Bovine adenovirus 3 core protein precursor pVII localizes to mitochondria, and modulates ATP synthesis, mitochondrial Ca^{2+} and mitochondrial membrane potential

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Viruses modulate the functions of mitochondria by translocating viral proteins to the mitochondria. Subcellular fractionation and sensitivity to proteinase K/Triton X-100 treatment of mitochondrial fractions of bovine adenovirus (BAdV)-3-infected/transfected cells suggested that core protein pVII localizes to the mitochondria and contains a functional mitochondrial localization signal. Moreover, mitochondrial localization of BAdV-3 pVII appears to help in the retention of mitochondrial Ca^{2+}, inducing a significant increase in the levels of ATP and maintaining the mitochondrial membrane potential (MMP) in transfected cells. In contrast, mitochondrial localization of BAdV-3 pVII has no significant effect on the levels of cytoplasmic Ca^{2+} and reactive oxygen species production in the transfected cells. Consistent with these results, expression of pVII in transfected cells treated with staurosporine decreased significantly the activation of caspase-3. Our results suggested that BAdV-3 pVII localizes to mitochondria, and interferes with apoptosis by inhibiting loss of the MMP and by increasing mitochondrial Ca^{2+} and ATP production.

INTRODUCTION

Mitochondria are vital organelles of the cell that regulate cellular functions and generate energy for all molecular processes (Hackenbrock, 1966; Mannella, 2006; Rapaport, 2003). In addition to energy production, mitochondria also play a central role in Ca^{2+} buffering, supply of metabolites, regulation of apoptotic factors, ageing and development (Chan, 2006; Hollenbeck & Saxton, 2005). Many viruses alter the structure and function of the mitochondria (Ohta & Siddiqui, 2002; Machida et al., 2010), inducing oxidative stress (Huh & Siddiqui, 2002; Machida et al., 2010), the mitochondrial membrane potential (MMP) and the production of ATP (Chang et al., 2009; Monné et al., 2007; Su & Hong, 2010) by translocating their proteins to the mitochondria.

Although adenovirus replicates in the nucleus of the cell, the possibility of its dependence on mitochondria appears logical. However, little is known about the role of mitochondria in adenovirus infections. Human adenovirus (HAdV) has been reported to localize to the mitochondria in cells infected with high-titre virus (Alesci et al., 2007), inducing damage to the mitochondrial architecture. Adenoviral early proteins localize to the mitochondria and either prevent or induce apoptosis (Degenhardt et al., 2000; Lomonosova et al., 2005). Adenovirus protein V interacts with p32 and localizes to the mitochondria (Matthews & Russell, 1998). Adenovirus death protein encoded by the E3 region of HAdV-5 induces oxidative stress and helps in the release of virus progeny from the virus-infected cell (Tollefon et al., 1996). A recent report suggested that HAdV-5 releases cathepsin B activity in the cytoplasm due to endosomal membrane rupture leading to the production of reactive oxygen species (ROS) (McGuire et al., 2011).

Bovine adenovirus (BAdV)-3, a member of the genus Mastadenovirus, is a non-enveloped icosahedral virus, which is being developed and evaluated as a vaccine delivery vector for animals (Zakhartchouk et al., 1999) and humans (Rasmussen et al., 1999). The complete DNA sequence and the transcription map of BAdV-3 genome...
have been reported (Reddy et al., 1998, 1999). Since mitochondria are major cellular organelles performing various functions, study of the mitochondria/virus interaction may provide new insights into the viral/host interactions. Adenovirus core protein VII is found as a precursor (pVII) in infected cells and as a mature protein (VII) in progeny virions (Hindley et al., 2007). Previous studies suggested that adenovirus core protein VII is involved in the import of the adenovirus genome into the nucleus (Wodrich et al., 2006), protecting it from the Mre11–Rad50–Nbs1 complex at early times after infection (Karen & Hearing, 2011), and may have a role in the packaging of DNA in capsids (Zhang & Arcos, 2005). Here, we demonstrated that BAdV-3 pVII localized to mitochondria and appeared to play a beneficial role by modulating mitochondrial functions, including apoptosis.

RESULTS

Isolation of mitochondria from bovine cells

To determine the localization of BAdV-3 proteins to mitochondria, the mitochondria-rich fraction was purified from mock- or BAdV-3-infected Madin–Darby bovine kidney (MDBK) cells and analysed for purity by Western blotting. Equal amounts of proteins from the mitochondrial fraction, cytoplasmic fraction and nuclear fraction of mock- or BAdV-3-infected cells were separated using 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with protein-specific antibodies (Fig. 1a). Anti-cytochrome oxidase (COX)-1 serum detected a specific protein in the mitochondrial fraction but not in the nuclear or cytoplasmic fractions of mock- or BAdV-3-infected cells. Anti-extracellular signal-regulated kinase (ERK) serum detected a specific protein in the nuclear fraction and cytoplasmic fractions of mock- or BAdV-3-infected cells. Anti-extracellular signal-regulated kinase (ERK) serum detected a specific protein in the nuclear fraction and cytoplasmic fractions of mock- or BAdV-3-infected cells but not in the mitochondrial fraction. However, anti-fibrillarin serum detected a specific protein

![Fig. 1](http://vir.sgmjournals.org)

**Fig. 1.** Western blot analysis of cellular fractions. Proteins from lysates of the indicated cellular fraction isolated from mock- or BAdV-3-infected MDBK cells were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed by Western blotting. (a) Anti-cytochrome oxidase (COX)-1 serum (mitochondrial marker), anti-fibrillarin serum (nuclear marker), anti-extracellular signal-regulated kinase (ERK) serum (cytoplasmic marker) and anti-heat shock protein 70 (Hsp70) serum (loading control). (b) Anti-pVII serum, anti-IVa2 serum, anti-penton serum and anti-hexon serum. Mitochondrial (Mito), cytosolic (Cyto) and nuclear (Nucl) fractions from uninfected (U) or infected (I) cells. Uninfected MDBK cells (Controls) and BAdV-3-infected MDBK cells collected at 24 or 48 h post-infection (24 or 48).

![Fig. 2](http://vir.sgmjournals.org)

**Fig. 2.** Analysis of mitochondrial (Mito.) fractions from BAdV-3-infected MDBK cells. Proteins from the lysates of cellular fractions isolated from BAdV-3-infected cells were treated as indicated and separated using 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed by Western blotting with (a) anti-hexokinase mAb and anti-cII mAb, and (b) anti-pVII serum, anti-hexon serum, anti-penton serum and anti-IVa2 serum.
Fig. 3. Analysis of the mitochondria-rich fraction from transfected cells. (a) Schematic diagram of plasmids. The names of the plasmids are given on the left of the panel. Proteins from the lysates of the mitochondrial (Mito.) fraction isolated from cells infected with WT BAdV-3 or transfected with indicated plasmid DNA were treated as indicated, and separated using 10% SDS-PAGE, transferred to nitrocellulose membranes and probed by Western blotting with protein-specific serum. (b) Vero cells co-transfected with pEY.VIImls and pOCT.DsRed (Harder et al., 2004) were visualized directly under a confocal microscope 48 h after transfection. Nuclei were stained with DAPI. The merger of EYFP-VIImls, OCT-DsRed and DAPI staining is shown.
Fig. 3. cont. (c). Amino acid homology of BAdV-3 pVII-like proteins. Alignment of deduced amino acid sequences of BAdV-3 pVII homologues with those of porcine adenovirus (PAdV)-3 (GenBank accession no. BAA76967), ovine adenovirus (OAdV)-A (GenBank accession no. AP_000010), canine adenovirus (CADV)-1 (GenBank accession no. AP_000055), HAdV-5 (GenBank accession no. AP_000207), HAdV-F (GenBank accession no. NP_040858), HAdV-52 (GenBank accession no. ABK35040), simian adenovirus (SADV)-48 (GenBank accession no. ADZ39809), HAdV-A (GenBank accession no. YP_002640218), Gorilla gorilla adenovirus B7 (GenBank accession no. ADQ38367) and HAdV-31 (GenBank accession no. CAO78634.1). Identical, strongly conserved and weakly conserved residues are indicated by an asterisk (*), semi-colon (;) and full point (.), respectively. The vertical arrow indicates a potential protease cleavage site.

in the nuclear fraction but not in the cytoplasmic or mitochondrial fractions of mock- or BAdV-3-infected cells. Anti-heat shock protein 70 (Hsp70) serum detected specific protein(s) in all fractions of mock- or BAdV-3-infected cells and was used as a loading control. These results suggested that the mitochondrial fractions purified from mock- or BAdV-3-infected cells were enriched highly in mitochondria.

**BAdV-3 proteins associate with mitochondria**

To determine if BAdV-3 proteins localize to the mitochondria, proteins from different cellular (mitochondrial, nuclear and cytoplasmic) fractions purified from mock- or BAdV-3-infected MDBK cells were separated using 10 % SDS-PAGE and analysed by Western blotting with selected BAdV-3 protein-specific antibodies (Fig. 1b). As seen in Fig. 1(b), anti-IVa2 serum, anti-penton serum and anti-hexon serum recognized specific proteins in BAdV-3-infected cells or purified mitochondrial, cytoplasmic and nuclear fractions of BAdV-3-infected cells. No such proteins could be detected in uninfected cells or purified mitochondrial, cytoplasmic and nuclear fractions of uninfected cells. Similarly, anti-pVII serum recognized specific proteins in BAdV-3-infected cells or purified mitochondrial and nuclear fractions of BAdV-3-infected cells. No such proteins could be detected in purified cytoplasmic BAdV-3-infected cells, uninfected cells or purified mitochondrial and nuclear fractions of uninfected cells.

**BAdV-3 pVII localizes to mitochondria in BAdV-3-infected cells**

Preliminary studies could not discriminate if the viral proteins localize to the mitochondria on their own due to the presence of the mitochondrial localization signal (MLS; integral membrane proteins and soluble proteins located in the inter-membrane space or matrix) or if they are loosely attached to outer mitochondrial membranes. However, analysis of BAdV-3 protein sequences (GenBank accession no. AF030154) by PsORT and WolfPsORT (Nakai & Horton, 1999), SherLoc (Shatkay et al., 2007) PreDator (Rost et al., 2004), TargetP (Emanuelsson et al., 2007) and MitoProt (Claros & Vincens, 1996) software identified a potential MLS in pVII and pIVa2 but not in hexon or penton.

To resolve the issue, mitochondria-rich fractions from BAdV-3-infected MDBK cells were treated with proteinase K and analysed by Western blotting using protein-specific antibodies. Proteinase K treatment degrades hexokinase protein (inserted in the outer mitochondrial membrane) but does not degrade complex II protein (inserted in the inner mitochondria membrane). As seen in Fig. 2(a), anti-hexokinase serum detects hexokinase protein in untreated mitochondria or BAdV-3-infected cells but not in proteinase K-treated mitochondria (Fig. 2a). As expected, anti-cII serum detects a complex II protein in untreated mitochondria, BAdV-3-infected cells and also in proteinase K-treated mitochondria (Fig. 2a). To demonstrate that the isolation procedure did not damage mitochondrial integrity, the purified mitochondria were treated with both proteinase K and 0.1 % Triton X-100, and analysed by Western blotting using protein-specific antibodies. This treatment renders proteins contained within the mitochondria (inner membrane and matrix) susceptible to protease treatment (Sardanelli et al., 2006). As expected, anti-cII serum did not detect a complex II protein in proteinase K/Triton X-100-treated mitochondria (which degrades proteins inserted both in the outer and inner membranes). These results reconfirmed and established that proteinase K treatment degrades the proteins exposed on the outer mitochondrial membrane but has no effect on the proteins inside the outer mitochondrial membrane. Next, the mitochondrial fraction isolated from BAdV-3-infected cells was treated with proteinase K in the absence or presence of Triton X-100. Treated and untreated mitochondria were analysed by Western blotting using BAdV-3 protein-specific antibodies. As seen in Fig. 2(b), anti-pVII serum detected pVII in proteinase K-treated but not in proteinase K/Triton X-100-treated mitochondria. In contrast, anti-hexon serum, anti-penton serum or anti-IVa2 serum did not detect protein-specific bands in proteinase K- or proteinase K/Triton X-100-treated mitochondria. Taken together, these results suggested that whilst IVa2 protein is exposed to the cytosol, pVII is contained within the mitochondria (inner membrane and/or matrix).

**BAdV-3 pVII localizes to mitochondria in transfected cells**

To determine if pVII could localize independently to the mitochondria, Vero cells were transfected with 0.4 μg cm⁻² of the indicated plasmid DNA. After 48 h of transfection, mitochondria-rich fractions isolated from the transfected cells were treated with proteinase K or
proteinase K/Triton X-100 and analysed by Western blotting using protein-specific antibodies. As seen in Fig. 3(a), pVII was detected in proteinase K-treated mitochondria but not in proteinase K/Triton X-100-treated mitochondria. These results confirmed earlier observations and suggested that BAdV-3 pVII localizes inside the mitochondria (inner membrane or matrix) independently of any other viral protein.

**BAdV-3 pVII contains a functional MLS**

Previous protein analysis predicted the presence of a potential MLS at the N-terminus of pVII (aa 1–54) (Fig. 3c). To determine if the MLS was targeting pVII to mitochondria, the DNA encoding the potential pVII MLS (aa 1–54) was fused in-frame to EYFP to create pEY.VIImls (Fig. 3). The mitochondria-rich fractions were isolated from the cells transfected with indicated plasmid DNA, treated with proteinase K or proteinase K/Triton X-100 and analysed by Western blotting using protein-specific antibodies. As seen in Fig. 3(a), both pVII and EY.VIImls fusion protein were resistant to proteinase K treatment but degraded after proteinase K/Triton X-100 treatment. These results suggest that targeting pVII to mitochondria involves the N-terminal 54 aa. Secondly, Vero cells were co-transfected with indicated plasmid DNA and analysed 48 h post-transfection by confocal microscopy. As seen in Fig. 3(b), a portion of recombinant EYFP co-localized with recombinant OCT-DsRed (which localizes only to mitochondria; Harder et al., 2004) in cells co-transfected with pEY.VIImls and pOCT.DsRed, suggesting that recombinant EYFP was localized to the mitochondria of the transfected cells. Interestingly, analysis of the N-terminal 54 aa of BAdV-3 pVII showed significant homology to corresponding proteins of other adenoviruses (Fig. 3).

**BAdV-3 pVII regulates ATP production**

Next, we determined if localization of pVII to mitochondria had any effect on the production of ATP in the transfected cells. To observe this, Vero cells were transfected with indicated plasmid DNA and ATP production was measured 48 h post-transfection. ATP concentration was found to be significantly higher in the cells transfected with pcDNA3.pVII DNA compared with that in the cells transfected with pcDNA3 or pcDNA3.100K (Fig. 4a). This indicated that pVII induced ATP production in the cells and played some role in ATP synthesis.

**BAdV-3 pVII regulates mitochondrial Ca2+ levels**

Next, we examined if pVII could regulate the Ca2+ buffering ability of mitochondria of transfected cells. Vero cells were transfected with individual plasmid DNAs, and mitochondrial and cytosolic Ca2+ levels were measured 48 h post-transfection using Fluo-4 AM and Rhod-2 AM, which are highly specific indicators of cellular and mitochondrial Ca2+, respectively. As seen in Fig. 5(a), the cells expressing pVII showed a significant increase in mitochondrial Ca2+ levels, whereas the cells expressing the BAdV-3 protein 100K did not show any significant increase in mitochondrial Ca2+ buffering activity. Thapsigargin treatment of the cells results in a global and transient increase in cytosolic calcium levels (Ong & Hausenloy, 2010), which allowed us to examine the ability of mitochondria to effectively uptake and sequester Ca2+. Following incubation of cells in thapsigargin, Ca2+ is released from the endoplasmic reticulum and is available for uptake by all organelles. In the absence of any stimulus, homeostasis directs Ca2+ back to normal depots (Fig. 5b). Observations were recorded at 100 s intervals to determine if released Ca2+ was taken up by the mitochondria of transfected cells. In normal cells, thapsigargin induces release of Ca2+, which subsides over time (homeostasis). The cells expressing pVII showed a significant sequestration and retention of mitochondrial Ca2+ even after 20 min post-treatment with thapsigargin (1 μM), whereas the cells expressing 100K showed no significant effect in mitochondrial calcium uptake post-treatment with thapsigargin (1 μM) (Fig. 5b). In addition, Vero cells expressing pVII or 100K (Fig. 5c) and exposed to 1 μM thapsigargin (Fig. 5d) showed no significant change in cytosolic Ca2+.

![Fig. 4. ATP production in transfected cells. (a) Vero cells were transfected with individual plasmid DNAs and ATP production measured 48 h post-transfection using the ATPLite 1 step kit and a multi-label reader. Measurements are given in arbitrary units (counts per second (c.p.s.)). Data represent the mean ± SEM of two independent experiments, each with three replicates. Means with a different letter are significantly different. *P<0.0001. (b) Proteins from lysates of these transfected cells were also separated using 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed by Western blotting with anti-β-actin mAb, anti-100K serum and anti-pVII serum.](image-url)
levels over the period of treatment. This showed that expression of pVII induced mitochondria to sequester and retain \(\text{Ca}^{2+}\) compared with those expressing 100K.

**BAAdV-3 pVII regulates the MMP**

To verify if alterations in mitochondrial \(\text{Ca}^{2+}\) caused any changes in the MMP, we measured the MMP in transfected cells using tetramethylrhodamine methyl ester (TMRM). Vero cells were transfected with indicated plasmid DNA and MMP changes were measured 48 h post-transfection (Fig. 6a). TMRM fluorescence levels decreased significantly in cells expressing 100K but not in the cells expressing pVII (Fig. 6a). To further confirm the role of pVII in maintaining the MMP, we performed a thapsigargin treatment experiment. As \(\text{Ca}^{2+}\) helps to maintain the MMP, thapsigargin treatment should not cause loss of \(\text{Ca}^{2+}\)-dependent MMP in these cells. As seen in Fig. 6(b), after thapsigargin treatment there was significant loss of the MMP in cells expressing 100K but not in cells expressing pVII. This indicated that expression of BAAdV-3 pVII helped the cells to maintain the MMP, whereas 100K had little or no effect on the maintenance of the MMP.
BAdV-3 pVII and ROS production in Vero cells

To assess mitochondrial function in the cells expressing BAdV-3 pVII or 100K, we measured mitochondrial ROS production in Vero cells transfected with indicated plasmid DNA. At 48 h post-transfection, the cells were incubated with dichlorofluorescein diacetate (DCF-DA) and the fluorescence was quantified (Fig. 7a). The cells transfected with pcDNA3.100K DNA showed a significant increase in the level of ROS production compared with those transfected with pcDNA3 or pcDNA3.pVII, indicating that expression of pVII did not increase oxidative stress levels in Vero cells.

BAdV-3 pVII inhibits caspase-3 activation

To confirm if pVII induces or inhibits apoptosis, a caspase-3 assay was performed. Caspase-3 is an active cell death protease involved in the execution phase of apoptosis (Zou et al., 1997). The caspase-3 assay employs a specific caspase-3 substrate, N-Ac-DEVD-N’-AFC, which upon cleavage by active caspase-3 generates a highly fluorescent product that can be measured using excitation and emission wavelengths of 400 and 505 nm. Vero cells were transfected with indicated plasmid DNA. After 48 h post-transfection, designated cells were treated with staurosporine (a caspase-independent direct activator known to induce apoptosis in cells by blocking the activity of kinases) for 4 h before measuring caspase-3 activity. The data were normalized by measuring the luciferase activity in each sample. As seen in Fig. 8(a), staurosporine treatment of cells transfected with pcDNA3 DNA showed significant activation of caspase-3. Interestingly, expression of pVII did not lead to the activation of caspase-3. In contrast, expression of pVII reduced significantly the activation of caspase-3 in staurosporine-treated cells, suggesting an anti-apoptotic role for pVII.

DISCUSSION

A number of viruses target mitochondria during the infection process and alter their functions (Ohta & Nishiyama, 2011). This involves usually the transport of specific viral proteins to the mitochondria, leading to the modulation of mitochondrial functions. We observed previously that BAdV-3 interacted with the mitochondria and altered mitochondrial structure during the course of infection. In the present study, we demonstrated that core protein pVII localizes into the mitochondria and modulates the mitochondrial physiology.

Like other viruses, BAdV-3 infection appears to affect mitochondrial functions, including ATP production, mitochondrial Ca^{2+} concentrations and the MMP. As localization of viral proteins to mitochondria may alter their function, it is tempting to speculate that one or more BAdV-3 protein(s) may be involved in interactions with the mitochondria to help in inducing these processes. Previous amino acid sequence analysis identified that pVII
contains a potential MLS. However, Western blot analysis of the mitochondrial fraction isolated from infected cells suggested that the all tested proteins appeared to be associated with the mitochondria. It is possible that some of these proteins associate non-specifically with mitochondria due to the effect of virus replication on the distribution of mitochondria in the infected cells. Electron microscopy analysis of infected cells at 12 h post-infection showed the presence of mitochondria in the close vicinity of protein synthesis factories in the infected cells (S. K. Anand & S. K. Tikoo, unpublished observations). These protein(s)-synthesizing factories might be synthesizing viral proteins, which might have been purified with the mitochondrial fraction during the purification process.

Of the four BAdV-3 proteins that were found to be associated with mitochondria, only pVII appeared not to be attached loosely, but localized to the mitochondria. Several lines of evidence support the suggestion that pVII localizes to the mitochondria due to the presence of a functional MLS. (1) pVII-specific proteins could be detected in Western blots of mitochondrial fractions of infected/transfected cells treated with proteinase K. (2) A potential MLS (aa 1–54) could localize the cytoplasmic protein EYFP to the mitochondria of transfected cells. (3) EYFP-VIImls fusion proteins could be detected in Western blots of mitochondrial fractions of transfected cells treated with proteinase K.

Localization of viral proteins in mitochondria (Nakai & Horton, 1999) has been implicated in altering various cellular processes, including subverting the host defence mechanisms to establish themselves and replicate (Castanier & Arnoult, 2011), altering Ca²⁺ homeostasis (Zhou et al., 2009), cellular metabolism (Maynard et al., 2010) and apoptosis (Danthi, 2011). Mitochondrial localization of BAdV-3 pVII induces a significant increase in the levels of ATP, indicating a positive role played by this protein during the course of infection.
Increased ATP is involved in the maintenance of ion gradients and thus the MMP across the mitochondrial membranes (Hollenbeck & Saxton, 2005). pVII also appears to help in the retention of mitochondrial Ca$^{2+}$. Positive effectors of oxidative phosphorylation (Balaban, 2009), it is conceivable that mitochondrial Ca$^{2+}$ retention helps cells to maintain a steady supply of ATP, thus helping to maintain the MMP. Although pVII helps in the increase and retention of mitochondrial Ca$^{2+}$, it has little or no effect on cytosolic Ca$^{2+}$. This may be due to the fact that other Ca$^{2+}$ stores such as the endoplasmic reticulum, which acts as a main storehouse of Ca$^{2+}$ in cells, may be releasing enough Ca$^{2+}$ to maintain cytosolic levels in spite of significant portions of Ca$^{2+}$ being retained by the mitochondria.

Viruses have devised different strategies, including inhibition of cell apoptosis, to facilitate their replication in infected cells. Proteins encoded by several DNA viruses, including vaccinia virus FIL (Wasilenko et al., 2003), Kaposi sarcoma herpesvirus K7 (Wang et al., 2002) or human cytomegalovirus vMIA (Goldmacher et al., 1999), block apoptosis by inhibiting the MMP at the mitochondrial level. Based on our observations, pVII appears to be an anti-apoptotic protein and may help in prolonging the life of the cells, thus helping BAdV-3 to complete its life cycle. Consistent with earlier observations is the fact that (1) pVII has little or no effect on ROS generation in the cells and thus may act as proapoptotic, and (2) expression of pVII in cells treated with staurosporine decreases significantly the activation of caspase-3.

Adenovirus mature core protein VII imports viral DNA in the nucleus (Hindley et al., 2007; Wodrich et al., 2006) and protects virion DNA in the nucleus from the DNA damage response at early times after infection (Karen & Hearing, 2011). As a potential MLS of pVII appears to be located at aa 25–171; Reddy et al., 1998) may contain the MLS (Y. Zhao & S. K. Tikoo, unpublished observations). We speculate that during early stages of infection when the virus needs viable cells, BAdV-3 pVII of infecting virus may localize to mitochondria and help to maintain the life of the cells.

An earlier report suggests that adenovirus pVII condenses viral DNA during progeny virus assembly in the nucleus (Hindley et al., 2007). Here, we demonstrate that BAdV-3 pVII also localizes to mitochondria, and appears to be involved in enhancing vital mitochondrial processes and prolonging the longevity of the cell at later stages of virus infection. Thus, pVII appears to be a multifunctional protein, which may be involved in different aspects of adenovirus infection.

**METHODS**

**Reagents.** Lipofectamine 2000 (Invitrogen), the mitochondria isolation kit for mammalian cells (Pierce), BCIP/nitro blue tetrazolium (NBT) reagent and staurosporine (Sigma), the Dual-Luciferase Reporter Assay System (Promega), ATPLite 1step kit reagents (PerkinElmer), Rhod-2 AM, Fluo-4 AM and TMRM (Molecular Probes), minimal essential medium (MEM; Invitrogen), and Dulbecco’s modified Eagle’s medium (DMEM; Sigma) were used as described by the manufacturer’s. Assays used either a multi-label counter (Victor X3; PerkinElmer) or confocal microscope (TCS-SP5; Leica).

**Cell lines and viruses.** MDBK cells were grown in MEM supplemented with 10% heat-inactivated FBS. WT BAdV-3 (WBR-1 strain) was propagated in MDMK cells in MEM supplemented with 2% FBS (Reddy et al., 1998). Vero cells were propagated in DMEM supplemented with 10% FBS.

**Antibodies.** Anti-penton and anti-hexon sera detect proteins of 62 and 98 kDa, respectively, in BAdV-3-infected cells (Kulkshreshtha et al., 2004). Anti-pVII serum recognizes two proteins of 22 and 20 kDa in BAdV-3-infected cells (Paterson, 2010). Anti-IVA2 serum recognizes a protein of 55 kDa in BAdV-3-infected cells (A. Gaba & S. K. Tikoo, unpublished observations). Anti-130K serum recognizes a protein of 130K in BAdV-3-infected cells (Kulkshreshtha & Tikoo, 2008). mAb specific to hexokinase and polyclonal antibodies specific to ERK2 and fibrillarin were purchased from Santa Cruz Biotechnology. mAb specific to COX-1 was purchased from Invitrogen, mAb specific to Hsp70 was purchased from Stressgen, mAb specific to mitochondrial complex II subunit was purchased from Mitosciences. mAb specific to β-actin was purchased from Sigma. Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG and AP-conjugated goat anti-mouse antibody were purchased from Jackson Immunoresearch.

**Plasmid construction.** pOCT.DsRed (Harder et al., 2004) was a gift from Dr H. McBride (University of Ottawa). The construction of pCDNA100K has been described previously (Kulkshreshtha & Tikoo, 2008). The other plasmids (supplementary File S1, available in JGV Online) were constructed using standard procedures.

**Isolation of mitochondria.** MDBK cells were infected with BAdV-3 at a m.o.i. of 5. Vero cells were transfected with individual plasmid DNAs (0.4 μg cm$^{-2}$) using Lipofectamine 2000. At indicated times post-infection or transfection, the cells were collected and used for isolation of mitochondria using the mitochondria isolation kit for mammalian cells as per the manufacturer’s instructions with some modifications. Approximately 2 × 10$^6$ MDMK cells (mock or infected) or Vero cells (mock or plasmid-transfected) were Dounce homogenized and pelleted at 300 g to collect the cell debris and nuclei. Supernatant 1 was collected, and the pellet containing the cell debris and nucleus was dissolved in nucleus isolation buffer [10 mM KCl, 10 mM MgCl$_2$, 10 mM Tris/ HCl (pH 7.4) and 10 mM DDT], further homogenized in a Dounce homogenizer, and finally centrifuged at 212 g to obtain the nuclear fraction. Supernatant 1 was centrifuged at 3200 g to pellet the mitochondria-enriched fraction. The resulting supernatant 2 left after isolation of the mitochondria-enriched fraction was used as the cytoplasmic fraction.

**Western blot analysis.** MDBK cells infected with WT BAdV-3 at an m.o.i. of 5 were harvested at indicated times post-infection. Vero cells were transfected with individual plasmid DNA (0.4 μg cm$^{-2}$) using Lipofectamine 2000 and harvested at indicated times post-transfection. Proteins from the lysates of cells, mitochondria, cytoplasmic or nuclear fractions were separated using 10% SDS-PAGE and analysed by Western blotting as described previously (Kulkshreshtha & Tikoo, 2008).

**Proteinase K treatment.** The isolated mitochondria were dissolved in buffer C of the mitochondria isolation kit with or without Triton X-100 containing proteinase K at a final concentration 150 μg ml$^{-1}$.
The treated samples were incubated for 30 min on ice before centrifugation at 6700 g for 15 min at 4 °C (Huh & Siddiqui, 2002). The pellet fraction(s) was subsequently analysed by Western blotting using protein-specific antibodies.

**Cellular ATP.** Vero cells grown in 96-well plates were transfected with indicated plasmid DNA (0.2 µg per well) using Lipofectamine 2000. At indicated times post-infection or transfection, the cells were treated with ATPLite 1step kit reagents. The emitted light, which is proportional to the ATP concentration, was recorded using a multi-label counter.

**Mitochondrial and cytosolic Ca2++.** Vero cells grown in 96-well plates were transfected with indicated plasmid DNA (0.2 µg per well) using Lipofectamine 2000. At indicated times post-infection or transfection, the cells were incubated with 5 µM mitochondrial Ca2+-sensitive dye Rhod-2 AM or 10 µM cytosolic Ca2+-sensitive dye Fluo-4 AM for 30 min at 37 °C. The cells were washed three times in Ca2+-free PBS or KRH buffer [129 mM NaCl, 5 mM NaHCO3, 4.8 mM KCl, 1.2 mM KH2PO4, 1 mM CaCl2, 1.2 mM MgCl2, 2.8 mM glucose and 10 mM HEPES (pH 7.4)] and equilibrated for 10 min. Fluorescence signals were detected using a multi-label counter using a 480/31 nm filter to excite the Fluo-4 AM fluorescence and 531 nm filter to excite Rhod-2 AM fluorescence.

The signals were detected at 535 (Fluo-4 AM) and 572 nm (Rhod-2 AM).

**MMP.** Vero cells grown in 96-well plates were transfected with 0.2 µg per well of indicated plasmid DNA using Lipofectamine 2000. At indicated times post-infection or transfection, the cells were incubated for 30 min with 100 nM TMRM in KRH-glucose buffer containing 0.02% pluronic acid, then washed and allowed to equilibrate for 20 min. Fluorescence signals were measured using a multi-label counter with a 531 nm excitation and 572 nm emission filter.

**Mitochondrial ROS.** Vero cells grown in 96-well plates were transfected with 0.2 µg per well of indicated plasmid DNA using Lipofectamine 2000. At indicated times post-transfection, the cells were incubated with 10 µM DCF-DA (Degli Esposti, 2002) and incubated for 30 min in KRH buffer. Finally, the cells were washed three times in KRH buffer and equilibrated for 10 min. Fluorescence signals were measured using a multi-label counter with 480/31 nm excitation and 535 (DCF-DA) and 580 nm emission filters.

**Confocal microscopy.** Vero cells seeded in two-well glass chamber slides were transfected with indicated plasmid DNA (0.8 µg per well) using Lipofectamine 2000. At indicated times post-transfection, the cells were mounted in Citifluor and visualized using a confocal microscope (TCS-SP5).

**Apoptosis assay.** Vero cells in one well (1 × 105 per well) of a 24-well plate were co-transfected with 0.8 µg per well of plasmid pRL, Renilla luciferase DNA and 0.8 µg per well of indicated plasmid DNA using Lipofectamine 2000. After indicated times post-transfection, selected samples were treated with 500 nM staurosporine for 4 h. The treated transfected cells were washed with PBS, lysed and incubated for 1 h in reaction buffer containing DEVD-afc substrate to determine the cleavage of caspase-3 by measuring the generated fluorescent product using a multi-label counter. Expression of Renilla luciferase was measured using the Dual-Luciferase Reporter Assay System. The results were normalized and plotted as mean of six independent readings.

**Statistical analysis.** Data were analysed by one-way ANOVA (Ascombe, 1948) using a general linear model procedure (SAS Enterprise Guide 4.2 under SAS 9.2 environment for Windows XP; SAS) for effect of treatment (DNA transfection). P>0.05 was considered non-significant. Tukey’s post-hoc tests for multiple comparisons were performed if the main effect was significant (P≤0.05). The values are expressed as mean ± SEM.

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