Conserved regions of bovine adenovirus-3 pVIII contain functional domains involved in nuclear localization and packaging in mature infectious virions

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Adenoviruses are non-enveloped DNA viruses that replicate in the nucleus of infected cells. One of the core proteins, named pVIII, is a minor capsid protein connecting the core with the inner surface of the capsid. Here, we report the characterization of minor capsid protein pVIII encoded by the L6 region of bovine adenovirus (BAdV)-3. Anti-pVIII serum detected a 24 kDa protein at 12–48 h post-infection and an additional 8 kDa protein at 24–48 h post-infection. While the 24 kDa protein was detected in empty capsids, only the C-terminal-cleaved 8 kDa protein was detected in the mature virion, suggesting that amino acids 147–216 of the conserved C-terminus of BAdV-3 pVIII are incorporated in mature virions. Detection of hexon protein associated with both precursor (24 kDa) and cleaved (8 kDa) forms of pVIII suggest that the C-terminus of pVIII interacts with the hexon. The pVIII protein predominantly localizes to the nucleus of BAdV-3-infected cells utilizing the classical importin α/β-dependent nuclear import pathway. Analysis of mutant pVIII demonstrated that amino acids 52–72 of the conserved N-terminus bind to importin α-3 with high affinity and are required for the nuclear localization.

INTRODUCTION

Adenoviruses are non-enveloped DNA viruses containing icosahedral capsid composed of major (hexon, penton, fibre) and minor (pIIIA, pVI, pVIII and pIX) structural proteins surrounding the core. The adenovirus core contains the genome (McConnell & Imperiale, 2004; Nemerow et al., 2009) and core proteins (V, VII, mu and terminal protein) that directly bind the viral DNA. The proteolytic cleavage of some structural proteins including pVIII by adenovirus-encoded protease (Anderson et al., 1973; Tremblay et al., 1983; Webster, et al., 1989) is required for maturation of newly formed virions.

Since assembly of adenovirus capsid takes place in the nucleus, structural proteins synthesized in the cytoplasm are transported to the nucleus. Migration of macromolecules from the cytoplasm to the nucleoplasm occurs through the nuclear pore complex (NPC) (Adam, 1999; Kohler et al., 1999). The NPC is composed of 50–100 different species of proteins called nucleoporins with an estimated total mass of ~125 MDa in higher eukaryotes. NPCs are incorporated into the nuclear envelope at sites where the inner and outer mitochondrial membranes are fused. They function as gatekeepers of the nucleus, performing the essential cellular role of mediating an exchange of proteins, nucleic acids and other factors between the cytoplasm and nucleoplasm (Theerthagiri et al., 2010).

The NPC permits a non-selective passive diffusion of molecules smaller than 40 kDa through the nuclear envelope via its aqueous channel of ~10 nm diameter (Paine et al., 1975). NPCs can accommodate active transport of particles as large as 25 nm in diameter or several million daltons in molecular mass (Feldherr et al., 1984). This directed transport requires energy and soluble factors and is triggered by short strings of amino acids called nuclear localization signals (NLS) (Davis, 1995; Gorlich & Mattaj, 1996). The archetype of NLS is the classical NLS, designated basic type NLS, which was first identified in nucleoplasmin and the simian virus 40 (SV40) large T antigen (Dingwall & Laskey, 1991). However, other types of NLS including non-classical type have been identified (Christophe et al., 2000).

The L6 region of the late transcription unit of bovine adenovirus (BAdV)-3 encodes three non-structural (22K,
33K, 100K) and one structural (pVIII) protein (Reddy et al., 1998). Earlier, we demonstrated that BAdV-3 33K interacts with 100K (Kulshreshtha & Tikoo, 2008) and is involved in viral capsid assembly and efficient capsid DNA interaction (Kulshreshtha et al., 2004). Recently, we demonstrated that cleavage of BAdV-3 100K by viral protease is not essential for replication of BAdV-3 (Makadiya, 2013). Here, we sought to characterize BAdV-3 pVIII, a minor 216 aa capsid protein containing two recognizable adenovirus protease specific cleavage sites (108IAGG and 143LGGG) (Reddy et al., 1998). Moreover, we describe the identification of domains involved in nuclear localization and incorporation in mature virions.

RESULTS

Sequence analysis of pVIII protein

Initially, to determine amino acid homology, the protein pVIII sequence encoded by different adenoviruses was analysed by the computer program BLOSUM 62 application, Clone manager version 9.3. As seen in Fig. 1, the N-terminus and C-terminus of pVIII appear conserved in pVIII encoded by 14 different adenovirus species including BAdV-3. Secondly, the BAdV-3 pVIII amino acid sequence was analysed using different online databases to predict intracellular localization signals. None of them predicted the presence of a classical NLS in BAdV-3 pVIII.

Analysis of expression of pVIII by Western blotting

To characterize BAdV-3 pVIII, protein-specific sera pVIIIa and pVIIIb were generated against peptides VIIIa (THV-EMPRNEVLEQLTSHGQIA) and VIIIb (LPYSQPPDSYPDQFIRHYNVYNSVSGYS), respectively. Sera collected after the final boost were analysed by Western blotting. As seen in Fig. 2(a), a mixture of anti-pVIIIa and anti-pVIIIb sera detected a 24 kDa protein between 12 and 48 h post-BAdV-3 infection. Interestingly, an additional 8 kDa protein could be detected only between 24 and 48 h post-infection (Fig. 2a). No such proteins could be detected in mock-infected cells (Fig. 2a, lane Cx).

Detection of pVIII in mature and immature BAdV-3

To determine the identity of the two proteins and if pVIII is part of the virions, immature and mature virions were purified from BAdV-3-infected Madin–Darby bovine kidney (MDBK) cells by double caesium chloride density-gradient centrifugation (Fig. 2b). Proteins from mature virions, empty capsids and lysates of mock- or BAdV-3-infected MDBK cells were analysed by Western blotting using anti-pVIIIa sera (Fig. 2c) or anti-pVIIIb sera (Fig. 2d). As seen in Fig. 2(c), anti-pVIIIa sera detected a band of 24 kDa in empty capsids (lane b) and BAdV-3 infected cells (lane c). No such protein could be detected in mature virions (lane d) or mock-infected MDBK cells (lane a). Anti-pVIIIb sera detected proteins of 24 kDa and 8 kDa in BAdV-3 infected cells (Fig. 2d, lane c; Fig. 2h). Only 24 kDa protein is detected in empty capsids (Fig. 2d, lane b; Fig. 2h). Similarly, only 8 kDa protein is detected in mature BAdV-3 virions (Fig. 2d, lane d; Fig. 2h), which corresponds to one of the adenovirus specific protease cleavage products (Fig. 2 h). No such protein was detected in immature virions using anti-pVIIIb (Fig. 2d, lane b). In addition, a higher molecular mass protein of ~110 kDa could be detected in BAdV-3 infected cells (Fig. 2d, lane c), purified empty capsids (Fig. 2d, lane b) or mature BAdV-3 virions (Fig. 2d, lane d). Based on mass spectrometry analysis, the ~110 kDa protein was identified as a hexon protein.

To determine if association between pVIII and hexon protein was through a disulfide bond, the samples were treated with 40 % (Fig. 2e, lane a), 30 % (Fig. 2e, lane b), 20 % (Fig. 2e, lane c) β-mercaptoethanol (ME) before analysing by Western blotting. As seen in Fig. 2(c), no significant differences could be detected in the expression pattern of the cleaved fragment of pVIII or the hexon (lanes a–c). To determine if the pVIII–hexon interaction was specific, proteins from mock- or BAdV-3-infected cells were analysed by Western blotting using glutathione S-transferase (GST)–pVIII-absorbed anti-pVIII sera. As seen in Fig. 2(f), preabsorption of anti-pVIII with GST–pVIII-purified protein abolished the detection of both pVIII and hexon protein in BAdV-3 infected cells (lane b). However, anti-pV serum (X. Zhao & S. Tikoo, unpublished data) detected a specific protein of 56 kDa in the same BAdV-3 infected cells (lane b). No such protein could be detected in mock-infected cells (lane c).

To determine the localization of pVIII in infected cells, proteins from nuclear or cytoplasmic fractions purified from BAdV-3 infected cells were separated by 15 % SDS-PAGE and analysed by Western blotting. As seen in Fig. 2(g), anti-pVIII detected a 24 kDa protein in both nuclear and cellular fractions of BAdV-3 infected cells. As expected, anti-fibrillarin serum detected fibrillarin protein in purified nuclear fractions but not in purified cytoplasmic fractions of BAdV-3 infected cells.

Sub-cellular localization of pVIII

To determine the intracellular localization of pVIII, MDBK cells were infected with BAdV-3 and analysed by immunostaining using anti-pVIIIa and b sera. As seen in Fig. 3, pVIII localized both in the cytoplasm and in the nucleus of BAdV-3 infected cells (Fig. 3a). No fluorescence was observed in uninfected MDBK cells (Fig. 3b). To examine if nuclear localization of pVIII requires any other protein, African green monkey kidney (Vero) cells were transfected with plasmid pEY–pVIII (1 μg per well in a two-well chamber slide) DNA and examined with a laser-scanning confocal microscope 36 h post-transfection. As seen in Fig. 3(c), pVIII localized in the nucleus and in the cytoplasm of transfected cells and appeared in the form of dots.

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To investigate the biochemical details of nuclear import of pVIII, we performed an in vitro nuclear import assay with digitonin-permeabilized MDBK cells. The plasma membranes of MDBK cells seeded on coverslips were permeabilized with 40 μg ml⁻¹ digitonin and transport mixes were prepared as indicated in Methods. GST–pVIII was used as a cargo protein and a well characterized GST–NLS–GFP (Adam et al., 1990) was used as a control.

As expected, GST–NLS–GFP, a control cargo containing classical importin α/β-dependent NLS was efficiently imported into the nucleus in the presence of the complete transport mixture (Fig. 4a). No such import could be detected in the absence of reticulocyte lysate (source of cytosolic nuclear transport factors) (Fig. 4b) or ATP regenerating system (Fig. 4c). Similarly, GST–pVIII was efficiently imported into the nucleus in the presence of the complete transport mixture (Fig. 4g). No such nuclear import could be detected in the absence of rabbit reticulocyte lysate (Fig. 4h) or ATP regenerating system (Fig. 4i). To determine if nuclear transport is mediated by a Ran-dependent mechanism, the nuclear import was analysed in the presence of the GTPase-deficient RanQ69L mutant [[preloaded with 10 mM guanosine triphosphate (MGTP)]. As seen in Fig. 4, addition of GTPase-deficient
Fig. 2. Western blots showing (a) proteins from the lysates of MDBK cells infected with WT BAdV-3 at an m.o.i. of 5 collected at indicated times post-infection were separated on 15% SDS-PAGE gels, transferred to PVDF membranes and probed using a mixture of anti-pVIIIa and α-pVIIIb antibodies. Uninfected MDBK cell lysates (Cx) were used as a negative control. β-Actin was used as a loading control. The sizes of markers are indicated on the left in kilodaltons. The pVIII protein(s) are indicated by arrows on the right. (b) CsCl₂ purification. Purified empty capsids and mature virion bands after double density-gradient centrifugation. (c–g) Analysis of pVIII of BAdV-3 in mature and immature virus. (c) Proteins from the lysates of mock-infected MDBK cells (lane a), BAdV-3 infected MDBK cells (lane c), CsCl₂ purified empty capsids (lane b) or mature virions (lane d) were separated by 15% SDS-PAGE and analysed by Western blotting using anti-pVIIIa. Size marker (M) bands in kilodaltons are indicated on the right. The hexon and pVIII proteins are indicated by arrows on the left. (d) Proteins from the lysates of mock-infected MDBK cells (lane a), BAdV-3 infected MDBK cells (lane c), CsCl₂ purified empty capsids (lane b) or mature BAdV-3
RanQ69L protein, which promotes disassembly of nuclear import complexes (Richardson et al., 1988), completely inhibited the nuclear import of GST–NLS–GFP (Fig. 4d) and GST–pVIII (Fig. 4j). In addition, nuclear import of both GST–NLS–GFP and GST–pVIII proteins was effectively inhibited when the experiment was performed on ice in the presence of the complete transport mix (Fig. 4f, l). Addition of wheat germ agglutinin (WGA), a lectin that binds glycosylated nucleoporins and inhibits NPC function also inhibited nuclear import of GST–NLS–GFP (Fig. 4e) and GST–pVIII cargo proteins (Fig. 4k).

Identification of nuclear localization domain of pVIII

To identify the domain(s) responsible for nuclear localization of pVIII, a panel of plasmids encoding mutant pVIII proteins fused in-frame to the EYFP gene was constructed (Fig. 5a). Vero cells (~70 % confluent) were transfected with individual plasmid DNAs and analysed by Western blotting (Fig. 5b) and confocal microscopy (Fig. 5c) at 36 h post-transfection. Deletion of amino acids 1–131 (pEY–pVIIId3) [Fig. 5c(iv)] and 1–72 (pEY–pVIIId4) [Fig. 5c(v)] resulted in the localization of pVIII in the cytoplasm of the transfected cells. In contrast, deletion of amino acids 73–216 (pEY–pVIIId1) [Fig. 5c(ii)] or 132–216 (pEY–pVIIId2) [Fig. 5c(iii)] resulted in the localization of pVIII in the nucleus of the transfected cells. Similarly, deletion of amino acids 1–36 and 73–216 (pEY–pVIIId5) [Fig. 5c(vi)] or 1–55 and 73–216 (pNLS–EYFP) [Fig. 5c(viii)] yielded nuclear localization of mutant proteins. In contrast, deletion of amino acids 57–216 (pEY–pVIIId6) [Fig. 5c(vii)] showed localization exclusively in the cytoplasm. This suggested that the potential NLS of pVIII appears to be located between amino acids 56–72. To assess if the identified NLS region of pVIII is able to direct the nuclear import of a heterologous cytoplasmic GFP–β-gal fusion protein (Wu & Tikoo, 2004), Vero cells were transfected with plasmid pNLSGFP–β-gal (amino acids 57–72 of pVIII fused in-frame with GFP–β-gal) or plasmid pCMVGFP–βgal DNAs and analysed by confocal microscopy at 36 h post-transfection. Amino acids 57–72 of pVIII [Fig. 5c(x)] were able to partially direct the heterologous protein into the nucleus, while pCMVGFP–βgal localized solely in the cytoplasm [Fig. 5c(ix)].

Interaction of pVIII with importins

Interaction of proteins with the members of the importin super family mediate nuclear transport through NPCs localized in the nuclear envelope. To determine the involvement and identification of the cellular import receptors, a GST pull-down assay was performed using mutant pVIII proteins (Fig. 6a). Importins α-1, α-3, α-5, β-7 and β-1 were expressed as GST fusion proteins in Escherichia coli (BL21) and purified (Fig. 6b). Purified GST alone or GST fusion proteins coupled to glutathione-Sepharose beads were individually incubated with radiolabelled pVIII synthesized in vitro in the presence of [35S]-methionine using plasmid pRSET–pVIII DNA, pcEY–pVIIIId1, pcEY–pVIIId4, pNLS–EYFP or pEYFPN1 and the TNT T7 Coupled Reticulocyte Lysate System. The protein bound to beads was separated by 10 or 15 % SDS-PAGE and visualized by autoradiography.

Fig. 3. Sub-cellular localization of pVIII. MDBK cells were infected with WT BAdV-3 at an m.o.i. of 5 (a) or were mock infected (b). Vero cells were transfected with 1 μg of plasmid pEY–pVIII DNA (c). At 36 h post-infection or 48 h post-transfection, the sub-cellular localization of pVIII in the cells was visualized by indirect immunofluorescence using α-pVIIla and β serum as the primary antibody and Cy-2 conjugated goat anti-rabbit antibody as the secondary antibody (a, b) or direct fluorescence (c) by laser-scanning confocal microscopy. The nuclei were stained with DAPI (a(ii), b(ii), c(ii)).
As seen in Fig. 6(c), pVIII interacts with importins α-1 (lane 3), α-3 (lane 2) and α-7 (lane 4). However, pVIII appears to interact preferentially with importin α-3. No such binding was observed when purified GST alone (lane 6), GST–importin α-5 (lane 5) or GST–importin b-1 (lane 1) bound to GS beads were used to pull down pVIII. Radiolabelled in vitro transcribed and translated (IVTT) pVIII was used as input control (lane 7).

Like pVIII (Fig. 6d, lane 2), GST–importin α-3 interacted with radiolabelled cEY–pVIIId1 containing a deletion of amino acids 73–216 (Fig. 6d, lane 3). No interaction was observed between GST–importin α-3 and cEY–pVIIId4 containing a deletion of amino acids 1–72 (Fig. 6d, lane 5). Finally, like pVIII (Fig. 6e, lane 4) or EY–pVIIId1 (Fig. 6e, lane 5), GST–importin α-3 bound to GST beads was able to bind to NLS–EYFP (EYFP fused to amino acids 57–72) (Fig. 6e, lane 2). No interaction was observed when GST–importin α-3 was used to pull down EYFP (Fig. 6e, lane 3) or GST alone was used to pull down NLS–EYFP (Fig. 6e lane 6).

**Fig. 4.** Nuclear import assay in digitonin-permeabilized cells. MDBK cells were permeabilized with 40 μg digitonin ml⁻¹ and incubated with GST–NLS–GFP (a–f) or GST–pVIII (g–l) in the presence (a, d, g, j) or absence (b, c, h, i) of indicated components of the transport mixture. The permeabilized cells were treated with WGA before addition of complete transport mixture (e, k) or the experiment with complete transport mix was performed at 4 °C. The pictures were taken with a laser-scanning confocal microscope.

### Nuclear import of pVIII in the presence of inhibitory peptides

To further confirm the involvement of importins in the nuclear import of pVIII, we performed nuclear import blocking assays using specific peptides that can selectively block the activities of importin α/β (Görlich et al., 1996a, b) or transportin 3 (Lai et al., 2001). Specific peptides representing the 41 aa importin β binding domain of importin α [IBB importin α (Görlich et al., 1996a)], importin α binding domain of Ycbp80 protein (Görlich et al., 1996b; Welch et al., 1999) and a 29 aa peptide containing eight RS repeats flanked by two arginine rich stretches (SR1; Lai et al., 2001) were synthesized and added individually to the transport mix with 1 mM concentration (50-fold molar excess). As expected, addition of Ycbp80 peptide (Fig. 7b) or IBB domain peptide (Fig. 7c) to the transport mixture blocked the transport of either GST–NLS–GFP or GST–pVIII. The transport of either GST–NLS–GFP or
**Fig. 5.** (a) Schematic representation of BAdV-3 pVIII. The numbers above the domains represent amino acid numbers in pVIII. Thick boxes represent BAdV-3 pVIII DNA, empty boxes represent EYFP/GFP DNA. Thick boxes with white dots represent β-gal DNA. Dotted lines represent deleted regions. The plasmid names are on the left. The protein names are on the right. (b) Proteins
GST–pVIII was not affected in the presence (Fig. 7d) or absence (Fig. 7a) of SR1 peptide to the transport mixture.

**DISCUSSION**

Adenovirus protein pVIII is the least characterized core protein, which is present as 120 copies per virion (Reddy et al., 2010). The pVIII protein appears to be involved in connecting the core of the virus with the inner surface of the adenovirus capsid (Rohn et al., 1997) thus playing a role in the stability of virion structure (Liu et al., 1985). A recent study suggests that porcine adenovirus-3 pVIII interacts with IVa2, which may suggest its role in genome packaging (Singh et al., 2005). Moreover, cleavage of core proteins including pVIII by adenovirus protease appears essential for maturation of progeny virions (Gastaldelli et al., 2008). Although pVIII, synthesized as precursor protein, is present in immature virions, only the cleaved form of pVIII protein is present in mature adenovirus virions (Chelius et al., 2002; Takahashi et al., 2006). The present study was performed to characterize and determine the functional domains of the BAdV-3 pVIII protein.

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**Fig. 6.** *In vitro* interaction of pVIII with transport receptors. (a) Schematic diagram of BAdV-3 pVIII. The solid box represents BAdV-3 pVIII DNA and the open box represents EYFP DNA. The open box with white dots represents the haemagglutinin (HA) tag. The amino acid numbers of pVIII protein are indicated. Plasmid names are on the left and protein names are on the right. (b) Coomassie blue staining of purified transport receptors. (c–e) GST pull-down assays. (c) *In vitro* transcribed and translated (IVTT) radiolabelled pVIII was incubated with importin α-1 (lane 3), α-3 (lane 4), α-5 (lane 5), α-7 (lane 6), β-1 (lane 1) or GST (lane 2). (d) IVTT radiolabelled individual WT pVIII (lane 2), cEY–pVIIId1 (lane 4), EYFP (lane 8) or cEY–pVIIId4 (lane 6) was incubated with importin α-3 (lanes 1, 3, 5 and 7, respectively) or IVTT radiolabelled pVIII with GST (lane 9). (e) IVTT radiolabelled NLS–EYFP (lane 1) or WT pVIII (lane 4) was incubated with importin α-3 (lanes 2 and 5) or IVTT radiolabelled NLS–EYFP and EYFP were incubated with GST (lane 6) or IVTT radiolabelled EYFP with importin α-3 (lane 3), respectively. The incubation was performed overnight at +4 °C on a nutator and pulled down with glutathione Sepharose beads. The proteins were separated by 10% SDS-PAGE. Subsequently, the gel was exposed to phosphor screen overnight and visualized on a Molecular Imager FX using Quantity One software. IVTT pVIII or GST (2 μl) were used as positive and negative controls, respectively.
The pVIII mRNA is predicted to encode protein of 216 aa, which is expressed as a 24 kD precursor pVIII protein detected first between 12 and 24 h post-infection. Like HAdV-5 (Vellekamp et al., 2001), the pVIII is also detected in the immature but not in mature BAdV-3 capsids. In contrast, 8 kDa cleaved pVIII could be detected in mature BAdV-3 capsids. This suggests that BAdV-3 protease cleaves pVIII at potential cleavage site 143LGGG generating the C-terminal 70 aa polypeptide, which is part of the mature BAdV-3 virion. Although mass spectrometry of purified virions has detected 112 N-terminal residues (Liu et al., 2003), 70 C-terminal residues (Takahashi et al., 2006) or both the N-terminal (112 residues) and C-terminal (70 residues) fragments in HAdV-5 (Blanche et al., 2001; Chelius et al., 2002), recent cryo-electron microscopy studies suggest that both N-terminal and C-terminal fragments are present in mature virions (Liu et al., 2010). It is possible that our inability to detect the N-terminal pVIII fragment in BAdV-3 virions could be due to the lower sensitivity of Western blot assay. Alternatively, it is possible that only a C-terminal 8 kDa protein of pVIII is present in mature BAdV-3 virions. Support for this comes from the fact that anti-pVIIIb sera raised against a peptide comprising amino acids 187–216 could detect the C-terminal (amino acids 147–216) 8 kDa cleavage fragment (Fig. 2d, lane d; Fig. 2e, lanes a–c) in purified virions. In contrast, anti-pVIIIa serum raised against a peptide comprising amino acids 85–109 detected the 24 kDa pVIII in empty capsids and in BAdV-3 infected cells, but no potential N-terminal (amino acids 1–112) cleavage fragment in mature virions (Fig. 2c, lane d).

High-resolution structural studies suggest that pVIII appears to interact with hexon and IIIa protein (San Martin et al., 2012) in the capsid of adenovirus. Both precursor and cleaved forms of BAdV-3 pVIII appear to be tightly associated with a 110 kDa hexon protein, which does not appear to involve the formation of disulfide bonds. A similar association has been observed between hexon and pVIII of the egg-drop syndrome virion (Rohn et al., 1997), which may be suggestive of providing virion stability during and after the biogenesis of the viral particles (Liu et al., 1985).

pVIII mainly localizes to the nucleus and appears in the form of multiple punctuate dots in transfected and infected cells. This distinct pattern of pVIII in the nucleus suggests the presence of the protein in virus replication compartments and its potential involvement in BAdV-3 replication. A similar pattern of expression has been indicated for ICP4, ICP8 and VP5 proteins of herpes simplex virus-1 (de Bruyn Kops et al., 1998) and BZLF1 proteins of Epstein–Barr virus (Daikoku et al., 2005), which are essential for viral replication.

The majority of proteins transported to the nucleus possess classical NLSs containing one or two clusters of basic amino acids. However, recent reports have identified non-classical NLSs in proteins that help them to locate to the nucleus (Christophe et al., 2000). Interestingly, analysis of the BAdV-3 pVIII protein sequence did not identify the presence of classical NLSs. Since proteins smaller than ~40 kDa can passively diffuse into the nucleus through NPCs (Lim et al., 2008), it is possible that detection of 24 kDa BAdV-3 pVIII in the nucleus could occur by passive diffusion. However, pVIII fused to a cytoplasmic EYFP protein was predominantly localized in the nucleus of transfected cells. This suggested that pVIII may contain a non-classical NLS, and is actively transported to the nucleus. Several observations support this conclusion. First, analysis of mutant BAdV-3 pVIII proteins suggested that amino acids 57–72 contain a potential NLS. Secondly, migration of pVIII to the nucleus is inhibited at 4 °C, in the absence of ATP. Thirdly, the nuclear import of pVIII is inhibited in the presence of WGA (which blocks active transport, but not the free diffusion of macromolecules through the NPC (Yoneda et al., 1987)).

Active transport involves binding of protein to the transportin and importin α/β heterodimer in the cytoplasm (Gorlich et al., 1995; Imamoto et al., 1995). This NLS recognition complex docks to the NPC (Newmeyer & Forbes, 1988; Richardson et al., 1988) and is subsequently

**Fig. 7. In vitro nuclear import blocking of pVIII with specific peptides.** Digitonin permeabilized MDBK cells grown on coverslips were incubated with the complete transport mix containing either Ycbp80 (b), IBB importin α (c), SR1 peptide (d) or complete transport mix without inhibitory peptides (a). The cells were analysed by laser-scanning confocal microscopy.
translocated through the pore by an energy-dependent, Ran-dependent mechanism (Moore & Blobel, 1993; Newmeyer & Forbes, 1988; Richardson et al., 1988; Yoneda et al., 1987). The inability of BAdV-3 pVIII to enter into the nucleus in the absence of reticulocyte lysate indicates the involvement of cytosolic import receptors for its transport. Interestingly, SR1 peptide, which inhibits transportin-3-mediated nuclear import, did not affect the nuclear localization of pVIII. In contrast, IBB importin peptide, which inhibits binding of importin α to importin β, and Ycbp80 peptide, which competes for binding to importin α, inhibit the nuclear transport of pVIII. These results suggest that nuclear transport of BAdV-3 pVIII is mediated by the importin α/β pathway.

In addition, the transport of BAdV-3 pVIII from the cytoplasm to the nucleoplasm is Ran dependent. Transport receptors use RanGTP binding as a means to regulate their interactions with cargoes or adaptor molecules (Jakel & Görlich, 1998). The Ran GTP gradient across the nuclear envelope has been proposed to control transport receptor–substrate interactions in a compartment-specific manner (Görlich et al., 1996b).

The presence of different isoforms of importin α may imply that each isoform imports specific substrates into the nucleus (Goodwin & Whitehouse, 2001). However, it is well described that all importin α isoforms can import most cargo proteins with comparable efficiency (Kohler et al., 1999). pVIII binds in vitro with importins α-1, α-3 and α-7, signifying the effective manipulation of more than one receptor to get an easy passage into the nucleus. Similar results have been reported for the nucleoprotein of influenza virus (Wang et al., 1997), EBNA-1 protein of Epstein–Barr virus (Kieff, 1996) and ORF57 protein of γ-2 herpes virus (Goodwin & Whitehouse, 2001) that have been shown to bind with importins α-1 and α-5.

In conclusion, our data demonstrates that pVIII is a nuclear protein associated with hexon that is expressed late in infection and, requires nuclear import factors and a non-conventional NLS(s) for localization to the nucleus. Moreover, while its N terminus contains domains involved in interaction with importins and nuclear localization, the 70 aa C terminus appears to be incorporated into mature virions.

**METHODS**

**Cell lines and viruses.** MDBK cells were grown in minimum essential medium (MEM; Sigma Aldrich) containing 5% FBS and 50 μg gentamicin ml⁻¹. Vero cells were grown in Dulbecco’s modified Eagle’s medium (Sigma Aldrich) containing 10% heat inactivated FBS (Sera Care Life Sciences) and 50 μg gentamicin ml⁻¹. For BAdV-3 purification, confluent MDBK cells grown in 150 mm flasks were infected with WT BAdV-3 at an m.o.i. of 5. Immature and mature BAdV-3 virions were purified by double caesium chloride density-gradient centrifugation as described earlier (Tollefsen et al., 1998). The purified mature virions and empty capsids were stored at −70 °C in PBS containing 10% glycerol.

**Peptide synthesis and production of polyclonal antibodies.** Peptides containing amino acids 85–109 (VIIIa) and 187–216 (VIIIb) of pVIII were synthesized based on intrinsic prediction of hydrophilicity and hydrophobicity of amino acid side chains using the Pioneer Peptide Synthesis system (Perkin Elmer). The peptides were individually conjugated to keyhole limpet haemocyanin as a carrier molecule. Rabbits were immunized with the individual conjugated peptides (500 μg per rabbit) emulsified with Freund’s complete adjuvant followed by two injections (conjugated peptide, 300 μg per rabbit) in Freund’s incomplete adjuvant 4 weeks apart. Serum was collected 10 days after the third injection and was tested for specificity by Western blotting.

For *in vitro* nuclear import blocking experiments, nuclear import protein inhibitory peptides: importin α binding domain of importin α (IBB importin α) (S5RMRKFKKKGGK DTAE LRRRVEVS ELRKA- KKD EQIL KRRNVC) (Görlich et al., 1996a), Ycbp80 (1MFNRKR-RGDFEDE ENYRDFP RMPK QRIP) (Görlich et al., 1996b) and SRI (CGGGRKKRQRSRSRSR SRSRSRRRR) (Lai et al., 2001) were synthesized using the Pioneer Peptide Synthesis system (Perkin Elmer).

**Construction of expression vectors.** Plasmids encoding GST-fused importin α-1, importin α-3, importin α-5, importin β-7 and importin β (kindly provided by Dr M. Köhler) have been described (Depping et al., 2008). The plasmids GST–NLS–GFP, (GST fused to NLS of SV40 T antigen) and GST–RanQ69L (dominant negative mutant of Ran fused to GST) were kindly provided by Dr Y. Yoneda (Tachibana et al., 2000). Plasmid pc3HA (pcDNA3 containing HA tag inserted at HindIII–EcoRI site) was a gift from Dr J. Wilson, University of Saskatchewan, Canada. The construction of other plasmids is described elsewhere (File S1, available in the online Supplementary Material).

**Mass spectrometry.** Proteins from mature BAdV-3, immature BAdV-3 or lysates of BAdV-3 infected MDBK cells were separated by 10% or 15% SDS-PAGE or 4–15% precast gradient gels (Bio-Rad). The separated proteins were electrothermally transferred onto PVDF (Bio-Rad) membranes by wet transfer. The membranes were then blocked with 10% skim milk at 4 °C overnight. Western blots were performed using α-pVIIIa and/or α-pVIIIb polyclonal primary antibodies (1:300) followed by alkaline phosphatase conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch) diluted 1:10000 or IRdye 680 donkey anti-rabbit IgG (Li-COR). For visualization of protein bands, the SDS-PAGE gels were stained with Coomassie blue for 30 min and de-stained with 40% methanol in 10% acetic acid. For mass spectrometry analysis, the desired protein bands were cut from the 4–15% precast gradient gels and sent for analysis to PBI, Saskatoon.

**Confocal microscopy.** The procedure is described elsewhere (File S1).

**Transfection assay.** Monolayers of Vero cells (60–70%) in two-well chamber slides were incubated with Opti-MEM I reduced serum media (Gibco) for 2 h prior to transfection. The cells were transfected with 1 μg per well of individual plasmid DNA using Lipofectamine 2000 (Invitrogen). After 36 h post-transfection, the cells were fixed and stained with 40% methanol in 10% acetic acid. For mass spectrometry analysis, the desired protein bands were cut from the 4–15% precast gradient gels and sent for analysis to PBI, Saskatoon.

**Recombinant protein expression and protein purification.** The expression and purification of GST and GST fusion proteins was carried out as described earlier (Imamoto et al., 1995; File S1).

**GST pull-down assay.** The assay was performed as described earlier (Paterson et al., 2012; File S1).
In vitro nuclear import assay. The in vitro nuclear import assay was carried out as described previously (Adam et al., 1999) with some modifications (Paterson et al., 2012). Briefly, the cells incubated with complete transport solution containing GST–NLS–GFP were processed as described by Paterson et al. (2012). The cells incubated with complete transport solution containing GST–pVIII were permeabilized using 0.05% Triton X-100 in PBS for 5 min, then stained with anti-pVIII or b serum followed by Cy3-conjugated goat anti-rabbit IgG. Finally, the cells were washed in PBS and mounted on slides in Vectashield mounting medium containing DAPI and viewed with a Zeiss LSM 5 laser-scanning confocal microscope.

Isolation of cellular fractions. The nuclear and cytoplasmic fractions of BAdV-3 infected cells were isolated as described previously (Anand et al., 2014).

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