstrated that a functional homology exists between PCV1 VP3 and CAV apoptin, confirming our hypothesis that PCV1 VP3 protein, like its apoptin homologue, possesses an ability to induce apoptosis. An independent study has confirmed this activity in other human and porcine cell lines (Chaiyakul et al., 2010). Additionally, we have demonstrated that PCV1 VP3 also possesses cell-type specific apoptotic activity like that of apoptin. Our studies discern that functional localization domains exist within PCV1 VP3 that confer default cytoplasmic localization in both cell types. Unlike apoptin, PCV1 VP3 does not relocalize to the nucleus of transformed cells prior to apoptosis. We have also observed that neither PCV2a VP3 nor PCV2b VP3 proteins adopt nuclear localization prior to induction of apoptosis (unpublished data). Previous studies indicate that, in the case of apoptin, this nuclear accumulation is critical; mutants that fail to localize to the nucleus also fail to induce apoptosis (Danen-van Oorschot et al., 2003; Heilman et al., 2006). These data suggest that PCV1 VP3 may act by a different mechanism that does not require localization-dependent activation.

The strong cytoplasmic localization of PCV1 VP3 is attributed to the C-terminal domain, which we discerned from truncation mutant localization studies to contain a functionally potent nuclear export signalling sequence. Tertiary structure

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Fig. 5. Subcellular localization of PCV1 VP3 does not differ between primary and transformed cells. (a) GFP-tagged WT PCV1 VP3 expressed in PFF and H1299 imaged 24 h post-transfection by confocal microscopy. (b) Nuclear/cytoplasmic fraction for each panel was calculated by green pixel intensity in both regions using ImageJ (**P<0.0005). Error bars indicate sd.

Fig. 6. Apoptotic induction and cell-type selectivity of PCV1 VP3. (a) Background apoptosis from medium alone (no DNA) was subtracted from all samples and apoptotic induction was normalized to CAV VP3 (apoptin) levels. Apoptosis is indicated as a percentage of apoptin-induced levels (CI 95%, **P<0.005, ***P<0.0005 when compared with an empty vector, here denoted control vector; n=10). (b) PCV1 VP3 induction of apoptosis in PFF and H1299 cells. Apoptosis assays were performed in parallel 24 h post-transfection. Apoptosis levels are indicated by relative fluorescence units (*P<0.05; error bar for PFF too small to visualize; PFF n=6; H1299 n=3). Error bars indicate sd.
predictions focused on this area indicate very high prediction scores for a globular domain of organized helical structure (data not shown). This indicates that this region may have been lost in CAV apoptin and PCV2 VP3, as opposed to gained in PCV1 VP3, where a less structured C-terminal region might be observed. Mutant studies that analyse the functionality of each domain independently may reveal interesting data potentially elucidating the role of this region.

Our apoptosis studies of PCV1 VP3 indicate that induction of apoptosis, like that by apoptin, occurs via a caspase 3/7-dependent pathway; however, our cell cycle data strongly suggest an alternative mechanism for upstream activation of cell death. A G1-mediated pathway to apoptosis for PCV1 VP3 is more consistent with a localization behaviour that is constitutively cytoplasmic as opposed to the G2/M arrest observed with apoptin, which has been shown to involve DNA damage pathways and association with the APC/C (Kucharski et al., 2009). Cytoplasmic localization of PCV1 VP3 does not preclude potential involvement with APC/C, however, as multiple forms of this complex exist, including a G1 iteration (activated by Cdh1) that regulates the SCF E3 ubiquitin ligase responsible for G1 exit (Bashir et al., 2004; Heilman et al., 2006). It is possible that the cell selection activity also ablate the multimerization capacity of the protein (Wang et al., 2004; Heilman et al., 2006 and unpublished data). Until localization (particularly nuclear export) can be uncoupled and independently evaluated, the clear role of localization of this protein cannot be assessed. If differential localization behaviour is eventually found to be superfluous, these homologous circovirus proteins may share a common mechanism for p53-independent transformed cell discrimination and cytotoxicity – a mechanism of great interest in the development of novel cancer therapeutics.

**METHODS**

**Sequence analysis.** ORFs were predicted within the PCV1 genome (GenBank accession number AY193712.1; Fig. 1) consisting of at least 100 codons by using MacVector. The nucleotide sequence of the third predicted ORF was translated and aligned with the protein sequence for CAV apoptin (UniProt Q99152), PCV2a VP3 (UniProt O56124) and PCV2b VP3 (UniProt B6V519). The PCV1 VP3 sequence was also searched for signal motifs and subcellular localization patterns using NetNES (la Cour et al., 2003), cNLS Mapper (Kosugi et al., 2009) and NLStradamus (Nguyen Ba et al., 2009).

**PCR amplification and purification.** PCR amplification of PCV1 ORF3 segments were performed using Promega GoTaq Green Master Mix under the following cycle conditions: melting at 95 °C for 30s, annealing at 55 °C for 30s, and extension at 72 °C for 30s. After 30 cycles, the reaction was held at 72 °C for 1 min for a final extension and then cooled to 10 °C, and stored at −20 °C. Forward and reverse primers were engineered to amplify the full-length third ORF as well...
as four truncation mutants representing the putative NES [PCV1-VP3(64–105)], tail [PCV1]-VP3(105–207), forward section [PCV1]-VP3(1–63) and a segment consisting of the NLS and tail sections [PCV1]-VP3(64–207) (Table 1). These putative signal sequences were identified using prediction algorithms described previously. Each engineered primer included an EcoRI restriction site in the forward primer and a BamHI restriction site in the reverse primer. Reaction products were purified by gel filtration on a 0.9 % agarose gel using Promega Wizard SV Gel and the PCR Clean-UP System.

Cloning and screening of ORF3. PCR products were ligated into pEGFP-C1 and p3 × FLAG-myc-CMV-26 Clontech expression vectors at an insert to vector ratio of 4:1. The ligation was performed with 3 μl T4 DNA ligase (Promega) in manufacturer-supplied buffer, and was incubated at 4 °C for 24 h. Overnight ligation products (5 μl) were used to transform JM109 competent Escherichia coli (50 μl, 108 c.f.u. μg−1). Transformed cells were plated on agar and incubated overnight at 37 °C. Colonies were screened for the gene of interest by lysis with a passive-lysis buffer (10 mM Tris-EDTA buffer pH 8.5, 10 mM NaCl, 3 mM MgCl2 and 0.5 % NP-40 in sterile double-deionized H2O), and EcoRI and BamHI restriction site digestion followed by 0.9 % agarose gel electrophoresis for 2 h at 90 V. Positive colonies were inoculated for mid-scale DNA purification using the Promega PureYield Plasmid Midiprep System.

Cell culture maintenance. H1299 human non-small cell lung carcinoma cell line (ATCC CRL 5803) was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FBS and 1 % PSF (100 U penicillin G sodium ml−1, 100 mg streptomycin sulfate ml−1, 0.25 mg amphotericin B ml−1). The cells were incubated at 37 °C with 5% CO2. Confluency was maintained at or below 95 % by passage with 0.25 % trypsin-EDTA. PFF were also maintained in the same manner.

Transient transfection. H1299 cells used for subcellular localization studies were transiently transfected with pEGFP-C1 constructs under the control of a constitutive cytomegalovirus (CMV) promoter. The cells were transfected using Qiagen Effectene Transfection Reagent kit following the manufacturer’s protocol. H1299 cells used for apoptosis assays were transiently transfected with p3 × FLAG-myc-CMV-26 constructs under the same constitutive CMV promoter. Cells were transfected in triplicate with 0.6 μg DNA diluted in 40 μl DMEM using 1.8 μl Promega FuGENE HD. The DNA–DMEM–FuGENE mixture was incubated at room temperature for 10 min before 10 μl each well was added to cells in a 96-well plate. PFF cells used for apoptosis assays were transfected with p3 × FLAG-myc-CMV-26 constructs using the Lonza Amaxa Nucleofector following the manufacturer’s protocol.

Fluorescence microscopy. H1299 cells were seeded in six-well plates containing circular coverslips and incubated overnight to obtain a confluency of 80 %. Cells were then transfected using the Qiagen Effectene Transfection Reagent kit with pEGFP-C1 constructs and stained with 15 μl mounting medium [50 % glycerol, 100 mM Tris pH 7.5, 2 % 1,4-diazabicyclo(2,2,2)octane (DABCO), 10 μg ml−1 DAPI] 24 h post-transfection. Cells were viewed by epifluorescence and confocal microscopy.

Apoptosis assays. H1299 cells were seeded at approximately 80 % confluency in a 96-well plate and transfected using Promega FuGENE HD with p3 × FLAG-myc-CMV-26 constructs. PFF cells were transfected using Lonza Amaxa Nucleofector with p3 × FLAG-myc constructs, then plated in a 96-well plate. Promega Apo-ONE homogeneous caspase 3/7 reagent and buffer were added 24 h post-transfection. Fluorescence readings (λex=499, λem=521) were made every hour for 48 h after a 24 h incubation and addition of caspase 3/7 buffer and reagent. Alternatively, apoptosis assays were performed in parallel with cell cycle analysis by seeding cells to reach approximately 80 % confluency in six-well format. Transfections were performed using Effectene Transfection Reagent (Qiagen) and after 48 h cells were washed once with medium and stained using the Image-iT LIVE red caspase detection kit (Life Technologies). After 60 min, cells were then fixed in 4 % paraformaldehyde in PBS for 15 min, washed in PBS and preserved in 70 % ethanol. Cells were imaged using a Zeiss Axiovert A1 epifluorescence microscope (×200 total magnification), and pixel areas and standardized detection thresholds were set using colour range selection in Photoshop CC (Adobe). Pixels in parallel green and red channels were quantified using the histogram function, and apoptotic indices were calculated as the ratio of red to green pixels.

Cell cycle analysis. H1299 cells were seeded to reach 80 % confluence and were transfected with GFP vector or GFP PCV1 VP3 constructs. At indicated time points post-transfection cells were harvested by transient trypsinization, washed in PBS and fixed in cold 4 % paraformaldehyde for 15 min. Cells were then collected, washed in PBS and fixed in 70 % ethanol for at least 1 h. Cells were then stained with propidium iodide solution (50 μg ml−1 propidium iodide, 2 mM MgCl2, 50 μg ml−1 RNase A in PBS) for 20 min at 37 °C and quantified by flow cytometry. GFP-positive cells were gated and analysed for DNA content by Watson (pragmatic) modelling using Flowjo 7.6.5 software.

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