Cleavage of bovine adenovirus type 3 non-structural 100K protein by protease is required for nuclear localization in infected cells but is not essential for virus replication

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The L6 region of bovine adenovirus type 3 (BAdV-3) encodes a non-structural protein named 100K. Rabbit antiserum raised against BAdV-3 100K recognized a protein of 130 kDa at 12–24 h and proteins of 130, 100, 95 and 15 kDa at 36–48 h after BAdV-3 infection. The 100K species localized to the nucleus and the cytoplasm of BAdV-3-infected cells. In contrast, 100K localized predominantly to the cytoplasm of the transfected cells. However, BAdV-3 infection of cells transfected with 100K–enhanced yellow fluorescent protein-expressing plasmid detected fluorescent protein in the nucleus of the cells, suggesting that other viral proteins may be required for the nuclear localization of 100K. Interaction of BAdV-3 100K with BAdV-3 33K protein did not alter the cytoplasmic localization of 100K. However, co-expression of BAdV-3 100K and BAdV-3 protease localized 100K to the nucleolus of the transfected cells. Subsequent analysis suggested that BAdV-3 protease cleaves 100K at two identified potential protease cleavage sites (aa 740–745 and 781–786) in transfected or BAdV-3-infected cells. The cleaved C terminus (107 aa) was localized to the nucleolus of the transfected cells. Further analysis suggested that the cleaved C terminus contains a bipartite nuclear localization signal and utilizes import receptor importin-α of the classical importin-α/β transport pathway for nuclear transport. Successful isolation of recombinant BAdV-3 expressing mutant 100K (substitution of alanine for glycine in the potential protease cleavage site) suggested that cytoplasmic cleavage of BAdV-3 100K by adenoviral protease is not essential for virus replication.

INTRODUCTION
Assembly of progeny adenovirus particles occurs in the nucleus and involves both adenovirus DNA and proteins (Berk, 2007). Although mature adenovirus virions contain only structural proteins, the production of infectious adenovirus virions also requires the involvement of a number of non-structural proteins. Three non-structural proteins, 33K, 22K and 100K, encoded by the late L4 region of human adenovirus type 5 (HAdV-5) are involved in different steps of adenovirus replication. Of these, the 100K protein is involved in inhibition of host cell protein synthesis (Cuesta et al., 2000), enhancing selective translation of adenovirus late mRNAs (Xi et al., 2004) and preventing apoptosis (Andrade et al., 2001). Moreover, 100K mediates the trimerization and transport of the hexon (Hong et al., 2005) for capsid assembly in the nucleus.

Like other viruses (Chambers et al., 1990; Gao et al., 1994; Kohl et al., 1988), production of infectious progeny adenovirus includes a final maturation step involving the cleavage of proteins in the assembled capsid by the adenovirus protease (Mangel et al., 2003; Weber, 1995). Usually, an inactive form of the protease present in immature virions is activated and cleaves selected virion capsid and core proteins present in the assembled virions, leading to the production of infectious progeny virions (Weber, 1976). Mature virions devoid of protease contain DNA but are non-infectious as they are unable to escape from the endosomes (Greber et al., 1996). Recently, 52/55K, a non-structural protein, has been reported to be cleaved by adenovirus protease during HAdV-5 virion maturation process (Pérez-Berna et al., 2014).
Bovine adenovirus type 3 (BAdV-3) is a non-enveloped icosahedral virus with a linear double-stranded genome of 34,446 bp (Reddy et al., 1998). Analysis of the genome sequence and transcriptional map suggests that, like HAdV-5 (Berk, 2007), the BAdV-3 genome is also organized into early, intermediate and late regions. Although the genome organization appears similar to that of HAdV-5, recent studies have identified features distinct to BAdV-3 including the organization of late region gene families (Reddy et al., 1998). Although cleavage of HAdV-5 100K, a non-structural protein, by viral protease has been demonstrated in vitro, adenovirus protease-specific cleavage of 100K proteins has not been reported in virus-infected/gene-transfected cells (Ruzindana-Umunyana et al., 2000, 2002). Here, we demonstrated that BAdV-3 100K is cleaved by the adenovirus protease in the cytoplasm of gene-transfected/virus-infected cells, but cleavage did not appear to be essential for BAdV-3 replication.

RESULTS

Expression of BAdV-3 100K

To determine the subcellular localization of the 100K protein in BAdV-3-infected cells, Madin–Darby bovine kidney (MDBK) cells on coverslips were infected with BAdV-3 (m.o.i. of 5) and analysed by indirect immunofluorescence using anti-100K serum (a mixture of N100 and C100). N100 recognizes the N-terminal aa 102–126 and C100 recognizes the C-terminal aa 776–790 of BAdV-3 100K. At 48 h post-infection, BAdV-3 100K protein localized in both the nucleus and cytoplasm of the infected cells [Fig. 1b(i)] suggesting that 100K localizes to the cytoplasm and nucleus of BAdV-3-infected cells.

To determine if nuclear localization of 100K was dependent of other viral proteins, the coding sequence of 100K was fused in frame with enhanced yellow fluorescent protein (EYFP) (Fig. 1a). As MDBK cells are not easily transfected with plasmid DNA, Vero cells were transfected with plasmid pb100K.EY DNA and examined at 48 h post-transfection by direct fluorescence microscopy. As seen in Fig. 1b(ii), 100K–EYFP fusion protein localized exclusively in the cytoplasm of transfected cells. The subcellular localization of 100K was also determined in Vero cells transfected with plasmid pHA.b100K DNA, with a haemagglutinin (HA) tag sequence fused in frame with 100K (Fig. 1a), by indirect immunofluorescence using anti-HA mAb. As seen in Fig. 1b(iii), the HA–100K fusion protein also localized exclusively in the cytoplasm of transfected cells.

Co-expression of 100K protein with other late proteins of BAdV-3

As nuclear localization of 100K may require the involvement of other viral proteins, initially we determined the localization of 100K–EYFP fusion protein in BAdV-3-infected cells. Cotton rat lung (CRL) cells (Du & Tikoo, 2010) were transfected with plasmid pb100K.EY DNA. At 24 h post-transfection, the cells were infected with BAdV-3 at an m.o.i. of 5. At 48 h post-infection, the cells were analysed by direct fluorescence microscopy. As seen in Fig. 1b(iv), the b100K.EY protein was localized in the nucleus of the BAdV-3-infected cell. To determine the role of other BAdV-3 proteins in nuclear localization of 100K in BAdV-3-infected cells, we performed co-transfection experiments. As an earlier report suggested that 100K interacts with 33K in BAdV-3-infected cells (Kulshreshtha & Tikoo, 2008) and the hexon in HAdV-2-infected cells (Hong et al., 2005), initially we investigated the localization of 100K in co-transfected cells expressing 100K and 33K or 100K and hexon proteins (Fig. 2a). As seen in Fig. 2, recombinant b100K.EY was localized exclusively in the cytoplasm of cells co-transfected with plasmids pDR.33K+pB100K.EY (Fig. 2b, panels a1–a4) or plasmids pDR.hexon+pB100K.EY (Fig. 2b, panels b1–b4) DNA. Similarly, 100K protein was localized exclusively in the cytoplasm of cells co-transfected with plasmid pc52K+pB100K.EY DNA (Fig. 2b, panels c1–c4), pEY.VIII+pHA.b100K (Fig. 2b, panels d1 and d4) or pDR.X+pB100K.EY (Fig. 2b, panels e1–e4). In contrast, co-transfection of cells with plasmid pDR.bProt+pB100K.EY (Fig. 2b, panels f1–f4) DNA resulted in the localization of recombinant b100K.EY protein (Fig. 2b, panel g2) in the nucleus and nucleolus of transfected cells (Fig. 2b, panels f2 and f4) without any alteration in the subcellular distribution of recombinant DR.bProt fusion protein (Fig. 2b, panel h1) in co-transfected cells (Fig. 2b, panels g1 and g4). These results suggested the role of the protease protein of BAdV-3 in the subcellular distribution of the 100K protein in infected cells.

Cleavage of BAdV-3 100K in transfected cells

To further confirm the localization and determine if 100K was cleaved in the presence of BAdV-3 protease, 293T cells were co-transfected with the indicated plasmid DNAs (Fig. 3a). At 48 h post-transfection, proteins from the lysates of transfected cells were separated by 12% SDS-PAGE and analysed by Western blotting using antibodies recognizing the N terminus (N100) or C terminus (C100) of BAdV-3 100K. As seen in Fig. 3b, N100 serum detected proteins of 130 kDa (b100K.EY) in cells co-transfected with plasmid pb100K.EY and control plasmid pDsRed-C1 DNAs (Fig. 3b, lane 2) and proteins of 115 and 105 kDa in cells transfected with plasmid pb100K.EY and pDR.bProt (Fig. 3b, lane 1). Similarly, C100 serum (Fig. 3c) detected proteins of 130 kDa (b100K.EY) in cells co-transfected with plasmid pb100K.EY+pDsRed-C1 DNAs (Fig. 3c, lane 2). In contrast, C100 serum detected a protein of 40 kDa (15K C terminus–EYFP fusion) in cells co-transfected with plasmid pb100K.EY+control plasmid pDsRed-C1 (Fig. 3b, lane 2; and c, lane 2).
To determine if BAdV-3 100K was cleaved in the presence of BAdV-3 protease and localized to the nucleus, proteins from the cytoplasmic and nuclear fractions of the cells co-transfected with the indicated plasmid DNAs were separated by 12% SDS-PAGE and analysed by Western blotting using protein-specific antibodies. Initially, we determined the purity of the cellular fractions. Anti-tubulin serum detected a protein of 50 kDa in the cytoplasmic fraction but not in the nuclear fraction of transfected cells (Fig. 3d). Similarly, anti-fibrillarin serum detected a protein of 36 kDa in the nuclear fraction but not in the cytoplasmic fraction of transfected cells (Fig. 3d). As expected both proteins were detected in total cell lysates of transfected cells. These results suggested that both the nuclear and cytoplasmic fractions were highly pure. Next we determined the localization of the b100K.EY fusion protein using anti-GFP serum. As seen in Fig. 3(d), the 140 kDa b100K.EY fusion protein could be detected in the cytoplasm but not in the nuclear fraction of cells co-transfected with plasmid pb100K.EY and pDsRED DNA. In contrast, a protein of 40 kDa could be detected in the nuclear but not in the cytoplasmic fraction of cells co-transfected with plasmid pb100K.EY and pDR.bProt DNA, suggesting that the C-terminal cleavage fragment of BAdV-3 100K localizes to the nucleus of transfected cells.

Fig. 1. Subcellular distribution of the BAdV-3 100K protein. (a) Schematic representation of BAdV-3 100K. The dotted box represents haemagglutinin (HA) tag sequence. The name of the plasmid is on the right and name of protein is on the left. (b) Confocal microscopy. Monolayers of MDBK cells infected with BAdV-3 were analysed 48 h post-infection by indirect immunofluorescence (IF) using anti-100K serum (N100+C100) (i). Vero cells transfected with plasmid pb100K.EY DNA were visualized 48 h post-transfection by confocal microscope (ii). Vero cells were transfected with the plasmid pHA.b100K DNA and analysed at 48 h post-transfection by indirect immunofluorescence using anti-HA antibody (iii). CRL cells (Du & Tikoo, 2010) were transfected with the plasmid pb100K.EY DNA. At 24 h post-transfection, the transfected cells were infected with BAdV-3 at an m.o.i. of 5 and analysed by direct fluorescence (DF) using confocal microscope at 48 h post-infection (iv). Nuclei were stained with DAPI (blue). Merged images are shown on the right.

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To determine if the 100K protein was also cleaved in BAdV-3-infected cells, MDBK cells were infected with BAdV-3 at an m.o.i. of 5. At 48 h post-infection, the proteins from the lysates of the infected cells were separated by 15% SDS-PAGE and probed by Western blotting using anti-100K sera. As seen in Fig. 4, N100 serum detected a protein of 130 kDa (Fig. 4a). In contrast, C100 serum detected a protein of 15 kDa in BAdV-3-infected cells (Fig. 4b). No such proteins could be detected in the mock-infected MDBK cells (Fig. 4). Moreover, cleavage of the 100K protein of BAdV-3 was detected in MDBK cells harvested at 24–48 h post-infection, suggesting that BAdV-3 100K is cleaved at late times post-infection.

Analysis of the BAdV-3 100K protein sequence for potential protease cleavage sites

Based on the cleavage of adenovirus structural proteins (Mangel et al., 2003) by adenovirus-encoded protease, at least two consensus protease cleavage sequences (M/I/L)XGX-G and (M/I/L)XGG-X (where X is any amino acid) have been identified (Diouri et al., 1996). Consensus adenoviral protease cleavage sequences include glycine residues essential for the cleavage of substrate proteins (Ruzindana-Umunya et al., 2000, 2002). Analysis of the amino acid sequence of BAdV-3 100K identified four
BAΔV-3 100K cytoplasmic cleavage and nuclear localization

(a) b100K.EY

DR.bProt

DsRed

EYFP

pb100K.EY

100K

EYFP

protease

DsRED

pDR.bProt

DsRED

pDsRed-C1

EYFP

pEYFP.N1

(b) N100 sera

(pb100K.EY + pDR.bProt)

(pb100K.EY + pDsRed-C1)

(pEYFP.N1 + pDsRed-C1)

Full-length

Cleaved

130

100

70

55

40

35

25

15

10

(d) pb100K.EY + pDR.bProt

pb100K.EY + pDsRed

pb100K.EY + pDR.bProt

Cyto

Nuc

Cyto

Nuc

Cell

Full-length

Cleaved

b100K.EY (uncleaved)

b100K.EY (cleaved)

Tubulin

Fibrillin
potential protease cleavage sites (Fig. 5a). However, instead of M/I/L, the first amino acid in the potential protease cleavage sites aa 740–745 and aa 744–748 was F and G, respectively.

**Mutational analysis of potential protease cleavage site(s) of the BAdV-3 100K protein**

To further characterize cleavage of the 100K protein by the BAdV-3 protease, we constructed plasmids (pbG706A.G708A, pbG742A.G744A, pbG746A.G747A, pbG783A.G784A and pbG742A.G783A) encoding mutant 100K proteins in which essential glycine residues of the potential protease cleavage sites of 100K were substituted with alanine residues (Fig. 5b). Cleavage of mutant 100K proteins by adenovirus protease was analysed by Western blotting of the lysates of the cells co-transfected with plasmid pDR.bProt and individual plasmid DNAs encoding WT or mutant 100K protein. As seen in Fig. 5(c), anti-GFP antibody detected a major protein of 40 kDa (potential cleavage at the aa 740–745 site) and a minor protein of 36 kDa (potential cleavage at the aa 781–786 site) in the cells co-transfected with plasmids pb100K.EY and pDR.bProt (Fig. 5c, lane 8). Similar proteins were detected in cells co-transfected with plasmids pbG706A.G708A and pDR.bProt (Fig. 5c, lane 1) or pbG746A.G747A and pDR.bProt (Fig. 5c, lane 5). Moreover, cleavage of 100K appeared to be efficient in cells expressing protease, as no uncleaved 100K protein could be detected (Fig. 5c, lanes 1, 5 and 8). In contrast, anti-GFP antibody detected only the 36 kDa protein in cells expressing protease and no other proteins were detected.
co-transfected with plasmids pbG742A.G744A and pDrbProt (Fig. 5c, lane 3). In addition, cleavage of 100K appeared to be inefficient, as uncleaved mutant 100K could be detected in these cells (Fig. 5c, lane 3). Only the 40 kDa protein could be detected in the cell lysates of cells co-transfected with plasmids pb100K.783/784 and pDR.bProt (Fig. 5c, lane 10), suggesting that BAdV-3 protease cleaves BAdV-3 100K. No cleaved fragments could be detected from the cell lysate co-transfected with plasmids pbG742A.G783A and pDR.bProt (Fig. 5c, lane 12).

To further characterize the cleavage of BAdV-3 100K, the subcellular localization of protease cleavage site mutant 100K proteins (Fig. 6a) was analysed in cells co-expressing BAdV-3 protease by confocal microscopy. As seen in Fig. 6(b) WT (b100K.EY) or single protease cleavage site mutant 100K proteins (b100K.742/744 or b100K.783/784) localized predominantly in the nucleus of transfected cells co-expressing the BAdV-3 protease. In contrast, the double protease cleavage site mutant (b100K.742/783) 100K localized predominantly in the cytoplasm of cells co-expressing the protease.

To confirm which cleavage fragment of 100K localized to the nucleus, we constructed three plasmids encoding mutant 100K proteins individually fused in frame to the gene encoding EYFP (Fig. 6c). The Vero cells were transfected with the individual plasmid DNAs and examined by

Fig. 5. Expression of BAdV-3 100K cleavage of mutant proteins in co-transfected cells. (a, b) Schematic diagram of BAdV-3 100K showing the four potential protease cleavage sites (a) and of the plasmid DNAs used (b). The origin of the DNAs is depicted. The glycine-to-alanine substitutions in the predicted protease cleavage sites are underlined in (b). The name of the plasmid is on the right side and the name of the protein is on the left side. (c) Proteins from lysates of the indicated plasmid DNA-transfected 293T cells harvested at 48 h post-transfection were separated by 15 % SDS-PAGE, transferred to nitrocellulose and analysed by Western blotting using anti-GFP antibody. The solid arrow indicates mature b100K.EY fusion protein and open arrows indicate the cleaved b100K.EY protein. Molecular mass markers (kDa) are shown on the left.
(a) b100K.EY  

(b) pb100K.EY + pDR.bProt

(b) pbG742A.G744A + pDR.bProt

(b) pbG783A.G784A + pDR.bProt

(b) pbG42A.G783A + pDR.bProt

(c) 1  

100K  

743  

EYFP  

p100b.EY

744  

100K  

p100b1.EY

850  

785  

850  

100K  

EYFP  

p100b2.EY

DaRed  

Nucleolin  

pRed.C1Nucleolin
BAdV-3 100K cytoplasmic cleavage and nuclear localization

(d) P100b.EY + pRed.C1.Nucleolin
(b) P100b1EY + pRed.C1.Nucleolin
(c) P100b2.EY + pRed.C1.Nucleolin

Bipartite

PDGRGRRGHPGERFQRHLHGRRRR

1 766 850 879 811 100K pHA.b100K
(b) bNLS–GFPβGal [GFP] βGal [p100.C1]
(c) bNLSdel–GFPβGal [GFP] βGal [p100.C2]
(G) GST-Imp IVTT[35S]100K + + + + +

(f) pHA.b100K p100.C1 p100.C2

DF/IF

DAPI

Merge

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Subcellular distribution of BAdV-3 100K cleavage site mutant proteins in co-transfected cells. (a) Schematic diagram of the plasmid DNAs. The origin of the DNAs is depicted. The glycine-to-alanine substitutions in the predicted protease cleavage sites are underlined. The name of the plasmid is on the right side and the name of the protein is on the left side. (b) Vero cells co-transfected with the indicated plasmid DNAs were analysed by direct fluorescence at 48 h post-transfection using laser-scanning confocal microscopy. (c) Schematic diagram of the plasmid DNAs. The origin of the DNAs is depicted. The amino acid numbers of BAdV-3 100K protein are shown. The name of the plasmid is on the right side. (d) Vero cells co-transfected with the indicated plasmid DNAs were analysed by direct fluorescence at 48 h post-transfection using confocal microscopy. (e) Schematic diagram of plasmid DNA. The origin of the DNAs is depicted. The amino acid numbers of BAdV-3 100K are shown. The amino acid sequence of the potential monopartite and bipartite NLSs are depicted. (f) Vero cells transfected with the indicated plasmid DNAs were analysed by direct fluorescence microscopy at 48 h post-transfection using confocal microscopy. DF, Direct fluorescence; IF, indirect fluorescence. (g) Purified GST alone (g) or GST fusion proteins of importin-α1, -α3, -α7 or -β1 were incubated with in vitro-transcribed and translated (IVT) [35S]-labelled 100K, pulled down with glutathione–Sepharose beads, analysed by 10 % SDS-PAGE and visualized by a phosphor screen.

Direct fluorescence. As seen in Fig. 6(d), EYFP fused to either aa 744–850 (cleavage fragment 1; 40 kDa) or aa 785–850 (cleavage fragment 2; 36 kDa) of 100K was localized to the nucleus and nucleolus of the transfected cells. However, EYFP fused to aa 1–743 of the 100K protein localized to the cytoplasm of the transfected cells. Taken together, these results suggested that the protease recognized two protease cleavage sites (aa 740–745 and 781–786) at the C terminus of the 100K protein of BAdV-3. Moreover, our results suggested that the cleaved C terminus of 100K localizes to the nucleus.

The C terminus of the 100K protein contains a nuclear localization signal (NLS)

Analysis of the BAdV-3 100K protein sequence using the PSORT II program identified a potential bipartite NLS at aa 789–811 (Fig. 6e). To determine if aa 789–811 containing

**Fig. 7.** Cleavage of adenovirus 100K by different proteases. (a, b) Proteins from the lysates of 293T cells transfected with the indicated plasmid DNAs were harvested at 48 h post-transfection, separated by 15 % SDS-PAGE, transferred to nitrocellulose and analysed by Western blotting using anti-GFP antibody. Solid arrows indicate the mature adenovirus b100K.EY fusion protein and open arrows indicate the cleaved product. Molecular mass markers (kDa) are shown on the left.
BAdV-3 100K cytoplasmic cleavage and nuclear localization

(a)

![Diagram showing the constructs pFBAV304a, pFBAV742, and pFBAV742/783 with CMV-EYFP and FRASAF peptides]

(b)

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Anti-100K (C100)

100K FL

100K CF

(c)

![Graph showing TCID50 values for BAV304a, BAV742, and BAV742/783]

TCID50 ml⁻¹

10¹ 10² 10³ 10⁴ 10⁵ 10⁶ 10⁷ 10⁸ 10⁹

BAV304a  BAV742  BAV742/783
basic residues are important for nuclear transport, aa 766–850 or 766–850 (containing deletion of aa 789–811) were individually fused in frame to the N terminus of a GFP–β-galactosidase fusion protein (GFP/βGal; Wu et al., 2004), to produce chimaeric bNLS–GFP/βGal and bNLSdel–GFP/βGal (Fig. 6e). As seen in Fig. 6(f), anti-HA serum detected 100K predominantly in the cytoplasm of plasmid pHA.b100K-transfected cells. As expected, bNLSdel–GFP/βGal fusion protein expressed in plasmid p100.C2-transfected cells localized predominantly to the cytoplasm of cells. In contrast, bNLS–GFP/βGal fusion protein expressed in plasmid p100.C1-transfected cells localized predominantly in the nucleus of the cells.

As importins are the receptors involved in the nuclear transport of some of adenoviral proteins (Köhler et al., 2001; Paterson et al., 2012), we performed a glutathione S-transferase (GST) pull-down assay using radiolabelled in vitro-transcribed and translated BAdV-3 100K protein. As seen in Fig. 6(g), GST–importin-α2 was able to bind radiolabelled 100K. No radiolabelled 100K could be detected when GST alone or GST fused to importin-α7, -α1 or -β1 was used to pull down radiolabelled protein. This result was suggestive of the presence of an NLS in the BAdV-3 100K protein sequence, which could bind with the importin-α3 nuclear import receptor and transport it to nucleus.

**Cleavage of the 100K protein in other mastadenoviruses**

To determine if the cleavage of 100K protein was conserved in other members of the genus Mastadenovirus, we constructed (i) plasmids expressing the 100K protein of HAdV-5 (pHA.h100K.myc.EY) or porcine adenovirus type 3 (PAdV-3) (pHA.p100K.myc.EY) as EYFP fusions proteins, and (ii) plasmids expressing the protease protein of HAdV-5 (pDR.hProt) or PAdV-3 (pDRpProt). Proteins from the lysates of the cells co-transfected with plasmid DNAs were separated by 12% SDS-PAGE and analysed by Western blotting. As seen in Fig. 7(a), anti-GFP serum detected a 40 kDa major cleavage product and a minor band of 36 kDa in cells co-transfected with plasmids pHA.b100K.myc.EY and pDRbProt. No such cleavage product could be detected in cells co-transfected with plasmids pHA.h100K.myc.EY and pDR.hProt, or pHA.p100K.myc.EY and pDR.pProt (Fig. 7a). These results suggested that only BAdV-3 100K was cleaved in the transfection assays.

To determine if BAdV-3 100K protein was cleaved by proteases encoded by other adenoviruses, the cells were co-transfected with plasmids pHA.b100K.EY and pDRbProt, pDR.hProt or pDRpProt expressing BAdV-3 protease, HAdV-5 protease and PAdV-3 protease, respectively, and analysed by Western blotting. As seen in Fig. 7(b), proteins of 40 and 36 kDa were detected in cells expressing BAdV-3 b100K.EY fusion protein and protease encoded by BAdV-3, HAdV-5 or PAdV-3. No such proteins could be detected in cells expressing BAdV-3 b100K.EY fusion protein alone.

**Analysis of recombinant BAdV-3 expressing protease cleavage site(s) mutant 100K protein**

To determine the role of 100K cleavage in BAdV-3 replication, we generated protease cleavage-resistant mutant viruses (Fig. 8a). The first recombinant virus, BAV742, contained a substitution of aa 742 and 744 from G to A in potential protease cleavage site 1 of 100K. The second recombinant, BAV742/783, contained substitution of aa 742, 746, 783 and 784 from G to A in potential protease cleavage site 2. Introduction of these amino acid substitutions altered the potential protease cleavage site of 100K, which was confirmed by Western blotting. As seen in Fig. 8(b), C100 serum detected a protein of 15 kDa in the WT BAdV-3 (BAV304a)-infected cells. No such protein could be detected in cells infected with BAV742 or BAV742/783.

Next, we determined the ability of mutant viruses to grow in MDBK cells. Virus-infected cells were harvested at the indicated times post-infection and freeze–thawed five times, and the cell lysates were analysed for virus titre by a TCID50 assay. As seen in Fig. 8(c), there was no significant difference in the growth of WT BAdV-3 or mutant BAV742 and BAV742/783.
DISCUSSION

Production of mature infectious adenovirus particle requires the cleavage of some virus structural proteins by virus-specific protease (Diouri et al., 1996). A recent report demonstrated that the 52/55K non-structural protein of HAdV-5 also acts as a substrate for adenovirus protease (Pérez-Berná et al., 2014). Here, we have reported the cleavage of 100K, a non-structural protein of BAdV-3 by viral protease in transfected and BAdV-3-infected cells. Moreover, we demonstrated that cleavage of BAdV-3 100K appears to be essential for nuclear localization of 100K but not for efficient virus replication. To the best of our knowledge, this is the first report of cytoplasmic cleavage of an adeno- viral non-structural protein by an adenoviral protease.

The 100K protein of BAdV-3 localizes to the nucleus in infected cells. Analysis of the 100K protein predicted a bipartite NLS at aa 789–811, which was sufficient to localize the GFPβGal fusion protein (Wu et al., 2004) to the nucleus in transfected cells. However, BAdV-3 100K protein localized to the cytoplasm in transfected cells, suggesting that the potential NLS is not functional when 100K is expressed alone in transfected cell. In contrast, 100K of HAdV-2/5 localizes to the nucleus in transfected cells (Koyuncu & Dobner, 2009). Interestingly, co-expression of 100K with protease but not hexon localized 100K to the nucleus of transfected cells. Although pV1 (Wodrich et al., 2003) or 100K (Hong et al., 2005) of adenovirus has been reported to be required for partial nuclear localization of the hexon, there appears to be no report suggesting the requirement of adenovirus protease for nuclear transport of an adenovirus protein.

Recent studies have demonstrated significant variation in the structure, subcellular localization and function of adenovirus protein homologues encoded by different members of the genus Mastadenovirus (Blanchette et al., 2013; Cheng et al., 2013; Stracker et al., 2005). Several lines of evidence suggest that significant differences may exist in 100K encoded by different mastadenoviruses. First, analysis of amino acid sequences identified two consensus protease cleavage sites (aa 740–745 and 781–786) in the 100K protein encoded by BAdV-3 but not in the 100K protein encoded by HAdV-5 or PAdV-3 (Fig. 7a). Secondly, the protease encoded by BAdV-3 can cleave the BAdV-3 100K protein in transfected cells. Moreover, cleavage of BAdV-3 100K protein could be detected in infected cells. Thirdly, cleavage of HAdV-5 or PAdV-3 100K protein by proteases encoded by HAdV-5 or BAdV-3 could not be detected in transfected cells. Fourthly, proteases encoded by HAdV-5 or PAdV-3 also cleaved BAdV-3 100K in transfected cells. Finally, BAdV-3 100K cleavage appears to be specific, as cleavage of mutant 100K containing altered protease cleavage sites could not be detected in transfected cells co-expressing protease.

The current model of adenovirus protease activation suggests the requirement of two viral co-factors, namely the C-terminal 11 aa of pV1 (pV1c) and adenoviral DNA (Mangel et al., 1993). Adenoviral protease is synthesized in the inactive form and can only be activated in the presence of co-factors in the nucleus of adenovirus-infected cells (Mangel et al., 2003). Interestingly, BAdV-3 protease appears to be active in the cytoplasm of infected cells (Fig. 7b) and cells expressing BAdV-3 100K. Similarly, HAdV-5 protease and PAdV-3 protease (Fig. 7b) appear to be active in the cytoplasm of the cells expressing BAdV-3 100K, although the efficiency varies for different protease recognition sites among proteases. It is possible that adenovirus protease can be active in the cytoplasm in the absence of the two viral co-factors. Support for this speculation comes from the fact that, at late times after adenovirus infection, cytokeratin 18 is cleaved by adenovirus protease in the cytoplasm (Mangel et al., 2003) in the presence of actin, which potentially acts as the co-factor pV1c.

In addition, our results are consistent with the notion that BAdV-3 100K may influence cleavage by mastadenovirus proteases. First, although BAdV-3 100K is cleaved by the indicated adenovirus proteases in co-transfected cells (Fig. 7), HAdV-5 or PAdV-3 100K is not cleaved by the indicated proteases in co-transfected cells. Secondly, the potential site recognized by the protease at aa 740–745 of BAdV-3 100K contains phenylalanine (F) at position 740 instead of residues methionine (M), isoleucine (I) or leucine (L) in the consensus protease cleavage sequences in the substrate proteins, namely (M/I/L)XGX-G and (M/I/L)XG-G-X. Our findings suggest the important aspect of adenoviral protease activation in BAdV-3 100K co-transfected cells, which can serve as a basis for further studies.

Although BAdV-3 100K protein alone was localized predominantly in the cytoplasm of the transfected cells, co-expression with the protease localized the 100K predominantly to the nucleus of the transfected cells. Further analysis suggested that the C-terminal protease-cleaved fragments of 100K were localized predominantly in the nucleus of the transfected cells co-expressing protease with WT 100K or the single protease cleavage site mutant 100K, but not with double protease cleavage site mutant of the b100K.EY fusion protein. It is possible that the C-terminal cleavage fragment of the b100K.EY fusion protein passively diffuses into the nucleus. Alternatively, it is possible that the C-terminal cleavage fragments contain NLS(s) and are actively transported to the nucleus using the importin-β/β import pathway. Support for this comes from the fact that: (i) analysis of the protein sequence identified a bipartite NLS (aa 789–811; Fig. 6) in the C-terminal domain of the BAdV-3 100K protein; (ii) aa 766–850 were sufficient to direct the nuclear import of a predominantly cytoplasmic fusion protein, GFPβGal (Fig. 8a, b); and (iii) aa 766–850 containing a deletion of aa 789–811 were not sufficient to direct the nuclear import of a predominantly cytoplasmic fusion protein GFPβGal (Fig. 8a, b).

Surprisingly, despite the presence of an NLS, 100K was not transported to the nucleus in transfected cells. We considered the possibility that other viral protein(s) may be

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required for transport of 100K to the nucleus. However, co-expression of 100K with other BAdV-3 late proteins except for the protease did not alter the localization of 100K. Alternatively, it is possible that 100K is retained in the cytoplasm by interacting with a cellular protein. Interestingly, the central domain (aa 498–588) of 100K interacts with the cytoplasmic dynein light chain TcTex 1 (DYNLT1) protein (N. Makadiya & S. K. Tikoo, unpublished data).

The significance of our finding about cleavage of 100K in BAdV-3 infection and localization of the C-terminal cleavage fragment(s) to the nucleus is not clear. Our results suggest that cleavage of 100K at aa 742 and 783 is not essential for efficient replication and generation of progeny BAdV-3. As the cleavage is detected at late times after BAdV-3 infection, we hypothesize that it may have a role in events occurring late in BAdV-3 infection including altered rRNA processing (Paterson, 2010), inhibition of cellular mRNA translation (Ayalew, 2014) and release of virus. However, there was no difference in rRNA processing or cellular mRNA translation in cells infected with WT or mutant BAdV-3 expressing protease cleavage-resistant 100K (data not shown). Moreover, there was no significant difference in the virus titres of WT or mutant BAdV-3. Although WT and BAdV-3 protease cleavage mutants grew to similar titres, it is possible that nuclear localization of the C-terminal cleavage fragment of 100K may help in lysis of infected cells, helping in the efficient release of progeny virus into the medium.

**METHODS**

**Antibodies.** Anti-100K serum raised against N-terminal peptide (aa 102–126) designated N100 and the C-terminal peptide (aa 776–790) designated C100 of BAdV-3 100K, respectively, recognized a cleavage fragment of 100K may help in lysis of infected cells, Western blot analysis. Cells infected with WT BAdV-3 at an m.o.i. of 5 or transfected with plasmid DNAs (10 µg per well of a six-well plate) were processed as described previously (Kulshreshtha et al., 2004).

**Cell fractionation.** Near-confluent 293T cells were co-transfected with the indicated plasmid DNAs. At 48 h post-transfection, the nuclear and cytoplasmic fractions of the cells were collected as described by Patel et al. (2010).

**Confocal microscopy.** The cells (2 x 10^5 cells per well) grown in four-chamber (Lab-Tek) poly-l-lysine-coated glass coverslips were infected with WT BAdV-3 at an m.o.i. of 1 or transfected with plasmid DNA (1 µg per well) and processed as described previously (Paterson et al., 2012).

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