Deletion of pV affects integrity of capsid causing defect in the infectivity of bovine adenovirus-3

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Members of the genus \textit{Mastadenovirus} including bovine adenovirus 3 (BAdV-3) encode a genus-specific unique protein named pV. The pV encoded by BAdV-3 is a protein of 423 aa showing 40.9\% identity to pV of human adenovirus 2. Here, we report the construction and analysis of recombinant BAdV-3 (BAV.dV) containing deletion of pV. The BAV.dV could only be isolated in CRL.pV cells expressing pV, suggesting that pV appears essential for the infection of BAdV-3. Analysis of BAV.dV suggested that despite affecting some late gene expression in virus-infected cells, there was no significant difference in the incorporation of viral proteins in the mature virions. Moreover, analysis of mature virions revealed degraded capsids leading to change in morphology and infectivity of BAV.dV. Furthermore, analysis of the genome sequence of different clones of BAV.dV passaged in different cell lines revealed no mutations in core proteins pVII and pX\_Mu suggesting that the replication defect may not be rescued. Our results suggest that pV is required for proper viral assembly of BAdV-3 as lack of pV produces aberrant capsids. Moreover, altered capsids lead to the production of non-infectious BAV.dV virions.

\textbf{INTRODUCTION}

Adenoviruses are non-enveloped icosahedral particles of 70–100 nM in diameter (Horne \textit{et al.}, 1959), which infect mammals, birds (Chiocca \textit{et al.}, 1996), reptiles (Benkó \textit{et al.}, 2002), frogs (Davison \textit{et al.}, 2000) and fish (Kovcs \textit{et al.}, 2003). Members of the genus \textit{Mastadenovirus} genus including human adenovirus (HAdV) infect mammals and encode unique proteins including pIX and pV (Davison \textit{et al.}, 2003). Earlier reports suggested that HAdV-5 pV acts as a bridge between the core and the capsid proteins in mature virions (Chatterjee \textit{et al.}, 1985; Lehmberg \textit{et al.}, 1999; Matthews \& Russell, 1998). Additional investigations have revealed that pV promotes viral assembly through nucleoplasmin 1 (Ugai \textit{et al.}, 2012) and is essential for virus replication in primary but not in cancer cells (Ugai \textit{et al.}, 2007).

Bovine adenovirus 3 (BAdV-3) is a non-enveloped icosahedral virus of 75 nm (body of virion) in diameter (Thompson \textit{et al.}, 1981), which contains a genome of 34 446 bp long organized into early (E), intermediate (I) and late (L) regions (Reddy \textit{et al.}, 1998). Previously, we reported that the core protein pVII encoded by L1 region of BAdV-3 localizes to the mitochondria using a mitochondrial localization signal, and interferes with apoptosis by altering some mitochondrial functions in infected cells (Anand \textit{et al.}, 2014). Recently, we reported that conserved regions of pVIII encoded by L6 region contain motifs involved in nuclear localization or packaging in mature virions (Ayalew \textit{et al.}, 2014). Similarly, conserved leucines (Kulshreshtha \textit{et al.}, 2015) and conserved arginines (Kulshreshtha \textit{et al.}, 2014) of 33K protein encoded by the L6 region appeared important in the binding and the activation of major late promoter, and in nuclear transport of 33K and BAdV-3 replication, respectively.

Though positional homologues are encoded by HAdV-5 and BAdV-3, the structure and function of the homologous proteins may not always be similar (Anand \textit{et al.}, 2014; Kulshreshtha \textit{et al.}, 2004; Li \textit{et al.}, 2009; Reddy \textit{et al.}, 1998). Recently, we demonstrated that unlike HAdV-5, BAdV-3 protease cleaves 100K protein, which is required for the nuclear transport in the infected cells but not for virus replication (Makadiya \textit{et al.}, 2015).

The L2 region of BAdV-3, a member of the genus \textit{Mastadenovirus}, encodes pV protein of 423 aa, which shows 40.9\% identity to pV encoded by HAdV-2 (Reddy \textit{et al.}, 1998). Recently, we have demonstrated that pV is detected as (a) a 55 kDa protein in CsCl gradient-purified BAdV-3 virions or in BAdV-3-infected cells and (b) is localized predominantly
in the nucleolus of the virus-infected cells, which is required for the replication of BAdV-3 (Zhao & Tikoo, 2016, unpublished results). Here, we report the role of pV in the replication of BAdV-3 in the infected cells.

RESULT

Isolation of BAV_dV in cotton rat lung cells

To determine if pV is essential for BAV304a (E3-deleted BAdV-3 containing CMV-EYFP cassette inserted in E3 region) (Du & Tikoo, 2010) replication, we constructed a plasmid pUC304A_dV containing BAdV-3 genome with deletion of pV [entire pV deleted (start to stop codon) with no effect on known transcription of genes on the opposite strand] and insertion of CMV-EYFP gene cassette in the E3-deleted region (Fig. 1a). Individual plasmid pUC304A_dV or pUC304A+ (containing BAdV-3 genome with insertion of CMV-EYFP gene cassette in E3-deleted region) (Fig. 1a) DNA were used to transfect VIDO DT1 cells [cotton rat lung (CRL) cells expressing I-SceI] (Du & Tikoo, 2010). At 6 days post-transfection, the EYFP expression and cytopathic effects (CPEs) were visible in the cells transfected with plasmid pUC304A+ DNA (Fig. 1b). However, repeated transfection of VIDO DT1 cells with plasmid pUC304A_dV DNA did show EYFP expression in a few cells, but not any CPEs even after 20 days post-transfection (Fig. 1b). Moreover, while the lysates from the cells transfected with plasmid pUC304A+ DNA produced CPEs in freshly infected VIDO DT1 cells, the lysates from the cells transfected with plasmid pUC304A_dV DNA did not produce any CPE or expression of EYFP in freshly infected VIDO DT1 cells (data not shown). These results suggest that pV is essential for the replication of BAV304a.

Construction of CRL_pV cells expressing BAdV-3 pV

To isolate a cell line expressing BAdV-3 pV, CRL cells were transduced with lentivirus expressing BAdV-3 pV and grown in the presence of puromycin as described earlier (Du & Tikoo, 2010). The puromycin-resistant clones were analysed initially for the expression of pV by Western blotting and immunofluorescence assay using pV-specific antiserum. Earlier analysis using anti-pV serum suggested that pV is expressed as 55 kDa in BAdV-3-infected cells and localizes predominantly in the nucleolus of BAdV-3-infected cells (Zhao & Tikoo, 2016, manuscript in preparation). As shown in Fig. 2(a), anti-pV serum detected a protein of 55 kDa in BAdV-3-infected cells.
cells. Similar protein could be detected in two puromycin-resistant clones using anti-pV serum (Fig. 2a, lanes 1, 2). No such protein could be detected in CRL cells (Fig. 2a, lane 4). Secondly, the sub-cellular location of pV in puromycin-resistant clones was analysed by confocal microscopy. As seen in Fig. 2(b), anti-pV serum detected protein predominantly localized in the nucleolus of pV expressing cells (CRL.pV1, CRL.pV2). No such protein could be detected in the nucleolus of CRL cells.

Isolation of BAV.dV in CRL.pV cells

To isolate pV-deleted BAV304a, CRL.pV cells were transfected with PacI-digested plasmid pUC304a.dV DNA and observed for the development of CPEs (Fig. 3a). As shown in Fig. 3(b), the CPE and EYFP expression was firstly observed at 7 days post-transfection, which increased by day 12. To confirm the identity of recombinant virus named BAV.dV, firstly, viral DNA was purified from infected CRL.pV cells, digested with KpnI and analysed by agarose gel electrophoresis. As shown in Fig. 3(c), BAV304a (lane 1) contains a fragment of 4.7 kb (appears as doublet with 4.5 kb), which was missing in BAV.dV. Instead, BAV.dV (lane 2) contains a fragment of 3.5 kb because of the deletion of pV gene. Secondly, the expression of pV in virus-infected CRL cells was analysed by Western blotting using anti-pV serum. As seen in Fig. 3(d), a 55 kDa protein could be detected in BAV304a-infected CRL cells (lane 1). Similar protein could be detected in uninfected CRL.pV cells (lane 3). No such band could be detected in BAV.dV-infected CRL cells (lane 2).

To determine the influence of pV on the formation of BAdV-3 particle, CRL cells or CRL.pV cells were infected with purified BAV.dV (grown in CRL.pV cells) at an m.o.i. of 2. At 48 h post-infection, the lysates of infected cells were used to purify virions by CsCl gradient centrifugation. No contamination could be detected in CsCl2-purified virions (Fig. S1, available in the online Supplementary Material). As seen in Fig. 3(e), deletion of pV predominantly produced population of virus at a density consistent with mature viruses. Moreover, no visible decrease in the production of the mature virus particles could be observed in BAV.dV grown in CRL.pV cells or CRL cells.

Growth of BAV.dV in CRL cells

To determine if BAV.dV can produce infectious viral particles in pV-negative CRL cells, viral growth characteristics of CsCl-purified BAV304a (grown in CRL cells) and BAV.dV (grown in CRL.pV cells) was analysed. Monolayers of CRL cells in a 24-well plate were infected with BAV304a or BAV.dV at m.o.i. of 2. The infected cells were harvested at different times (0, 6, 12, 24, 36 and 48 h) post-infection. After freeze-thawing three times, the samples were titrated by TCID50 in CRL.pV cells. As shown in Fig. 3(f), BAV304a and BAV304R (BAV.dV revertant) (Fig. S1) grew to a titre of $-10^8$ TCID50 ml$^{-1}$ at 48 h post-infection of CRL.pV cells.

In contrast, there was no detectable increase in the titre of BAV.dV. Similarly, no detectable increase in BAV.dV titre could be observed in Madin–Darby bovine kidney (MDBK) cells (data not shown). The particle to TCID50 ratio was 1 : 10 (BAV304a in CRL cells), 1 : 1000 (BAV.dV in CRL.pV cells) and 1 : 100 000 (BAV.dV in CRL cells).

Analysis of protein expression in BAV.dV-infected cells

To analyse if the expression of viral proteins is modulated in BAV.dV (containing deletion of pV)-infected cells, monolayers of CRL cells were infected with BAV304a or BAV.dV (grown in CRL.pV cells) at m.o.i. of 2. At 24 h post-infection, the cells were harvested and lysed. The proteins from the cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed by protein-specific antiserum and secondary antibodies conjugated with fluorophores. Finally, the membranes were scanned and analysed by Odyssey CLx Imaging System. As expected (Fig. 4a, b), the expression of pV could be detected in BAV304a-infected CRL cells, but not in BAV.dV-infected CRL cells. No appreciable difference could be detected in the expression of early DNA-binding protein (DBP) in CRL cells infected with BAV304a or BAV.dV. However, compared with BAV304a, reduced expression of some late proteins, particularly 100K, pX and pVII, and fibre were observed in BAV.dV-infected cells. Moreover, both precursor and cleaved form of pVII could be detected in BAV304a- or BAV.dV-infected cells.

Analysis of BAV.dV DNA replication

The CRL cells were infected with purified BAV304a or BAV.dV (grown in CRL.pV cells) at an m.o.i. of 2. At 12, 24 or 36 h post-infection, the cells were collected, washed with PBS and used to extract DNA as described previously (Farina et al., 2001). The DNA isolated from equal number of cells was digested with restriction enzyme BmtI. Analysis of restriction enzyme-digested DNA (Fig. 4c) suggested that there was not much difference in the replication of BAV304a and BAV.dV in CRL cells.

Analysis of protein incorporation in BAV.dV viral particles

To determine the incorporation of pV in the progeny virions, proteins from purified virions were separated by 10% SDS-PAGE, transferred to nitrocellulose and analysed by Western blotting using anti-pV serum. As seen in Fig. 5 (a), anti-pV detected a protein band of 55 kDa in purified BAV304a grown in CRL cells (lane 1). A protein band of similar molecular weight could be detected in purified BAV.dV grown in CRL.pV cells (lane 3). However, no such protein could be detected in BAV.dV grown in CRL cells (lane 2). Moreover, there was no much difference in the incorporation of the viral proteins in purified BAV304a or BAV.dV virions (grown in CRL cells or CRL.pV cells). As...
seen in Fig. 5(a), hexon and fibre proteins were efficiently incorporated in purified BAV304a grown in CRL cells (lane 1), purified BAV.dV grown in CRL cells (lane 2) or purified BAV.dV grown in CRL.pV cells (lane 3).

Anti-pVII serum detected both precursor and cleaved form of pVII in BAV304a-infected CRL cells (Fig. 5b, lane 2) or BAV.dV-infected CRL cells (Fig. 5b, lane 2) or CRL.pV cells (Fig. 5b, lane 6). As expected, a protein consistent with the cleaved form of pVII could be detected in purified BAV304a grown in CRL cells (Fig. 5b, lane 1), purified BAV.dV grown in CRL cells (Fig. 5b, lane 3) or grown in CRL.pV cells (Fig. 5b, lane 5).

**Analysis of BAV.dV by transmission electron microscopy**

To examine if the deletion of pV affects the formation of BAdV-3 particles, CRL cells were infected with BAV304a or BAV.dV (grown in CRL.pV cells) at an m.o.i. of 2. At 24 h post-infection, the cells were collected, processed and analysed by transmission electron microscopy (TEM). As seen in Fig. 6(a), BAV304a (panels 3, 4) appeared to produce more viral particles than BAV.dV (panels 5, 6) in infected CRL cells. Moreover, BAV304a particles were uniform and loosely arranged (panel 3). In contrast, BAdV.dV particles appeared to be clustered...
together and appeared tightly organized in rows (panel 5). Analysis of the enlargement of selected areas of TEM images suggested that BAV304a are clearly of typical icosahedral in shape (panel 4). However, BAV.dV showed less clear morphology and did not possess clear icosahedral shape (panel 6). No such virions could be detected in mock-infected CRL cells (Fig. 6a, panels 1, 2).

Next, we analysed the CsCl-purified BAV304a or BAV.dV (grown in CRL cells) by TEM. The analysis of mature BAV304a virions detected intact capsids with typical icosahedral shape (Fig. 6b, panels 1, 2). In contrast, most of the BAV.dV particles appeared circular in shape with partially degraded capsids (Fig. 6b, panels 3, 4).

**Thermostability of BAdV.dV**

To determine if the deletion of pV alters viral thermostability, purified viral particles in PBS containing 10 % glycerol were incubated at different temperatures for 3 days or incubated at different temperatures (−80, 4 and 37 °C) for 0, 1, 3 and 7 days. Finally, the infectivity was measured by
TCID<sub>50</sub> assay. As seen in Fig. 7, there appears no difference in the thermostability or dynamics of viral inactivation of BAV304a or BAV.dV grown in CRL.pV cells. In contrast, both thermostability and dynamics of viral inactivation of BAV.dV grown in CRL cells appear significantly different from BAV304a.

**DISCUSSION**

The production of infectious progeny adenovirus requires a maturation step involving the cleavage of capsid and core proteins by adenovirus protease (Anderson et al., 1973). However, the significance of cleavage of each precursor protein in determining the infectivity is not clear (Mangel & San Martín, 2014). Analysis of viral protein expression in BAV.dV-infected cells, suggesting that pV may be involved in the regulation of late gene expression probably by acting on major late promoter (Leong et al., 1990). Similar results have been reported earlier for HAdV-5 pV (Ugai et al., 2006). Further analysis of BAV.dV demonstrated no significant change in the infectivity and DNA replication of HAdV-5 expressing pV-GFP (Le et al., 2006; Puntener et al., 2011). These results suggest that deletion of BAV.dV may not significantly alter the virus assembly, but instead make the virion capsid more fragile leading to the detectable changes in virion morphology and infectivity.

Unlike primary cells (Ugai et al., 2012), HAdV-5 pV is not required for virus replication and formation of infectious virus particles in cancer cells (Ugai et al., 2012). This is due to apparent thermostable mutations (G13E and R17I) in the less conserved region of core protein X/Mu, which compensate for the lack of pV (Ugai et al., 2007). Moreover, analysis of CsCl gradient-purified pV-deleted HAdV-5 grown in cancer cells showed increased incorporation of protein X/Mu in mature virions. In contrast, pV appears essential for the replication of BAdV-3 CRL or MDBK cells. Despite conservation of arginine residue at amino acid 20 of BAdV-3 pV (Ugai et al., 2007), analysis of DNA sequence of different clones of BAV.dV grown (different passages) in CRL or MDBK cells did not reveal any mutation in the core proteins X/Mu or pVII (data not shown). Because of unavailability of reagents, the incorporation of the X/Mu could not be analysed in CsCl gradient-purified BAV.dV grown in CRL cells. Our results suggest that deletion of pV does not introduce compensatory mutations in core proteins X/Mu or pVII.

In summary, we have demonstrated that BAdV-3 pV is essential for the infection of BAdV-3 in CRL (primary) and MDBK (continuous) cells. Analysis of BAV.dV suggested that pV appears to be required for maintaining the integrity of the capsid more fragile leading to the detectable changes in virion morphology and infectivity.
capsid structure and helps in stability of the BAdV-3 capsid. However, lack of pV did not introduce any compensatory mutations in other core proteins X\Mu or pVII. Moreover, pV may have a role in the proteolytic cleavage of pVII.

**METHODS**

**Cells and viruses.** MDBK, CRL (Papp et al., 1997), VIDO-DT1 (CRL cells expressing I-SceI) (Du & Tikoo, 2010) and CRL-pV cells (described below) were cultivated in minimal essential medium (MEM) (Sigma)
supplemented with 10% heat-inactivated FBS (Invitrogen). The HEK293T cells (ATCC® 3RL-3216™) were cultivated in Dulbecco’s modified eagle’s medium (DMEM) (Sigma) with 10% FBS. BAV304a was propagated in CRL cells and BAV.dV was propagated in CRL-pV cells.

Antibodies. Production and characterization of anti-DBP (Kulshreshtha et al., 2004) and anti-fibre (Kulshreshtha et al., 2004) antisera, which detect a protein of 48 and 102 kDa in BAdV-3-infected cells, respectively, has been described. The anti-pX serum detects a protein of 25 kDa, anti-hexon serum detects a protein of 103 kDa and anti-pVII serum detects proteins of 22 and 20 kDa (Paterson, 2010) in BAdV-3-infected cells. Production and characterization of anti-pV serum, which recognizes a protein of 55 kDa in BAdV-3-infected cells, will be described elsewhere (Zhao & Tikoo, 2016, unpublished results). Rhodamine (TRITC-) conjugated goat anti-rabbit antibody (Jackson ImmunoResearch), Alexa Fluor 680-conjugated goat anti-rabbit antibody (Invitrogen), anti-β-actin mAb (Sigma-Aldrich) and IRDye800-conjugated goat anti-mouse antibody (Rockland) were purchased.

Construction of plasmid pUC304A.dV. A 6.4 kb EcoRV–BstI107I DNA fragment of plasmid pUC304A+ (E3-deleted BAdV-3 containing CMV-EYFP inserted in E3 region) was isolated, blunt end repaired by T4 polymerase and ligated to a 2.1 kb EcoRV–BstI107I fragment of plasmid pMCS1 (Thanbichler et al., 2007) creating plasmid pMCS-pV. To delete pV from pMCS-pV, a 465 bp fragment was amplified by using primers dV-F1-F: 5’-TGATCCGGGCAATCCGGG-3’; dV-F1-R: 5’-TGATCCGGGCAATCCGGG-3’ and plasmid pMCS-pV DNA as a template. Similarly, a 602 bp fragment was amplified by PCR using primers dV-F2-F: 5’-GCCGATTAACCCGATCGCTCGCCGGCATCGCAAGCAGG-3’; dV-F2-R: 5’-GCCGATTAACCCGATCGCTCGCCGGCATCGCAAGCAGG-3’ and plasmid pMCS-pV DNA as a template. In the third PCR, these two fragments were annealed and used as DNA template to amplify the 1040 bp DNA fragment without pV by overlapping PCR by using primers dV-F1-F and dV-F2-R. Finally, a 622 bp EcoRI–Hpal DNA fragment of the third PCR product was isolated and ligated to EcoRI–Hpal-digested plasmid pMSC-pV creating plasmid pMSC.dV.

A 1.6 kb BflI fragment (containing kanamycin-resistant gene) of plasmid pUC4K (Taylor & Rose, 1988) was isolated and ligated to BflI-digested plasmid pMCS.dV to create plasmid pMSC-dV.Kan. The recombinant plasmid pUC304-dV-Kan was generated by homologous recombination in Escherichia coli BJ5183 between the plasmid pUC304A+ DNA and a 6.4 kb EcoRV–BstI107I DNA fragment of plasmid pMCS-dV.Kan. Finally, plasmid pUC304.dV-Kan was digested with BflI and the large fragment was religated to create plasmid pUC304A.dV.

Construction of pV expressing cell line CRL.pV. Earlier, we successfully used lentivirus system to isolate VIDO DT1 cells expressing I-Scel endonuclease (Du & Tikoo, 2010). To isolate the cell line stably expressing BAdV-3 pV, we used the second-generation replication defective lentivirus system containing plasmid pTrip-puro, plasmid pSPAX expressing human immunodeficiency virus Gag/Pol proteins and plasmid pMD2.G, expressing vesicular stomatitis virus G protein. Briefly, a 1.2 kb DNA fragment containing the BAdV-3 pV gene was ligated to EcoRV-Xhol-digested plasmid pTrip-pV-Puro (containing a puromycin-resistant marker), creating plasmid pTrip-pV-Puro. The HEK293T cells were co-transfected with plasmid (pTrip-pV-Puro, pSPAX2 and pMD2.G) DNAs. At 48 h post-transfection, the lentivirus in media was collected and used to transduce CRL cells with 8 µg ml⁻¹ polybrene. At 24 h post-transduction, the transduced cells were transferred to 10 cm² dishes. After 24 h, media were replaced by fresh selection media containing 5 µg puromycin ml⁻¹ of puromycin. The puromycin-resistant cell clones were picked, propagated in puromycin-containing media and tested for the expression of BAdV-3 pV.

Western blot analysis. Twenty micrograms of proteins from purified virus, virus-infected cell lysates or pV-expressing cell lysates were...
separated by 10% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad) and analysed by Western blotting using protein-specific anti-serum (anti-β mAb 1:5000, other polyclonal serum 1:400) and Alexa Fluor 680- or IRDye800-conjugated antibodies (1:10 000). The membranes probed with fluorophore-conjugated secondary antibody were scanned and analysed by Odyssey® CLx Imaging System (LI-COR).

Confocal microscopy. Monolayers of CRL-pV cells in two-well chamber slides were fixed with 3.7% paraformaldehyde and permeabilized with 0.1 M PBS containing 0.5% Triton X-100. After blocking with 5% goat serum, the cells were stained with rabbit anti-pV serum and rhodamine (TRITC)-conjugated goat anti-rabbit IgG (Jackson Immunoresearch). Finally, the cells were mounted by mounting buffer (Vector Laboratories) containing DAPI and observed under a confocal microscope TCS SP5 (Leica).

Isolation of recombinant BAV.dV. VIDO DT1 (Du & Tikoo, 2010) or CRL-pV (this report) cells in six-well plates were transfected with PacI-digested plasmid pUC304A.dV or pUC304A+ using Lipofectamine 2000 (Invitrogen). At 4 h post-transfection, the medium was replaced with fresh MEM containing 2% FBS. The cells showing CPE were harvested and freeze-thawed three times. Finally, the recombinant virus was propagated in CRL-pV cells and purified by CsCl gradient centrifugation (Tollefson et al., 1999).

Virus purification. Briefly, CRL or CRL-pV cells in T-150 flasks (Corning) were infected at an m.o.i. of 1. At 48 h post-infection, infected cells were collected by centrifugation and resuspended in 15 ml 10 mM Tris/HCl with 10% glycerol at 1 h, the lower band representing mature virus was collected and subjected to the top of a CsCl density gradient. After centrifugation at 35 000 r.p.m. for 1 h, the lower band representing mature virus was collected and subjected to the second centrifugation overnight. Finally, the virus band was collected, dialysed in 10 mM Tris/HCl for three times and stored in 10 mM Tris/HCl with 10% glycerol at −80°C.

Virus DNA replication. The CRL cells were infected with BAV304a or BAV.dV at an m.o.i. of 2. At 12, 24 and 36 h post-infection, the infected cells were washed in PBS and used to purify low molecular weight DNA as described previously (Farina et al., 2001). Equal amount of DNA was digested with BsmI restriction enzyme and separated by agarose gel electrophoresis and analysed by Gel Doc™ XR+ System (Bio-Rad).

Virus single cycle growth curve. CRL cells in 24-well plates were infected with BAV304a or BAV.dV at m.o.i. of 2. At different times post-infection, the infected cells were harvested, freeze-thawed three times and titrated by TCID50 in CRL-pV cells as described elsewhere (Kulshreshtha et al., 2004).

Viral thermostability assay. To determine if deletion of pV alters BAV.dV thermostability, 1×106 (from CRL-pV) or 1×105 (from CRL) infectious virus particles purified from CRL-pV or CRL in 10% glycerol containing PBS were incubated at different temperatures (−80, −20, 4, 25 and 37°C) for 3 days. To analyse the dynamics of viral inactivation, purified 1×106 (from CRL-pV) or 1×105 (from CRL) infectious virus particles in 10% glycerol containing PBS were incubated at different temperatures (−80, 4 and 37°C) for 0, 1, 3 and 7 days. Finally, the infectivity of the treated virus particles was measured by TCID50 in CRL-pV cells.

Transmission electron microscopy. CRL cells were infected with BAV304a or BAV.dV at an m.o.i. of 2. At 24 h post-infection, the cells were collected and fixed in 2.5% glutaraldehyde, and post-fixed with 1% OsO2 in 0.1 M PBS. After dehydration with a graded ethanol series and propylene oxide, the samples were infiltrated with a mixture of propylene oxide and EMBed-812 embedding medium and polymerized in embedding capsules at 60°C for 24–48 h. The pellets were sectioned by using a Reichert ultratome microtome, and sections were stained with 2% uranyl acetate and lead citrate. Finally, the stained sections were viewed using a Philips CM10 TEM.

To analyse CsCl gradient-purified virions by TEM, 300 mesh formvar/carbon copper grids were placed on droplets of purified BAV304a or BAV.dV for 2 min. After rinsing with water three times (20 s per wash), the grids were stained with 0.5% phosphothungstic acid for 1 min. Finally, the grids with negatively stained virions were viewed using a Philips CM10 TEM.

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