Bovine adenovirus type 3 containing heterologous protein in the C-terminus of minor capsid protein IX

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Abstract

Earlier, we detected pIX of BAdV-3 as a 14-kDa protein in purified virions. Analysis of BAdV-3 pIX using different region antibodies revealed that the N-terminus and central domain of the pIX contain immunogenic sites and are not exposed on the surface of BAdV-3 virion. This suggested that the C-terminus of BAdV-3 pIX (125 amino acid) may be exposed on the virion and may be used as a site for incorporation of heterologous peptides or proteins. We constructed recombinant BAV950 containing a small peptide (21 amino acid), including the RGD motif or recombinant BAV951 containing enhanced yellow-green fluorescent protein (EYFP) fused to the C-terminus of pIX. Western blot analysis demonstrated that the chimeric pIX-RGD was incorporated into virion capsids. Incorporation of the RGD motif into the pIX resulted in significant augmentation of BAdV-3 fiber knob-independent infection of the integrin-positive cells, suggesting that RGD motifs are displayed on the surface of virion capsids and are accessible for binding to integrins. Analysis of BAV951 revealed that the chimeric pIX is incorporated into virion capsids and EYFP containing the C-terminus of pIX is exposed on the surface of the virion. Moreover, insertion of chimeric pIXs was maintained without change through successive rounds of viral replication. These results suggested that in contrast to major capsid proteins (hexon, penton, fiber), the minor capsid protein IX can be used for the incorporation of targeting ligands based on either small peptides or longer polypeptides.

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The Adenoviridae family currently includes at least 70 serotypes of animal adenoviruses and 51 serotypes of human adenoviruses (HAdV) (Mei et al., 2003). Adenoviruses can infect a great variety of post-mitotic cells, even those associated with highly differentiated tissues. The adenovirus capsid consists of three major structural proteins, hexon (II), penton base (III), and knobbed fiber (IV), along with many other minor structural proteins, namely VI, VIII, IX, and IIIa (Stewart, 2002). Although the complex process of entry and intracellular transit involves several structural proteins, it appears that the penton base and the fiber proteins are responsible for the early steps of virus entry pathway. Initially, the C-terminal knob domain of the fiber protein binds with the primary cellular receptor (Louis et al., 1994; Wickham et al., 1993). A fiber receptor for majority of HAdVs has been identified as the coxsackie/adenovirus receptor (CAR) (Bergelson et al., 1997; Roelvink et al., 1998; Tomko et al., 1997). Following binding to the fiber receptor, the RGD motif within the penton base interacts with αVβ integrins and facilitates virus internalization via receptor-mediated endocytosis (Greber et al., 1993; Mathias et al., 1994; Wickham et al., 1993). As the RGD motif is not present in the fiber or the penton base protein of wild-type BAdV-3, the integrin-positive cells are not efficiently transduced by the virus (Wu and Tikoo, 2004).

To provide the therapeutic effect where needed, the adenovirus vector should be capable of delivering genes to specific cells. Because the pattern of viral receptor expression varies among different tissues (Fechner et al., 1999), the natural tropism of the adenovirus needs to be changed. Genetic engineering of HAdV capsid proteins to incorporate targeting ligands has been used to modify the tropism of HAdV vectors (Wickham, 2000). Several peptide ligands have been inserted in major capsid proteins, namely fiber...
(Belousova et al., 2002; Dmitriev et al., 1998; Mizuguchi et al., 2001; Wickham et al., 1996b, 1997; Xia et al., 2000), hexon (Vigne et al., 1999), or penton (Wickham et al., 1996a) of HAdV. However, the structure of capsid proteins has hampered the effectiveness of this approach by limiting the tolerance of capsid proteins towards insertion of longer peptides. Thus, this targeting approach may not be useful for many candidate receptors.

Protein pIX is a minor structural component of the virion, which acts as a capsid cement thus enhancing the thermal stability of the virion (Colby and Shenk, 1981). It influences the transcriptional activity of viral promoters (Rosa-Calatrava et al., 2001) and is essential for the packaging of full-length viral DNA (Ghosh-Choudhury et al., 1987). Earlier studies indicated that C-terminal region of pIX is located on the surface of the capsid (Akalu et al., 1999; Dmitriev et al., 2002), suggesting that it could be used for insertion of targeting ligands. Recently, the minor capsid protein pIX of HAdV-5 has also been used for insertion of small heterologous peptide sequences (Dmitriev et al., 2002).

The pIX protein of BAdV-3 is 125 amino acids long and shows homology of 16–28% to the pIX proteins of other adenoviruses (Reddy et al., 1998). Unlike HAdV (Berk and Sharp, 1978), the BAdV-3 pIX transcript is 3’ co-terminal with E1A and E1B transcripts. The pIX of BAdV-3 is detected as a 14-kDa protein in virus-infected cells and is the component of virion capsid (Reddy et al., 1999a). To assess the feasibility of incorporating heterologous sequences into BAdV-3 pIX, we constructed and characterized recombinant BAdV-3 encoding the chimeric pIX containing either the RGD motif or the enhanced yellow-green fluorescent protein (EYFP) fused to the C-terminus of the protein.

Results

Characterization of BAdV-3 pIX

To characterize the pIX protein in detail, protein-specific antisera were raised by immunizing rabbits with synthetic peptides coupled to a carrier. To determine if antisera raised against the pIX peptide recognize the protein, Western blot analysis was performed. As expected, M1 antisera (aa 61–80) recognized a protein of 14 kDa in BAdV-3-infected cells (Fig. 1A, lane 2) and purified virions (Fig. 1A, lane 3). No

![Figure 1](https://example.com/figure1.png)

Fig. 1. Western blot analysis of pIX in wild-type BAdV-3-infected cells. Proteins from mock-infected MDBK cells (lane 1), wild-type BAdV-3-infected MDBK cells (lane 2), or purified BAdV-3 virions (lane 3) were separated by 12.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The separated proteins were probed in Western blots by anti-pIX M1 (A) or N1 (B) sera. (C and D) Localization of pIX on capsid surfaces of wild-type BAdV-3 particles by immunogold electron microscopy using N1 (C) or M1 (D).
such band could be observed in mock-infected cells (Fig. 1A, lane 1). Similarly, N1 antisera (aa 13–32) recognized a protein of 14 kDa in BAdV-3-infected cells (Fig. 1B, lane 2) and purified virions (Fig. 1B, lane 3), but not in mock-infected cells (Fig. 1B, lane 1). However, no such band could be detected in BAdV-3-infected cells or purified virions using either C1 (aa 80–99) or C2 (aa 90–110) antisera.

To determine the antigenic regions of pIX exposed on the virus particle, immune electron microscopy was performed using purified virions and pIX-specific antisera. As seen in Fig. 1, partially disrupted capsids of virions were labeled with N1 (Fig. 1C) and M1 (Fig. 1D) sera. However, no labeling was detected when N1 or M1 sera were incubated with complete BAdV-3. These results suggested that antigenic sites in the N terminus (amino acid 13–32 and 61–80) of pIX are not exposed in the virion.

Analysis of BAdV-3 pIX sequence

Although the protein sequence identities between BAdV-3 pIX and other adenoviruses are not high (Reddy et al., 1998), we could clearly identify two sequences common in pIX of all known adenoviruses (Fig. 2A). One of them is the conserved element that can be located at the N-terminus of the protein. In HAdV-2 and -5, this sequence is necessary for pIX incorporation into the capsid (Rosa-Calatrava et al., 2001). Another sequence is a putative leucine-zipper, comprising four leucine and one valine residues, each spaced six residues apart. Studies on HAdV revealed the requirement of this sequence for transactivation of the promoters (Lutz et al., 1997) and formation of nuclear inclusions (Rosa-Calatrava et al., 2001).

Construction of pIX-modified BAdV-3

Because the N-terminal domain appears to be embedded in virion capsid, we choose to incorporate heterologous peptide or protein into the C-terminus of BAdV-3 pIX. We used the HpaI site (nt 3560 of BAdV-3 genome) in the 3' end of the pIX gene to incorporate heterologous sequences into the BAdV-3 pIX C-terminus. This resulted in the removal of the last four amino acids of the wild-type BAdV-3 pIX. The plasmids used for constructing chimeric pIX are depicted in Fig. 3. The genes encoding pIX-RGD and pIX-EYFP were inserted individually into the BAdV-3 genome using the homologous recombination machinery of Escherichia coli (Chartier et al., 1996). The PacI-digested pFBAV950, pFBAV951, or pFBAV3 plasmid DNAs were transfected into VIDO R2 cells (HAdV-5 E3-transformed fetal bovine retina cells; Reddy et al., 1999b) and produced cytopathic effects in 14 days. The infected cell monolayers showing 50% cytopathic effects were collected, freeze–thawed, and recombinant viruses were plaque purified and propagated in Madin Darby bovine kidney (MDBK) cells. The recombinant BAdV-3s were named BAV950 (pIX-RGD) and BAV951 (pIX-EYFP). The amino acid sequence of the modified pIX in BAV950 is presented in Fig. 2B. The last four amino acids on the C-terminus of pIX were replaced with the 21 amino acid sequence containing the RGD motif and a short spacer with the GS repeats. Amino acid sequence of modified pIX in BAV951 is presented in Fig. 2C. The last four amino acids in the C-terminus of pIX were replaced with the 242 amino acid of EYFP. The viral DNA was extracted and analyzed by PCR using primers P91, 5'-CTAATCGATACATGTAC ACTG-3' (nt 3057–3077); and P92, 5'-CCAACCGGTTGTGGAAAATC-3' (nt 4450–4469). As expected, a 1393-bp PCR fragment could be

A. MAEEGRIYYPVTARLPKWSGSVQDKTGSNMLGGVVPNNQAHRTETVGETATRDNLAEGARRPEDQTTPDMLVEDSLGGLKRRMDLLEESNQQSTLATNRLR TGLAAAYVQANLVVGGQVNPFF

Leucine zipper

B. MAEEGRIYYPVTARLPKWSGSVQDKTGSNMLGGVVPNNQAHRTETVGETATRDNLAEGARRPEDQTTPDMLVEDSLGGLKRRMDLLEESNQQSTLATNRLR TGLAAAYVQANLVVGGQVQVATMSKGEEFTGVPILVELEDVNGHKFSVSGE GEGDATYGKLTFLKICCGTGLPVVPWFTLVTFYGLQCFARYPDHKMQHDFFK SAMPEGYYQVQRTIFFKDGNYKTRAEVKFEGDVLNRIELKGDFKEDGNILGH KLEYNYNSHNYVMADKQGKNGIKVNFKMRHNIEDGSGVLADHYQONTPIGDPGV LLPDNYLHSLQOSALSDPNEKRDHVMLLEFVTAAGITLGMDELYK

C. MAEEGRIYYPVTARLPKWSGSVQDKTGSNMLGGVVPNNQAHRTETVGETATRDNLAEGARRPEDQTTPDMLVEDSLGGLKRRMDLLEESNQQSTLATNRLR TGLAAAYVQANLVVGGQVQVATMSKGEEFTGVPILVELEDVNGHKFSVSGE GEGDATYGKLTFLKICCGTGLPVVPWFTLVTFYGLQCFARYPDHKMQHDFFK SAMPEGYYQVQRTIFFKDGNYKTRAEVKFEGDVLNRIELKGDFKEDGNILGH KLEYNYNSHNYVMADKQGKNGIKVNFKMRHNIEDGSGVLADHYQONTPIGDPGV LLPDNYLHSLQOSALSDPNEKRDHVMLLEFVTAAGITLGMDELYK

Fig. 2. Amino acid sequences of pIX. (A) Wild-type BAdV-3 pIX. The conserved sequence element and putative leucine zipper are underlined. (B) BAV950 chimeric pIX. RGD-containing peptide sequence is underlined. (C) BAV951 chimeric pIX. The EYFP sequence is underlined.
Fig. 3. Schematic diagram of the plasmids. The map of the plasmids depicting the location of different genes and restriction enzyme sites is shown. The map is not drawn to the scale. The restriction enzyme sites (HpaI in pBAVN and A in pFBAV3) are shown in bold.
detected in wild-type BAdV-3 DNA (Fig. 4A, lane 1). As a result of the insertion of heterologous sequences, PCR fragments of 1456 and 2125 bp could be detected in passage 1 of BAV950 (Fig. 4A, lane 2) and passage 1 of BAV951 (Fig. 4A, lane 4) DNAs, respectively. Similar PCR fragments could also be detected in passage 10 DNA of BAV950 (Fig. 4A, lane 3) or BAV951 (Fig. 4A, lane 5).

Incorporation of modified pIX into BAdV-3 capsid

The wild-type BAdV-3, BAV950, and BAV951 were purified by CsCl gradient centrifugation. The virion proteins denatured by boiling in sodium-dodecyl sulphate (SDS) and 2-mercaptoethanol were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting using anti-pIX M1 sera (Reddy et al., 1999a). As expected, anti-pIX M1 sera recognized a protein of 14 kDa in wild-type BAdV-3 (Fig. 4B, lanes 1 and 3). In contrast, anti-pIX M1 sera recognized a protein of 16 kDa in BAdV950 (Fig. 4B, lane 2). The increase in the molecular mass of pIX in BAV950 is consistent with the addition of 17 extra amino acids, thus confirming the presence of RGD sequences in pIX of BAdV950 capsids.

The anti-pIX M1 sera recognized a protein of 41 kDa in CsCl-purified BAV951 (Fig. 4B, lane 4). A protein of similar molecular weight could also be detected in CsCl-purified BAV951 (Fig. 4B, lane 6), but not in wild-type BAdV-3 (Fig. 4B, lane 5), using anti-EYFP sera. These results confirmed the presence of EYFP in pIX of BAV951 capsids. To determine if EYFP incorporated into modified pIX is displayed on the surface of BAV951 particles, immunoelectron microscopy was performed using CsCl-purified BAV951 virions and anti-EYFP sera. As can be seen in Fig. 4C, the BAV951 virions were labeled with EYFP-specific antibodies and gold-tagged protein A, whereas the BAdV-3 virions did not react with the anti-EYFP sera. In addition, CsCl-purified BAV951 showed green fluorescence when visualized under fluorescent microscope (Fig. 4D).

Infection efficiency of pIX-modified BAdV-3

To determine whether the incorporation of RGD in modified pIX is displayed on the surface of the BAV950 virion, we evaluated the infection efficiency of BAV950 of integrin-containing cells (HeLa and A549). The cells were infected with BAdV-3 or BAV950 at a multiplicity of...
infection of 10 TCID<sub>50</sub>/cell (one TCID<sub>50</sub> is defined as the highest dilution of the virus showing cytopathic effects in 50% of the replicates). After 2 h of adsorption, the cells were washed twice with PBS and media were changed. At 48 h post infection, the cells were trypsinized and harvested. Total DNA was extracted from the cells and DNA aliquots were subjected to real-time PCR analysis. As seen in Fig. 5, compared to BAdV-3, we found 10-fold more viral DNA in BAV950-infected A549 or HeLa cell lines.

To determine if this increased infection of integrin-positive cells was due to the RGD motif in modified pIX of BAV950, integrin-positive human A459 cells in 12-well plate (2 x 10<sup>5</sup> cells/well) were incubated with wild-type BAdV-3 or BAV950 at a multiplicity of infection of 10 TCID<sub>50</sub>/cell in serum-free media. After 30 min of incubation, the media were aspirated and cell monolayer was overlaid with minimum essential medium (MEM) containing 5% fetal bovine serum (FBS) and 0.7% agarose. After 3 days of incubation at 37 °C, the agarose overlay was removed and the cells were fixed with 100% methanol. The cells were stained with anti-BAdV-3 DBP antibodies and infectious units of BAdV-3 were counted as described earlier (Zhou et al., 2001). As seen in Fig. 6A, compared to the wild-type BAdV-3, 3.5-fold more infectious units of the BAV950 virus

Fig. 5. Quantitation of viral genomes. Total DNA was extracted from virus-infected cells 48 h postinfection. The number of viral genomes present in A459 and HeLa cells was estimated by real-time PCR as described in the text.

Fig. 6. Accessibility of pIX-incorporated RGD ligand for binding. (A) Monolayers of A549 cells in 12-well plates were incubated with purified BAdV-3 fiber knob protein or MEM for 10 min. Wild-type BAdV-3 or BAV950 was then added at a multiplicity of infection of 10 for 30 min at room temperature. (B) Monolayers of A549 cells in 12-well plates were incubated with RGD containing peptide (QAGTFALRGDNPQG) or MEM for 10 min at 4 °C. Wild-type BAdV-3 or BAV950 was then added at a multiplicity of infection of 10 for 30 min at 4 °C. Finally, the viruses were aspirated and the cells were overlaid with media containing 0.7% agarose. After 3 days of incubation at 37 °C, the agarose overlay was removed and the cells were fixed with 100% methanol. The cells were stained with anti-DBP and infectious units of BAdV-3 were counted as described (Zhou et al., 2001). Each bar represents the cumulative mean ± SD of triplicate determinations.
were detected in A549 cells. To confirm that increased infection efficiency was due to binding of BA V950 to the integrins, we infected A549 cells in the presence of soluble BAdV-3 fiber knob protein (70 μg/ml). As seen in Fig. 6A, the presence of BAdV-3 fiber knob protein caused 90% reduction in the detection of BAdV-3 infectious units in A549 cells. However, there was 18% reduction in the detection of BA V950 infectious units in A549 cells (Fig. 6A). To confirm that the increased binding of BA V950 was due to the RGD motif, we infected A549 cells in the presence of RGD containing peptide (QAGTFALRGDNPQG). The presence of RGD peptide caused 68% reduction in the detection of BAdV950 infectious units in A549 cells. However, there was no significant difference in the detection of BAdV-3 infectious units in A549 (Fig. 6B).

Viral growth

To estimate virus yield, MDBK cells in 12-well plate (2 × 10^5 cells per well) were infected with BAdV-3, BA V950, or BA V951 at a multiplicity of infection of 1 TCID_{50}/cell. At 72 hpi, cells and culture media were harvested, freeze–thawed, and the viruses were titrated on MDBK cells as described in the text.

Discussion

Recombinant HAdVs from subgroup C have proved to be highly efficient in transducing foreign genes to a variety of cells and tissues. However, this promiscuous tropism of HAdV vectors results in uncontrolled gene transfer to both target and nontarget cells, thus compromising the efficacy of gene delivery (Wickham, 2000). In addition, certain cell types are not efficiently infected with HAdV vectors. To overcome these limitations, mutagenesis (to alter native tropism) and genetic modification (insertion of peptide ligands) of major capsid proteins (fiber, hexon, and penton) have been employed to redirect the HAdV vector to a cell of interest (Dmitriev et al., 1998; Mizuguchi et al., 2001; Vigne et al., 1999; Wickham et al., 1996a, 1996b, 1997; Xia et al., 2000). However, structural properties of major capsid proteins have put constraints on the length of the inserted peptide ligand (Dmitriev et al., 1998; Wickham, 2000; Wickham et al., 1997). An alternate to this strategy may be the modification of minor capsid proteins (Dmitriev et al., 2002). Here, we report the (a) construction and characterization of recombinant BAdV-3 expressing chimeric pIX, (b) stable incorporation of chimeric pIX (pIX-RGD) into mature BAdV-3 virions resulting in increase of BAdV-3 infection efficiency of integrin-positive human A549 and HeLa cells, and (c) stable incorporation of modified pIX containing heterologous polypeptide (EYFP) into virions.

Earlier, we have demonstrated that the pIX of BAdV-3 is detected as a 14-kDa protein in virus-infected cells and is the component of virion capsid (Reddy et al., 1999a). Analysis of BAdV-3 pIX using antibodies produced against different regions suggested that the N-terminus (amino acid 13–32) and central domain (amino acid 61–80) of pIX contain immunogenic sites. However, it is possible that the C-terminus may contain immunogenic sites that were not present in overlapping synthetic peptides. While immunogenic sites of HAdV-3 pIX are both in the N- and the C-terminus of the protein, the immunogenic sites of HAdV-2 pIX appear to be located primarily in the N-terminus of the protein (Akalu et al., 1999).

Despite differences in the accessibility of antigenic determinants of pIX in intact HAdV virions, it appears that the topography of pIX is identical in icosahedral capsids of different adenoviruses (Akalu et al., 1999; Dmitriev et al., 1998). While the C-terminal domain of pIX is located on the surface of the virion, the N-terminal domain is hidden inside the virion (Akalu et al., 1999; Dmitriev et al., 2002). Immunogold electron microscopy of CsCl-purified BAdV-3 virions using pIX-specific sera indicated that antigenic regions in the N-terminus (amino acid 13–32 and 61–80) of pIX are not exposed in the virion. This suggested that like other adenoviruses, the C-terminus of pIX may be exposed on the surface of the BAdV-3 capsid.

To confirm the orientation of pIX and to determine if it can be used for the incorporation of heterologous peptide, we isolated recombinant BA V950 encoding modified pIX (containing 21 amino acid peptides including the RGD motif fused to the C-terminus of pIX). Western blot analysis demonstrated that the chimeric pIX is stably expressed in BA V950-infected cells and is incorporated into viral capsids. As the RGD motif is not present in the fiber or the penton base protein of wild-type BAdV-3, the integrin-positive cells are not efficiently transduced by the virus (Wu and Tikoo, 2004). However, BA V950 transduced integrin-positive cells more efficiently than wild-type...
BAV951 virions. Moreover, the genome of BAV951 appeared to be stable after several passages. These results suggest that genetic modification of adenovirus pIX incorporating high-affinity polypeptide ligands including single chain antibodies could be possible without affecting viral assembly.

A number of features of BAV95-3 including the absence of preexisting antibodies in humans and resistance to neutralization by human sera containing HAdV-5 neutralizing antibodies (Wu and Tikoo, manuscript in preparation) make it attractive for use as a vector in human vaccination. However, BAV95-3 transduces nonbovine cells including human cells poorly (Wu and Tikoo, 2004). As such, attempts have been made to alter the tropism of BAV95-3 by using modified fiber protein (Wu and Tikoo, 2004). Availability of pIX-mediated targeting will help not only in developing improved BAV95-3-based vectors for human vaccination, but also for developing improved BAV95-3 vectors for bovine vaccination.

Materials and methods

Cells and virus

MDBK and VIDO R2 (HAdV-5 E1-transformed fetal bovine retina cells; Reddy et al., 1999b) cells were grown in Eagle’s minimum essential medium (MEM) with 5% fetal bovine serum (FBS). The WBR-1 strain of BAV951 was propagated in MDBK cells in Eagle’s MEM containing 2% FBS (Reddy et al., 1999a).

Antibodies and proteins

Rabbit anti-EYFP sera were purchased from Clontech. Rabbit antisera produced against the BAV95-3 pIX peptide [M1 (61EGARRPEDQ TPYMLVE38)] have been described (Reddy et al., 1999a). To raise antisera against different regions of pIX, three synthetic peptides representing the N-terminus [N1 (13RLPKWSQSVDDKTGSMN L32)] and C-terminus [C1 (60LGGKRRMDDLIEENQ QLLA39) and C2 (60LGGQNLATINRLTGLAA110)] were synthesized. Rabbits were first injected with 0.5 mg peptide, conjugated to keyhole limpet hemocyanin in Freund’s complete adjuvant, and subsequent two injections were given using 0.2 mg of peptide conjugated to egg white ovalbumin in Freund’s incomplete adjuvant at 4 weeks interval.

The construction, expression, and purification of BAV-3 fiber knob protein will be described elsewhere (Wu and Tikoo, manuscript in preparation). Briefly, the knob domain of BAV-3 was expressed in baculovirus expression system using pAcSG2 vector (Pharmingen International) and purified as described by the manufacturer. The concentration of the purified knob was determined by the Bradford protein assay (Bio-Rad) using bovine serum albumin as a standard.

Construction of recombinant plasmids

A 12-kb NorI fragment (containing vector backbone and nucleotide 1–10051 of BAV-3) isolated from plasmid pFBAY3 (Fig. 3) was religated, creating plasmid pBAVN (Fig. 3). A 8-kb AgeI fragment isolated from plasmid pBAVN was religated to create plasmid pBAVNdA (Fig. 1). A 731-bp AgeI–NorI fragment [containing the gene for enhanced yellow-green fluorescent protein (EYFP)] isolated from plasmid pEYFP-NI (Clontech) was blunt end repaired with Klenow and ligated to HpaI-digested pBAVNdA (Fig. 3) creating plasmid pBNdAYFP. To incorporate the RGD peptide coding sequence into the 3’ end of pIX, two overlapping synthetic oligonucleotides (sense oligo: 5’-GGAT- CAGGATCAGGTTCAGGGATGCTGCTGCCTGCG- GACTGCGCGGCATGTTTTCGGTTTAAGTT-3’, antisense oligo: 5’-AATATACCGAAAAACAAATCGC-GGACAGTGCAGGCAGGCACTCCCTGAAACTG- GATCCCTGATCC-3’) were used to make the DNA sequence containing the RGD motif and a shorter encoding five GS repeats (Wickham et al., 1997). The oligonucleotides were annealed to form a DNA duplex and ligated to HpaI-digested pBAVNdA (Fig. 3) creating plasmid pBNdAYFP. To incorporate the RGD peptide coding sequence into the 3’ end of pIX, two overlapping synthetic oligonucleotides (sense oligo: 5’-GGAT- CAGGATCAGGTTCAGGGATGCTGCTGCCTGCG- GACTGCGCGGCATGTTTTCGGTTTAAGTT-3’, antisense oligo: 5’-AATATACCGAAAAACAAATCGC-GGACAGTGCAGGCAGGCACTCCCTGAAACTG- GATCCCTGATCC-3’) were used to make the DNA sequence containing the RGD motif and a shorter encoding five GS repeats (Wickham et al., 1997). The oligonucleotides were annealed to form a DNA duplex and ligated to HpaI-digested plasmid pBNdAYFP and pBNdARGD, creating plasmid pBAV-NotYFP and pBAVNotRGD, respectively.

The recombinant BAV-3 genomes containing pIX fused to RGD (pFBAY950) or EYFP (pFBAY951) were generated by homologous DNA recombination in E. coli BJ5183 (Chartier et al., 1996) between BsaBI–Pmel-digested pFBAY3 (Fig. 3) and a 10.7-kb PaeCl–NotI fragment of pBAVNotYFP or between BsaBI–Pmel-digested pFBAY3.
(Fig. 3) and a-10 kb PacI–NotI fragment of pBAVNotRGD, respectively.

Construction of the recombinant BA\textit{d}V-3

Monolayers of VIDO-R2 cells (5 \times 10^5 cells) in 60-mm dish were transfected with 8 \mu g of PacI-digested pFBAV950, pFBAV951, or pFBAV3 by a Lipofectin method. The cytopathic effects appeared 14 days after transfection. The cells showing 50% cytopathic effects were collected, freeze–thawed two times, and the recombinant virus was plaque purified and propagated on MDBK cells. The insertion of foreign sequences into the viral genome was analyzed by PCR using viral DNA as a template. The primers used for analysis were P91 [5’-CTAATCGATACATGTCAC TG-3’] (3057 bp of BA\textit{d}V-3 genome) and P92 [5’-CCAACCG-GTTGTGGAAAATC-3’] (4450 bp of BA\textit{d}V-3 genome).

Western blotting

For Western blot, proteins from CsCl-purified virions (5 \mu g) were separated by 12% sodium-dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Nonspecific binding sites on the membranes were blocked with 1% BSA fraction V. The membranes were probed with rabbit polyclonal anti-sera against pIX of BA\textit{d}V-3. The membranes were incubated for 1 h with appropriately diluted protein-bearing sera. After several washing steps, the grids were incubated with gold-tagged protein A for 1 h at room temperature. Finally, the grids were stained with 2% phosphotungstic acid and examined by transmission electron microscopy. The blot was developed using alkaline phosphatase color development kit (Bio-Rad).

Immunogold electron microscopy

For immunogold electron microscopy, CsCl-purified virions were adsorbed to nickel grids. After adsorption, the grids were incubated for 1 h with appropriately diluted protein-specific sera. After several washing steps, the grids were incubated with gold-tagged protein A for 1 h at room temperature. Finally, the grids were stained with 2% phosphotungstic acid and examined by transmission electron microscopy.

Real time PCR

Total DNA was extracted from virus-infected A549 or HeLa cells using QIA\textsc{gen} DNAeasy Tissue Kit. Aliquot of 70 ng of total DNA was used in the Real Time PCR analysis with Platinum qPCR supermix (Invitrogen). The primers were from the BA\textit{d}V-3 hexon gene sequence: RTP-1: 5’-TACAGTAA GTGGCGTTGTA-3’ and RTP-2: 5’-CTATCAAATAGGCCGCCTA-3’. The 5’ end-labeled FAM and 3’-labeled TAMRA probe were used in the PCR reaction. The sequence of the probe was CCGCCTAAC-CACGAACACACCTACG. Dilutions of pFBAV3 DNA were used for absolute quantification of viral genomes in the DNA sample.

Virus-binding assay

Monolayers of A549 cells in 12-well plates were incubated with purified BA\textit{d}V-3 fiber knob protein (70 \mu g/ml) or MEM for 10 min. Wild-type BA\textit{d}V-3 or BA\textit{v}950 was then added at a multiplicity of infection of 10 for 30 min at room temperature. Alternatively, monolayers of A549 cells in 12-well plates were incubated with RGD containing peptide (500 \mu g/ml) or MEM for 10 min at 4 °C. Wild-type BA\textit{d}V-3 or BA\textit{v}950 was then added at a multiplicity of infection of 10 for 30 min at 4 °C. The viruses were aspirated and the cells were overlaid with MEM containing 5% FBS and 0.7% agarose. After 3 days of incubation at 37 °C, the agarose overlay was removed, the cells were fixed with 100% methanol. Finally, the cells were stained with anti-DBP and infectious units of BA\textit{d}V-3 were counted as described (Zhou et al., 2001). Each bar represents the cumulative mean \pm SD of triplicate determinations.

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