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Role of Protein VII in the Production of Infectious Bovine Adenovirus-3 Virion

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Abstract: Bovine adenovirus (BAdV)-3 genome encodes a 26 kDa core protein designated as protein VII, which localizes to the nucleus/nucleolus. The requirement of a protein VII-complementing cell line for the replication of VII-deleted BAdV-3 suggests that protein VII is required for the production of infectious progeny virions. An analysis of the BAV.VIId^{+} virus (only phenotypically positive for protein VII) detected no noticeable differences in the expression and incorporation of viral proteins in the virions. Moreover, protein VII does not appear to be essential for the formation of mature BAV.VIId^{+}. However, protein VII appeared to be required for the efficient assembly of mature BAV.VIId^{-} virions. An analysis of the BAV.VIId^{-} virus (genotypically and phenotypically negative for protein VII) in non-complementing cells detected the inefficient release of virions from endosomes, which affected the expression of viral proteins or DNA replication. Moreover, the absence of protein VII altered the proteolytic cleavage of protein VI of BAV.VIId^{-}. Our results suggest that BAdV-3 protein VII appears to be required for efficient production of mature virions. Moreover, the absence of protein VII produces non-infectious BAdV-3 by altering the release of BAdV-3 from endosomes/vesicles.

Keywords: BAdV-3; adenovirus protein cleavage; adenovirus protease cleavage sites; adenovirus assembly; endosomes; DNA packaging; mature infectious virions

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1. Introduction

Bovine adenovirus (BAdV)-3 virions are non-enveloped icosahedral particles 90–100 nm in diameter, containing a non-segmented, double stranded linear DNA genome of 34,446 bps [1]. The viral capsid is composed of major (hexon, fibre and penton) and minor (VI, VIII, IIIa and IX) capsid proteins [2,3]. While major capsid proteins are involved in initial virus–cell interactions and the induction of immune responses, minor capsid proteins are involved in stabilizing the virion structure by interacting with major capsid proteins and other minor proteins [2]. The viral core contains adenovirus DNA and associated viral proteins (V, mu, terminal protein, cysteine protease, IVa2 and VII), which connect the viral core with viral capsid [2,3].

Adenoviral core protein VII, a highly basic protein, localizes to the nucleus, nucleolus and mitochondria [4,5]. While un-cleaved protein VII localizes to the nucleolus of infected cells [5] and is detected in immature virions [6], proteolytically cleaved protein VII localizes to the nucleus of infected cells [5] and is detected in mature infectious virions [6]. Adenovirus protein VII appears to perform multiple functions [7], including nuclear transport of a viral genome [8,9], the prevention of inducing an innate immune response [10] and participating in the endosomal escape of uncoated virions [11,12].

Although reports have suggested that adenovirus capsid assembly and DNA packaging may occur simultaneously [13], the sequential model, where the insertion of the adenovirus genome occurs into preformed capsids presumably through a unique portal vertex [14], appears to be an accepted model.

An earlier report suggested that the interaction of un-cleaved core protein VII with newly synthesized adenovirus genome condenses viral DNA into an adenovirus capsid [15]. However, an analysis of the Ad5-VII-LoxP virus grown in Cre recombinase-expressing cells demonstrated that protein VII is not required for the packaging of DNA into virions or virus assembly [11]. Moreover, the absence of protein VII in mutant virus particles leads to the inefficient escape of virions from the endosomes [11].

Earlier reports demonstrated differences between proteins encoded by HAdV-5 and BAdV-3 [4,16–18]. Since protein VII encoded by HAdV-5 and BAdV-3 showed 45% homology, we investigated the function of BAdV-3 protein VII in viral replication. Here, we demonstrate that BAdV-3 protein VII is required for the efficient viral assembly and release of virions from endosomes.

2. Materials and Methods

2.1. Cell Lines and Viruses

VIDO DT1 (cotton rat lung cells [19] expressing endonuclease *I-SceI*) [20], GT23 (fetal bovine retina cells transformed with bovine TERT and SV40 large T antigen; this study) and KT21 (GT23 cells expressing BAdV-3 protein VII; this study) cells were propagated at 37 °C and 5% CO_2 in minimal essential medium (MEM; Sigma, Tokuo, Japan). The Human embryonic kidney (HEK 293T) (ATCC CRL-11268/17) cells were propagated in Dulbecco's modified minimal essential medium (DMEM; Sigma). All media were supplemented with heat-inactivated 10% fetal bovine serum (FBS; SAFC industries, Sigma), 10 mM HEPES (Gibco, Ottawa, ON, Canada), 0.1 mM nonessential amino acids (NEAA; Gibco), and 50 µg/mL gentamicin (Bio Basics, Toronto, ON, Canada). BAV304a (BAdV-3 E3 region replaced with EYFP [expressing enhanced yellow fluorescent protein] under CMV promoter) [20], recombinant BAV.VIId (this study), recombinant BAV.VIIdR (this study) viruses were propagated in GT23 and KT21 cells in MEM supplemented with 2% fetal bovine serum.

2.2. Antibodies

The production and characterization of antibodies against BAdV-3 protein VII [4], DNA binding protein (DBP) [21], protein VIII [22] and protein IVa2 [23] have been described previously. The production and characterization of sera against protein VI and termi, nal protein (TP) will be described elsewhere. FITC-conjugated goat anti-rabbit antibody, alkaline phosphatase (AP)-conjugated goat anti-mouse IgG and alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG were purchased from Jackson Immune Research. Anti-tubulin mouse monoclonal antibody was purchased from Sigma, Canada.

2.3. Construction of Recombinant Plasmids

Plasmid pC.HA (plasmid pcDNA3 containing HA tag) was provided by Dr. Joyce Wilson, University of Saskatchewan, Canada. Plasmid pMCS [24], plasmid pTRIP-hygro [20], plasmid pC.HA.VII [25] and plasmid pUC304a [20] have been described earlier. Plasmid pMD2.G and pSPAX were gifts from Robert Brownlie, VIDO, University of Saskatchewan, Canada. The following plasmids were constructed as per standard procedures using restriction enzymes and ligation [26]. The identity of plasmids was confirmed by DNA sequencing. The details of the construction of plasmids used in this study are described below.

(i) ***pMCS-VIId:*** A 840 bp DNA fragment was amplified by PCR using primers FW1*SphI*—RV1*SbfI* (Table 1) and plasmid pUC304a [20] DNA as a template. Similarly, a 1916 bp DNA fragment was amplified by PCR using primers FW2*SbfI*—RV2C*HpaI* (Table 1) and plasmid pUC304a DNA as a template. In the third PCR, these two PCR products were reannealed and used as DNA template to amplify a 2764 bp PCR

product by overlapping PCR using primers FW1*SphI*—RV2C*HpaI* (Table 1). The final PCR product (2764 bp) was digested by *SphI-HpaI* and ligated to *Sph*I—*Hpa*I-digested plasmid pMCS DNA (2229 bp) to create plasmid pMCS-VIId (4895 bp).

(ii) ***pMCS-VIIdKan:*** A 1.2 kb DNA fragment was isolated from plasmid pUC4K (REF) DNA and ligated to *Sbf*I-digested plasmid pMCS-VIId DNA, creating plasmid pMCS-VIIdKan plasmid (6225 bp).

(iii) ***p304a.Kan.VIId:*** A 2764 bp *Sph*I-*Hpa*I DNA fragment of plasmid pMCS-VIIdKan DNA was isolated and recombined with plasmid pUC304a DNA by homologous recombination using *Escherichia coli* BJ5183 to create plasmid p304a.Kan.VIId.

(iv) ***p304a.VIId:*** The plasmid p304a.Kan.VIId DNA was digested with *Sbf*I and the larger fragment was re-ligated to create plasmid p304a.VIId. The identity of the plasmid was confirmed by RE analysis and plasmid DNA sequencing.

(v) ***p304a.VIIdR:*** A 3272 bp DNA fragment was amplified by PCR using primers FW1*SphI*—RV2*HpaI* (Table 1) and plasmid pUC304a [20] DNA as a template. The plasmid p304a.VIId was digested with *Sbf*I and recombined with 3272 bp PCR product by homologous recombination using *Escherichia coli* BJ5183 to create plasmid p304a.VIIdR.

(vi) ***pTrip.VII:*** A 516bp DNA fragment (encoding protein VII) was amplified by PCR using primers *NheI*.pVII.pTrip.Fw—pVII.*SalI*.Rv.Epi (Table 1) and plasmid pUC304a [20] DNA as a template. The PCR product was digested with *NheI-SalI* and ligated to *NheI-SalI*-digested plasmid pTrip-hygro DNA (containing a hygromycin resistant marker), creating plasmid pTrip.VII.

(vii) ***pTrip.SV40LT***: A 2508 bp fragment was amplified by PCR using primers pLOX.Ttag—NheI—F and pLOX.Ttag--EcoRI--R—NEW (Table 1) and plasmid pLOX.Ttag (Addgene) DNA as a DNA template. The PCR amplified DNA fragment was digested with *NheI-EcoRI* and ligated to *NheI-EcoRI*-digested plasmid pTrip.puromycin, creating plasmid pTrip.SV40LT.

(viii) ***pTrip.bTert***: Cellular RNA was extracted from MDBK cells using RNeasy Mini Kit (QIAGEN) as per the kit protocol and 1st strand cDNA was synthesized by PrimeScript 1st strand cDNA Synthesis kit (Takara catalogue# 6110A). A 3378 bp bovine TERT (bTert) amplified by PCR using primers bTert-F-XbaI and bTert-R-EcoRI (Table 1) was digested with *XbaI-EcoRI* and ligated to *XbaI-EcoRI*-digested plasmid pTrip-puromycin DNA, creating plasmid pTrip.bTert.

Table 1. List of primers.

Primer Name	Sequence
FW1SphI	ATTGCATGCGGACATCGTGTTGCTGCCGGGCTGTG
RV1SbfI	GCAAAAAAAAGTAGCAGACATTCCTGCAGGGTTCCTGACAGTCCCTGGTG
FW2SbfI	CACCAGGGACTGTCAGGAACCCTGCAGGAATGTCTGCTACTTTTTTTTGC
RV2CHpaI	ATTGTTAACAAGCTGTGTGGGGCTGGCGCCGGCTCT
Fw seq all	TAAGCAGACAGGGGCACAGCAG
Rv seq all	CAGTCGGCGGGCTTTTATTGAAG
NheI.pVII.pTrip.Fw	CGCGCTAGCGCCGCCATGGATTACCCATATGAC
pVII.SalI.Rv.Epi	GAG GTCGACTCAAACGGTGTTGCTGACCGTAG
Q adeno Fwd	CAGGTGCCAGTCAAGATTAC
Q adeno Rev	ATGGCCGACTGAGTCATAAG
Actin Fwd	CTAGGCACCAGGGCGTAATG
Actin Rev	CCACACGGAGCTCGTTGTAG
bTERT-F-XbaI	TATTTCTAGAATGCCGCGCGCGCCCAG
bTERT-R-EcoRI	GGCCGAATTCTCAGTCCAAGATGGTCTTGAAGTC
pLOX.Ttag—NheI—F	GCCGCTAGCATGGATAAAGTTTTAAACAGAGAG
pLOX.Ttag--EcoRI--R—NEW	GAAGAATTCCGGGGCCGCTAACAACAAC

2.4. Transfection

The cells were transfected with individual plasmid DNA (5 μg/10^6 cells) using Lipofectamine™ 3000 (Invitrogen, Waltham, MA, USA) as per manufacturer's instructions. After 4–6 h post transfection, the transfection media was replaced with fresh media containing 2% FBS, and the cells were incubated for the indicated times for further analysis.

2.5. Western Blot

Proteins from the lysates of virus infected or plasmid transfected cells were separated by 12–20% SDS-PAGE, transferred to nitrocellulose membrane or methanol-treated PVDF membrane (Bio-Rad, Hercules, CA, USA) and detected by Western blotting as described [16] using protein-specific antiserum and fluorophore-conjugated secondary antibodies.

2.6. Indirect Immunofluorescence Assay

Virus-infected or plasmid DNA-transfected cells were fixed with 3.7% paraformaldehyde for 15 min before permeabilizing with 0.5% Triton X-100. The permeabilized cells were blocked with 5% FBS for 1 h before incubating with a protein-specific primary antibody followed by fluorophore-conjugated secondary antibodies. Finally, the cells were mounted with mounting medium (VectaShield) containing 4′,6-diamidino-2-phenylindole (DAPI) and imaged under Zeiss LSM 5 Laser scanning confocal microscope.

2.7. Complementation Assay

VIDO DT1 [20] cells in 6-well plates were co-transfected with indicated plasmids DNA (5 μg/10^6 cells) using Lipofectamine™ 3000 (Invitrogen) as per the manufacturer's instructions. The transfected cells were observed under fluorescent microscope daily for the appearance of fluorescent-focus-forming units. The average number of GFP positive cells were quantified as fluorescent focus unit (FFU) per field.

2.8. Transformation of Fetal Bovine Retina Cells

Low passage fetal bovine retina cells (FBRCs) isolated from the eyeballs of bovine fetus (collected from a slaughterhouse) were seeded in a six-well plate at 70% confluency. To produce lentiviruses, the recombinant plasmid pTrip.bTert DNA or plasmid pTrip.SV40LT DNA (18 μg each) were co-transfected with the packaging plasmids psPAX2 (9 μg) and pMD2.G (9 μg) using a turbofect transfection reagent (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's instructions. The lentivirus particles produced in the medium supernatant were collected at 48 h and 72 h post transfection. Both the collections of individual recombinant lentiviruses were pooled and filtered through 0.45 μm filter membrane (Fisher Scientific, Hampton, NH, USA). The filtered individual lentiviral supernatant was used together at MOI 3 to transduce the primary fetal bovine retina cells (FBRCs) in the presence of 8 μg/mL polybrene. After 10 days of culturing, the transformed cells appeared which were further selected with 10 μg/mL puromycin. The puromycin-resistant morphologically transformed cell clone was established from a single focus and named GT23.

2.9. Construction of BAdV3 Protein VII-Expressing Cell Line

A second generation replication-defective lentivirus expressing BAdV-3 protein VII was generated by transfecting 293 T cells with a mixture of plasmid pTrip.VII DNA, plasmid pSPAX (expressing HIV Gag/Pol proteins) DNA and plasmid pMD2.G (expressing vesicular stomatitis virus G protein) DNA. After 48 or 72 h post transfection, media containing the lentivirus in the supernatant was collected. Monolayers of GT23 cells were transduced with a lentivirus expressing BAdV-3 protein VII in media containing 8 μg/mL polybrene. After 48 h post transduction, the cells were grown in media containing 500 μg/mL of hygromycin. The selection media was changed every 3 days until the appearance of hygromycin-resistant clones. Finally, selected hygromycin resistant clones were propagated and analyzed for the expression of BAdV-3 protein VII by Western blot. A hygromycin-resistant clone ex-

pressing BAdV-3 protein VII, designated KT21, was selected. An analysis of KT21 cells by immunofluorescence assay suggested that the number of cells expressing bovine protein VII appeared decreasing after 10th passage.

2.10. Isolation of Mutant BAdV-3

The monolayers of KT21 cells (this report) in six-well plates were transfected with 2–5 μg of plasmid p304a.VIId DNA using Lipofectamine 3000 reagent (Invitrogen). At 4–6 h post transfection, the medium was replaced with fresh MEM containing 2% FBS. The cells were observed daily for cytopathic effect (CPE). The cells showing cytopathic effect and green fluorescence were collected, freeze–thawed and the mutant virus, designated BAV.VIId^{+}, was purified.

2.11. Virus Purification

The cells in T-150 flasks were infected at an M.O.I. of 1. At 48 h post-infection, the infected cells were collected by centrifugation and resuspended in 15 mL 10 mM Tris/HCl. After freeze–thawing three times, the cell lysates were subjected to CsCl gradient centrifugation, as described earlier [27]. Finally, the mature viruses were collected after the second gradient centrifugation, dialyzed in 10 mM Tris/HCl three times and stored in 10 mM Tris/HCl, containing 10% glycerol at −80 °C.

2.12. Fluorescent Focus Assay

The monolayers of VIDO DT1 cells grown in 24-well plates were infected with BAV304a or BAV.VIId viruses at MOI of 5. At 48 h post infection, GFP-positive cells/foci were counted for each well using a fluorescence microscope. The average number of GFP-positive cells was quantified as the number of fluorescent focus units (FFUs) per field as described [28].

2.13. Virus Single Cycle Growth Curve

The monolayers of cells in 24-well plate were infected with BAV304a or BAV.VIId at a MOI of 2. The infected cells were harvested at 6, 12, 24, 36 and 48 h post infection, lysed by freeze–thawing three times, and used to determine the virus titer by determining fluorescent foci units per ml (FFU/mL).

2.14. Virus DNA Replication

The cells were infected with BAV304a or BAV.VIId at MOI of 2. At 6, 12, 24, 36, and 48 h post-infection, the infected cell pellets were collected by centrifugation and genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. The viral genome copy number was determined by quantitative real-time PCR as described previously [28], using primers for viral DNA (Q adeno Fwd, Q adeno Rev) and actin (Actin Fwd and Actin Rev; Table 1). Viral genomic replication was calculated as the value of the viral genome copy number divided by the actin copy number. For comparison, the value of each virus was normalized to that of BAV304a at 6 h post infection and is shown as the relative viral genome copy number. The experiment was performed independently two times, and each sample was tested in duplicate.

2.15. Transmission Electron Microscopy (TEM)

The cells were infected with BAV304a or BAV.VIId at MOI of 2. At 48 h post-infection, the cells were collected and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C. The fixed cell pellets were processed for TEM, as described earlier [29]. Finally, the stained sections were viewed using a Hitachi HT7700 transmission electron microscope. CsCl gradient-purified BAV304a and BAV.VIId viruses were stained negative and observed by TEM as described [28].

To detect the early stages of virus cell interaction, the monolayers of GT23 cells were incubated with individual purified viruses at a MOI of 10 for 1 h at 4 °C, and further incubated for 1 h or 5 h at 37 °C. The infected cells were processed for TEM analysis as described earlier [28].

2.16. Statistical Analysis

Data were analyzed by GraphPad Prism, version 10.1.1 (323) (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences among the groups were calculated using unpaired *t*-test. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1. Construction of Protein VII-Deleted BAdV-3

To determine the role of protein VII in virus replication, initially, we attempted to isolate a mutant BAdV-3 containing a deletion of protein VII. Briefly, using the homologous recombination machinery of BJ5183 cells [30], we constructed two full length plasmids (Figure 1A): (a) plasmid p304a.VIId, a full-length genomic clone of BAdV-3 containing a deletion of protein VII, and (b) plasmid p304a.dR containing wild-type protein VII inserted back in plasmid p304a.VIId DNA (to demonstrate that the observed effect is because of the deletion of only the protein VII gene, and not due to an alteration in another gene). The identity of these plasmids was determined by an analysis of restriction enzyme *Not*I-digested DNA using agarose gel electrophoresis. As seen in Figure 1B, a unique 656 bp fragment was present in *Not*I-digested plasmid pUC304a DNA and plasmid p304a.VIIdR DNA, but not in *Not*I-digested plasmid p304a.VIId DNA.

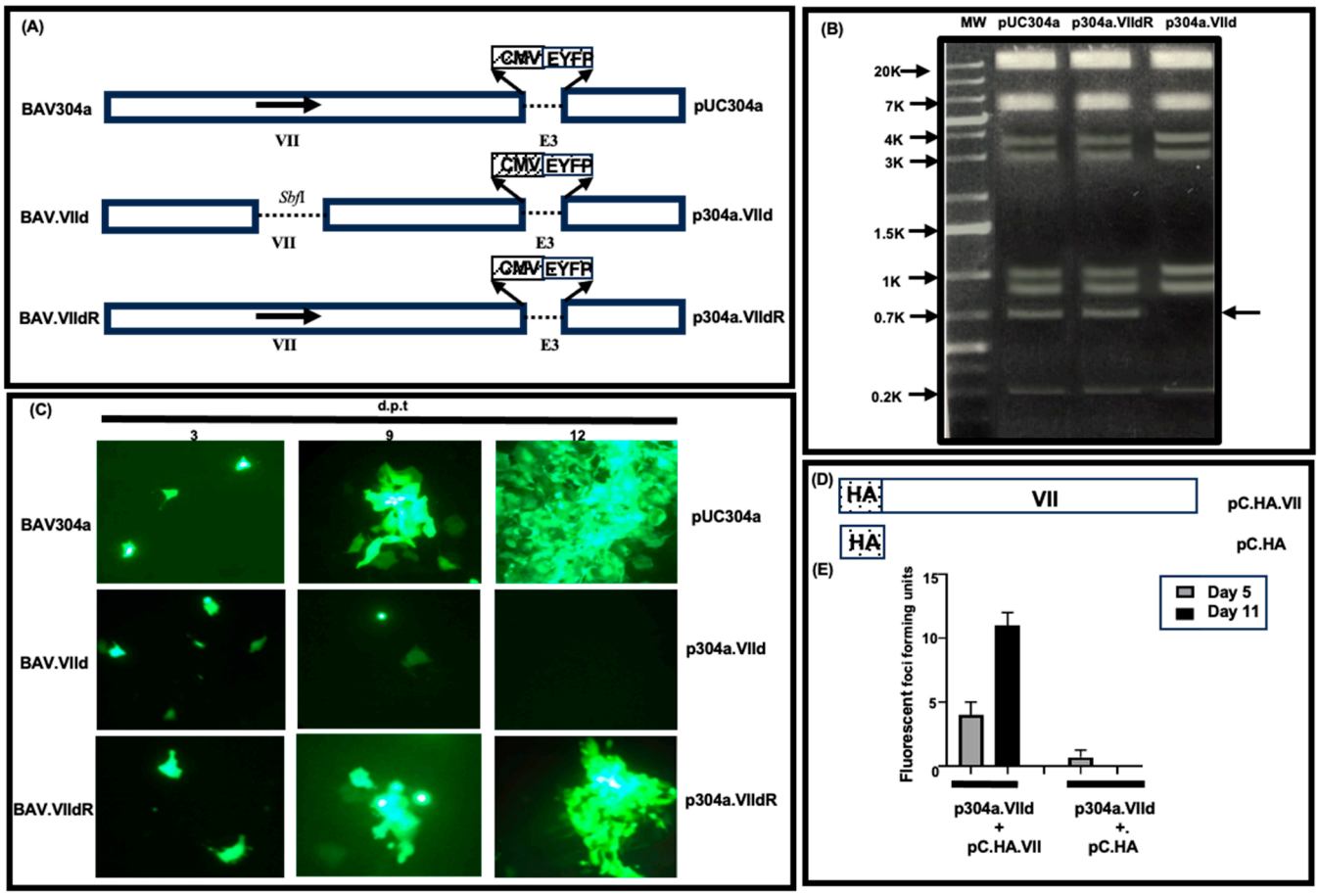

Figure 1. (**A**). Schematic representation of bovine adenovirus-3 DNA in plasmids. The horizontal arrow shows the direction of transcription; the dotted line shows deleted region; CMV (human

cytomegalo virus immediate early protein promoter; EYFP (enhanced yellow fluorescent protein). The name of the virus is depicted on the left of the panel; the name of the plasmid is depicted on the right of the panel. (**B**). Restriction enzyme analysis. Purified plasmid DNAs were digested with *Not*I enzyme and analyzed by agarose gel electrophoresis. (**C**). Fluorescent microscopy. VIDO-DT1 cells, transfected with indicated plasmid DNAs, were observed for the appearance of cytopathic effects and fluorescent foci. The numbers on the top depict the days post transfection (d.p.t). The name of the virus is depicted on the left of the panel. The name of the plasmid is depicted on the right of the panel. (**D**). Schematic representation of plasmids. The name of the plasmid is depicted on the right. (**E**) Complementation assay. VIDO-DT1 cells were co-transfected with indicated plasmid DNAs and fluorescent cell foci were counted at the indicated times post transfection. The results depict the average of three experiments.

To isolate mutant BAdV-3, VIDO DT1 cells (cotton rat lung fibroblasts expressing *I-SceI* endonuclease) [20] were transfected with indicated plasmids DNAs and observed daily for the development of the cytopathic effects (CPE). The cells transfected with plasmid pUC304a DNA or plasmid p304a.VIIdR DNA showed CPE and increased EYFP expression at 11 days post transfection (Figure 1C), However, no EYFP expression or cytopathic effect could be observed in cells transfected with plasmid p304a.VIId DNA at 11 days post transfection (Figure 1C).

To confirm if protein VII is essential for viral replication, we performed a complementation assay as described [28]. VIDO DT1 cells in 6-well plates were co-transfected in triplicates with plasmid p304a.VIId + pC.HA.VII DNAs or plasmid p304a.VIId + pC.HA DNAs (Figure 1A,D). The transfected cells were observed under a fluorescent microscope daily for the appearance of fluorescent-focus-forming units. VIDO DT1 cells co-transfected with plasmid p304a.VIId + pC.HA DNAs did not show an increase in the number of FFUs from day 5 to day 11 post transfection. In contrast, VIDO DT1 cells co-transfected with plasmid p304a.VIId + pC.HA.VII DNA resulted in an increase in the number of FFUs from day 5 to day 11 post transfection (Figure 1E).

3.2. Generation of a Cell Line Expressing BAdV-3 Protein VII

Initially, fetal bovine retina cells were transformed with a lentivirus expressing the bovine Tert gene and a SV40 large T antigen. The transformed cell line was designated as GT23 (Figure 2A, panel b). To develop a cell line expressing BAdV-3 protein VII, GT23 cells were transduced with the lentivirus expressing BAdV-3 protein VII, as described earlier [20]. The hygromycin-resistant clones appeared 7 days after transduction. The clones were expanded and analyzed for the expression of BAdV-3 protein VII by Western blotting using anti-VII serum. As shown in Figure 2B, anti-VII serum detected a protein of 26 kDA in BAV-3 infected GT23 cells. A similar protein band could be detected in the hygromycin-resistant GT23 cell clone. No such protein could be detected in mock infected GT23 cells. Moreover, an immunofluorescent assay using anti-VII serum detected protein VII predominantly in the nuclei of the cells. No such fluorescence could be detected in GT23 cell nuclei. This cell line expressing protein VII was designated as KT21.

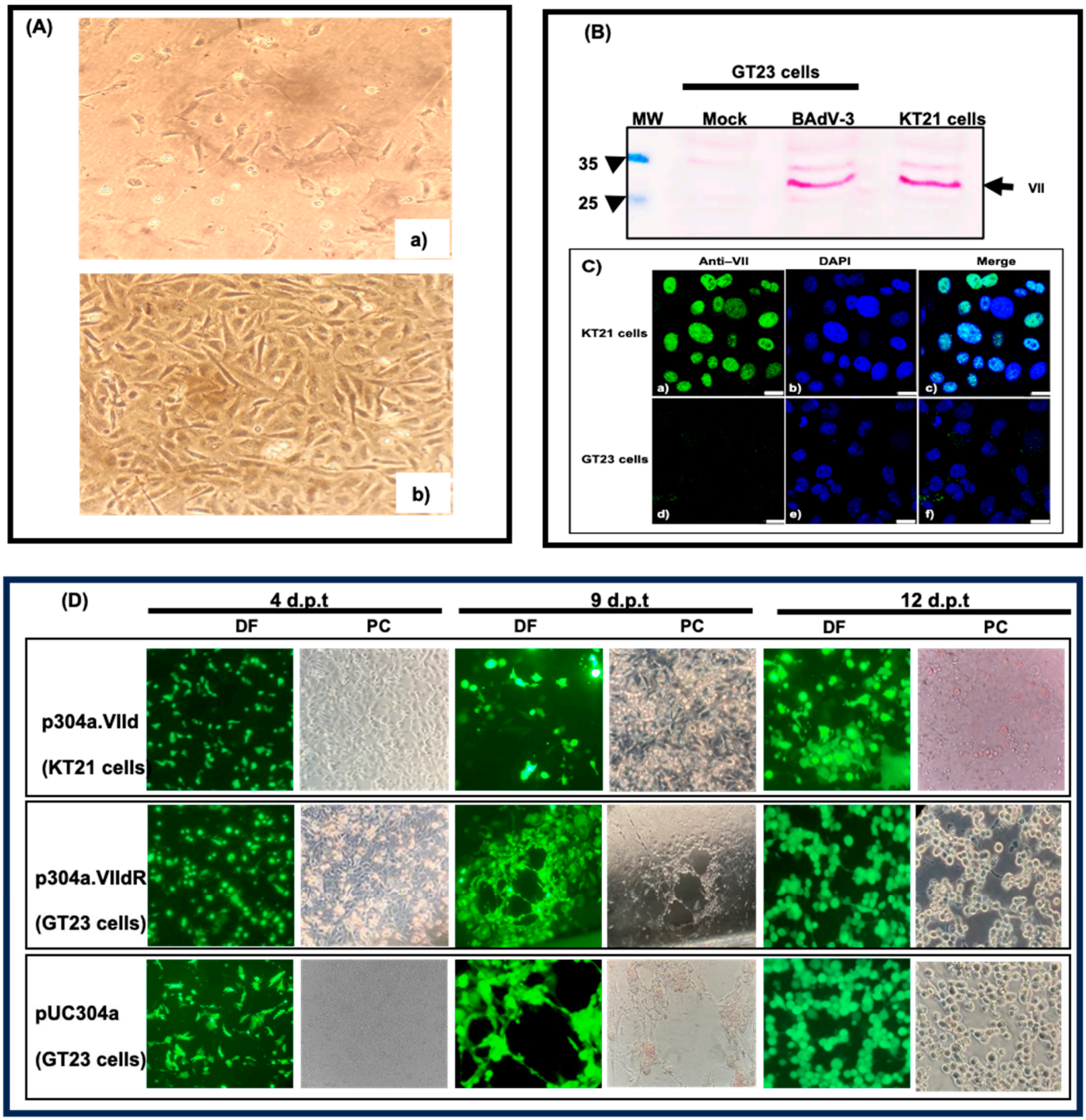

Figure 2. Isolation of protein VII-deleted BAdV-3 (BAV.VIId^{+}). (**A**). GT23 cell line. Fetal bovine retina cells were transformed with replication-defected lentiviruses expressing bovine Tert gene and SV40 large T antigen, as described in Materials and Methods. Objective 10×. (**A**) Morphology of untransformed (panel **a**) and transformed (panel **b**) fetal bovine retina cells. (**B,C**). Analysis of KT21 cells. (**B**). Proteins from cell lysates of GT23 cells, BAdV-3-infected GT23 cells or KT21 cells were separated by SDS PAGE, transferred to nitrocellulose and analyzed by Western blot using anti-VII sera. The molecular weight markers are shown on the left of the panel. (**C**). Monolayers of indicated cells were fixed with 3.7% paraformaldehyde and the expression of protein VII was visualized by indirect immunofluorescence using anti-VII sera using confocal microscope. The nuclei were stained by DAPI. Scale bars are shown as white lines (**D**). The monolayers of GT23 or KT21 cells were transfected with indicated plasmid DNAs and visualized for the development of cytopathic effects and expression of EYFP. The numbers on the top depict the days post transfection (d.p.t). DF (direct fluorescence); PC (Phase contrast). Objective 10× (magnification).

3.3. Isolation of Mutant BAV.VIId$^+$

The monolayers of KT21 cells in six-well plates were transfected with 2–4 µg of *Pac*I-digested plasmid p304a.VIId DNA (Figure 1A) using Lipofectamine 3000 reagent (Invitrogen). Similarly, the monolayers of GT23 cells were individually transfected with 2–4 µg of *Pac*I-digested plasmid pUC304a DNA or plasmid p304a.VIIdR DNA using Lipofectamine 3000 reagent (Invitrogen). The cells were observed daily for the appearance of EYFP-expressing cells and cytopathic effects (CPE). At 12 days post transfection (Figure 2D), the cells showing EYFP expression and CPE were harvested and grown as described earlier [28,29,31].

The monolayers of KT21 cells were infected with the mutant virus at a MOI of 2. At 48 h post infection, the cells were harvested, freeze–thawed, and the lysates of infected KT21 cells were used to purify virions by CsCl density gradient (Figure 3A). The purified mutant virus was designated as BAV.VIId$^+$ (grown in KT21 cells) (Table 2).

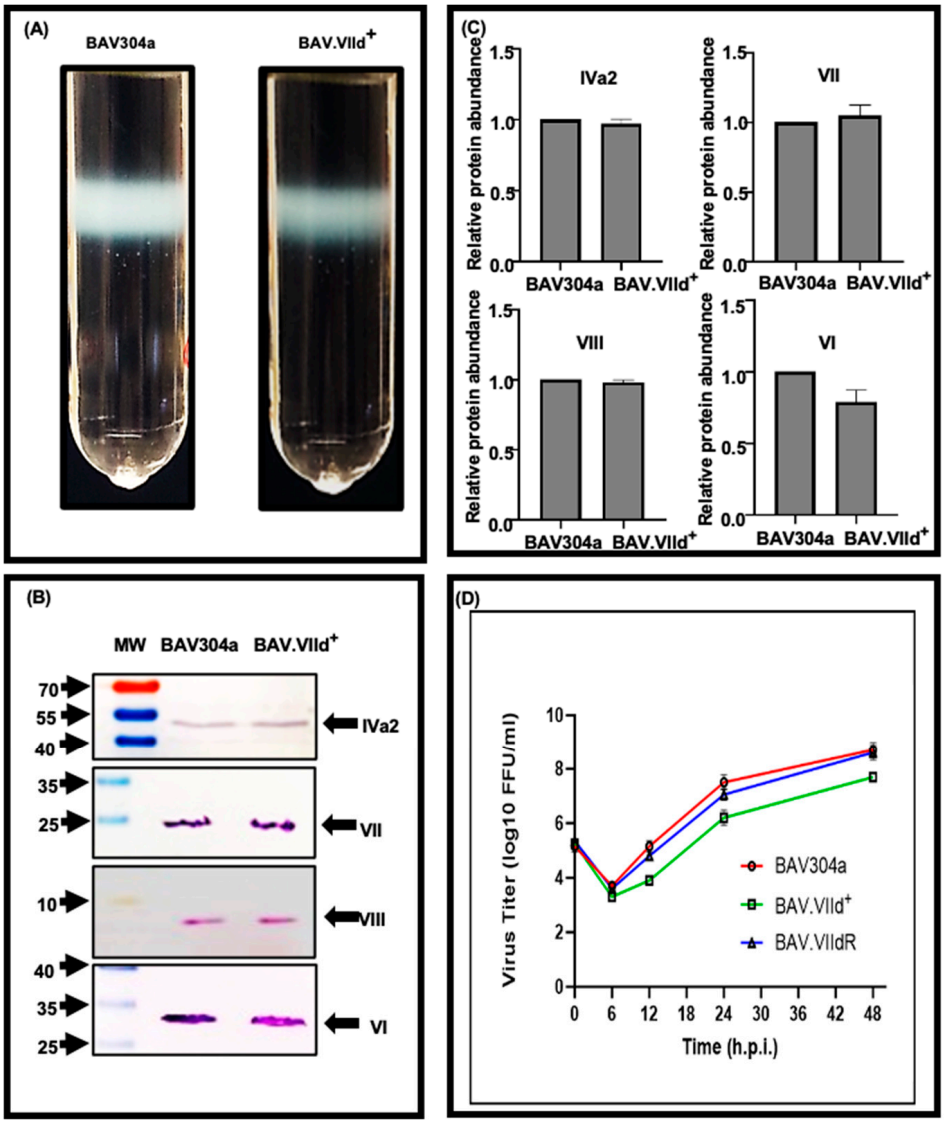

Figure 3. Analysis of BAV.VIId$^+$. (**A**). CsCl gradient purification. Virus was purified from lysates of infected cells using CsCl density gradient purification. Mature virion bands are shown after double density gradient purification. (**B**). Western blot. The proteins from purified BAV304a and BAV.VIId$^+$ were separated by SDS-PAGE gels, transferred to nitrocellulose and analyzed by Western blot using protein-specific sera. The molecular weight markers are shown on the left of the panel. The names of the proteins are shown on the right of the panel. (**C**). The values of panel (**B**) were analyzed by

GraphPad Prism, version 10.1.1 (323) (GraphPad Software, Inc., La Jolla, CA, USA). The values represent the mean of two independent experiments and error bars indicate SD. (**D**). Virus titer. The KT21 cells infected with indicated viruses were harvested at different times post infection, freeze–thawed three times and used to determine the titer by fluorescent foci assay. h.p.i (hours post infection).

Table 2. Summary of virus characteristics.

Virus	Characteristics
BAdV304a	Bovine adenovirus-3 containing EYFP gene flanked at 5′ end by HCMV promoter inserted into E3 deleted region
BAV.VIId	BAV304a containing deletion of protein VII gene
BAV.VIIdR	BAV.VIId containing protein VII gene inserted back at normal location
BAV.VIId^{+}	Protein VII gene deleted BAV304a grown in KT21 cells [(protein VII complementing cells; protein VII expressing cells) (genotypically negative but phenotypically positive for protein VII)]
BAV.VIId	BAV.VIId^{+} grown in GT23 cells [(no-complementing; not expressing protein VII) (Both genotypically and phenotypically negative for protein VII)]

3.4. Analysis of CsCl Purified BAV.VIId^{+}

To determine the incorporation of selected viral proteins in CsCl gradient-purified BAV.VIId^{+} virions, proteins from purified virions were separated by 15% SDS-PAGE and analyzed by Western blotting using protein-specific anti-sera. As seen in Figure 3B, no significant difference appears in the incorporation of viral protein IVa2 (panel IVa2), protein VII (panel VII), protein VIII (panel VIII) and protein VI (panel VI) in BAV.VIId^{+} virions. The relative amount of protein in BAV304a or BAV.VIId^{+}-infected cells is plotted (Figure 3C).

3.5. Analysis of Mutant BAV.VIId^{+} in KT21 (VII^{+}) Cells

To determine the growth of mutant BAV.VIId^{+} in KT21 cells, monolayers of cells in 24-well plates were infected with indicated viruses at MOI of 2. The infected cells harvested at 6, 12, 24, 36 and 48 h post infection were lysed by freeze–thawing three times and titrated as fluorescent-forming-unit per ml (FFU/mL) in KT21 cells. As seen in Figure 3D, BAV304a or BAV.VIIdR grew to a titer$10^{8.5}$ FFU/mL. However, BAV.VIId^{+} grew to a titer of $10^{6.8}$ FFU/mL.

To determine the expression of viral proteins, monolayers of KT21 were infected with BAV304a or BAV.VIId^{+} at MOI of 2. At 48 h post infection, the cell lysates were analyzed by W estern blotting using protein-specific anti-sera. As seen in Figure 4 (panels A,B), there appears no significant difference in the expression of early (DNA-binding protein [DBP]), intermediate (protein IVa2) or late (protein VII and protein VIII) proteins in KT21 cells infected with BAV304a or BAV.VIId^{+}.

Next, we determined the virus DNA replication in KT21 cells using quantitative real time PCR (qPCR) as described earlier. KT21 cells were infected with indicated viruses at a MOI of 2. At 6, 12, 24, 36 and 8 h post-infection, genomic DNA was extracted and analyzed by qPCR. As seen in Figure 4C, the replication of the viral genome appeared similar at different times post infection in KT21 cells infected with BAV304a or BAV.VIId^{+}.

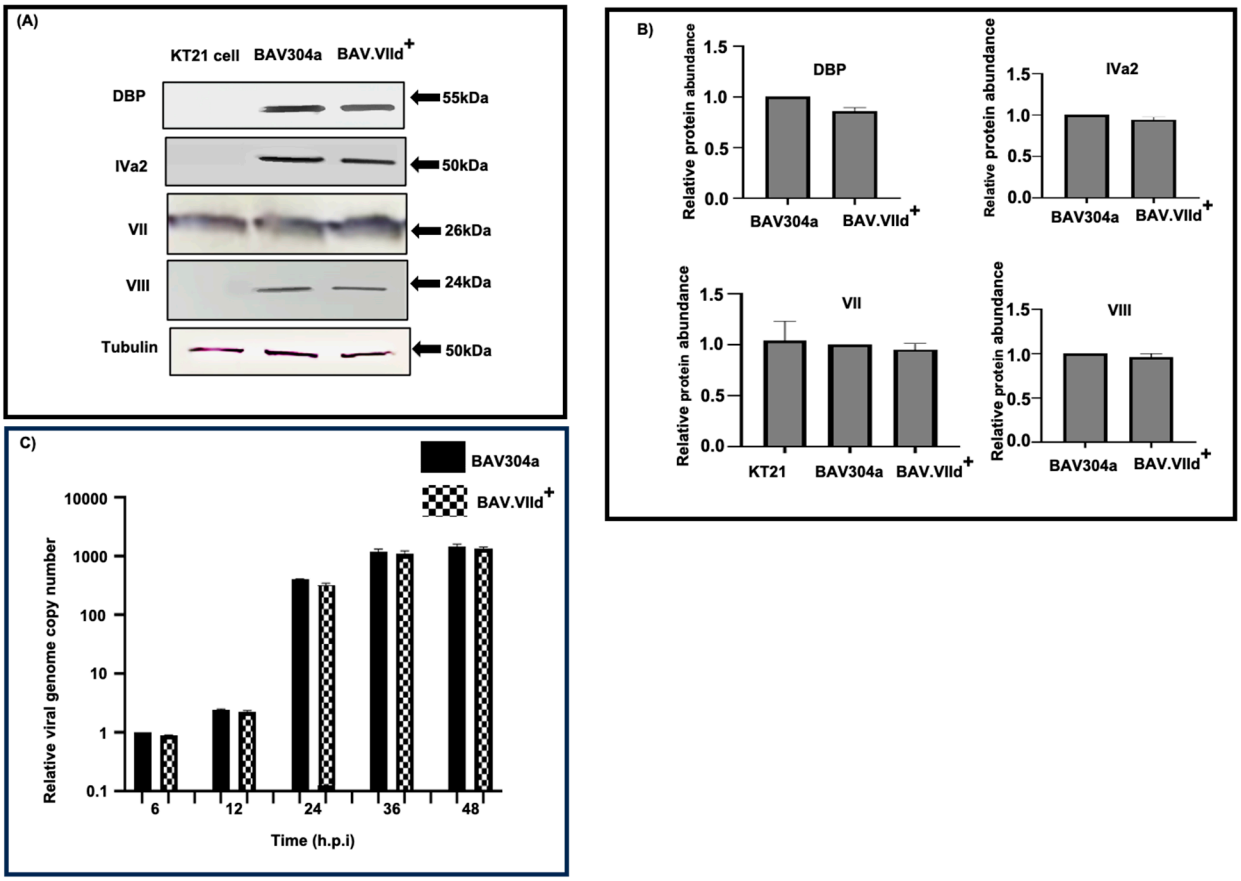

Figure 4. Analysis of BAV.VIId$^+$ in KT21 cells. (**A**). Western blot. The proteins from the lysates of KT21 cells or indicated virus-infected cells were separated by SDS-PAGE, transferred to nitrocellulose membrane and analyzed by Western blot using protein-specific antibodies. The molecular weight markers are shown on the right of the panel. The names of the proteins are shown on the left of the panel. (**B**). The values of panel **B** were analyzed by GraphPad Prism, version 10.1.1 (323) (GraphPad Software, Inc., La Jolla, CA, USA). The values represent the mean of two independent experiments and error bars indicate SD. (**C**). Viral genome replication. The GT23 cells were infected with equivalent amounts of the indicated virus in triplicate. At indicated times post infection, the infected cells were collected, and genomic DNA was isolated. The viral genome copy number was determined by quantitative PCR and divided by the actin copy number for normalization. For comparison, the normalized genome copy number values for each virus at each time point were compared to the value of wild-type virus at 6 h.p.i (h post infection).

3.6. Analysis of Mutant BAV.VIId$^+$ in GT23 (VII$^-$) Cells

To determine the expression of viral proteins in GT23 cells, monolayers of cells were infected with BAV304a or BAV.VIId$^+$ at MOI of 2. At 48 h post infection, the cell lysates were analyzed by Western blotting using protein-specific anti-sera. As expected, (Figure 5A), no protein VII expression could be detected in BAV.VIId$^+$-infected GT23 cells. Moreover, there appears no significant difference in the expression of early (DNA-binding protein [DBP]), intermediate (protein IVa2) or late (protein VIII) proteins in GT23 cells infected with BAV304a or BAV.VIId$^+$ (Figure 5A,B).

Finally, we determined the virus DNA replication in GT23 cells using quantitative real time PCR (qPCR) as described earlier. GT23 cells were infected with indicated viruses at a MOI of 2. At 6, 12, 24, 36 and 48 h post-infection, genomic DNA was extracted and analyzed by qPCR. As seen in Figure 5C, the replication of the viral genome appeared similar at different times post infection in GT23 cells infected with BAV304a or BAV.VIId$^+$.

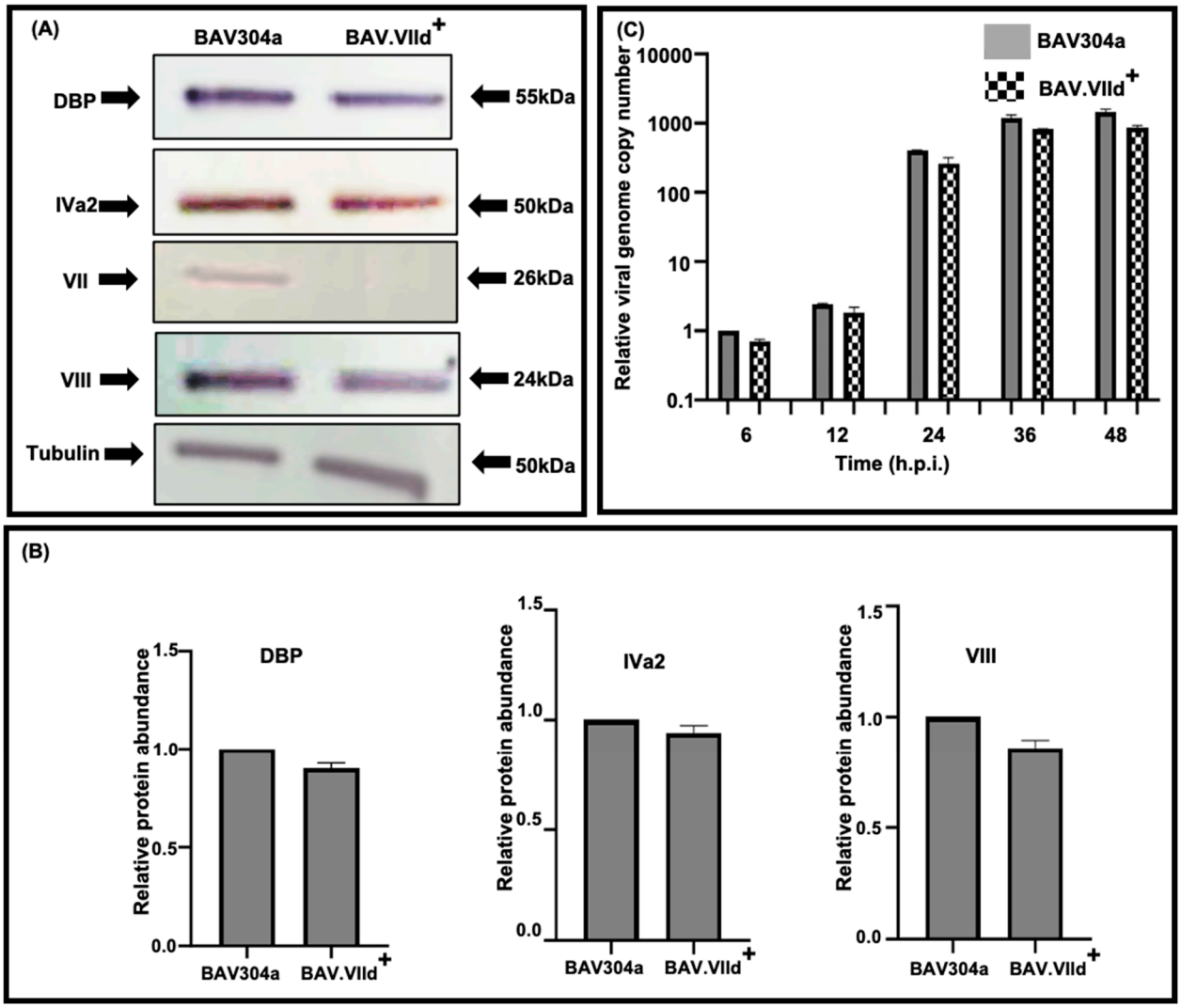

Figure 5. Analysis of BAV.VIId^{+} in GT23 cells. (**A**). Western blot. The proteins from the lysates of indicated virus-infected GT23 cells were separated by SDS-PAGE, transferred to nitrocellulose and analyzed by Western blot using protein-specific antibodies. The molecular weight markers are shown on the right of the panel. The names of the proteins are shown on the left of the panel. (**B**). The values of panel **B** were analyzed by GraphPad Prism, version 10 (GraphPad Software, Inc., La Jolla, CA, USA. The values represent the mean of two independent experiments and error bars indicate SD. DBP (DNA binding protein). (**C**). Viral genome replication. The GT23 cells were infected with equivalent amounts of the indicated virus in triplicate. At indicated times post infection, the infected cells were collected, and genomic DNA was isolated. The viral genome copy number was determined by quantitative PCR as described in Figure 4 legend. h.p.i (hours post infection).

3.7. Analysis of CsCl-Purified BAV.VIId^{-}

The monolayers of GT23 cells were infected with mutant virus at a MOI of 2. At 48 h post infection, the cells were harvested, freeze–thawed and the lysates of infected GT23 cells were used to purify virions by CsCl density gradient (Figure 6A). The purified mutant virus was designated as BAV.VIId^{-} (grown in GT23 cells) (Table 2).

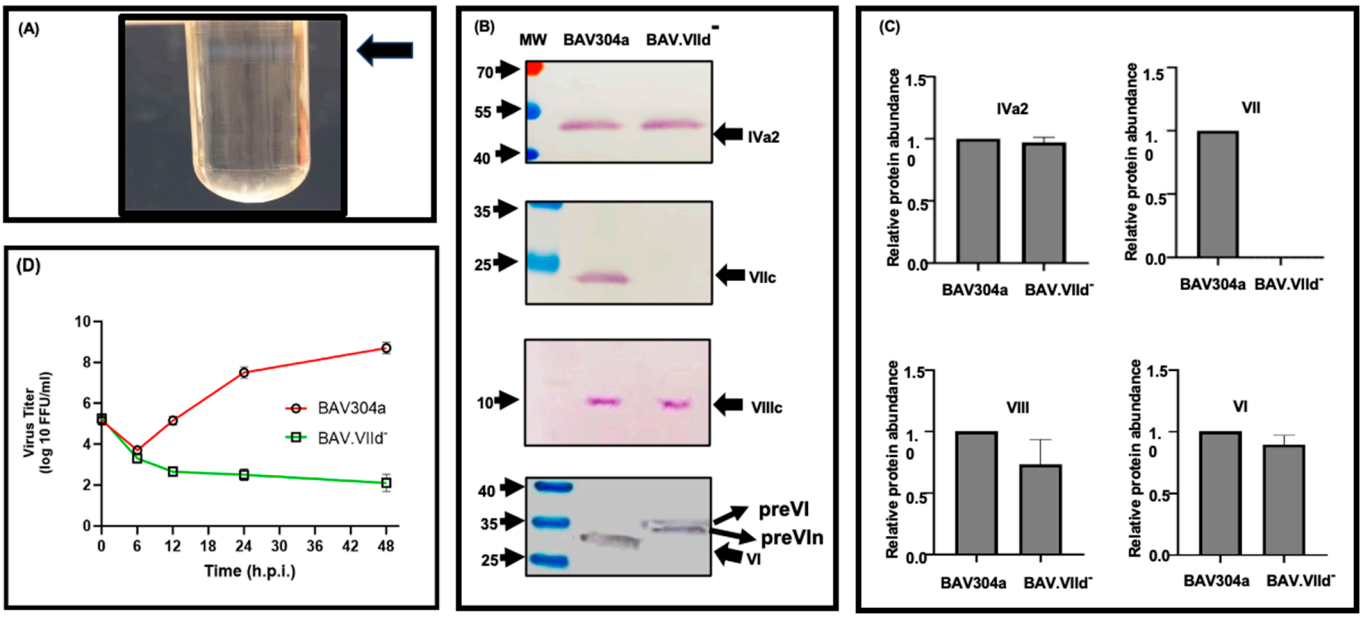

Figure 6. Analysis of BAV.VIId$^-$. (**A**). CsCl gradient purification. The virus was purified from the lysates of GT23-infected cells using CsCl density gradient purification. Mature virion bands are shown after double density gradient purification. The arrow represents the location of the virus band (**B**). Western blot. The proteins from purified BAV304a and BAV.VIId$^-$ were separated by SDS-PAGE gels, transferred to nitrocellulose and analyzed by Western blot using protein-specific sera. The molecular weight markers are shown on the left of the panel. The name of the proteins is shown on the right of the panel. (**C**). The values of panel **B** were analyzed by GraphPad Prism, version 10 (GraphPad Software, Inc., La Jolla, CA, USA). The values represent the mean of two independent experiments and error bars indicate SD. (**D**). Virus titer. The GT23 cells infected with indicated viruses were harvested at different times post infection, freeze–thawed three times and used to determine the titer by fluorescent foci assay.

To determine the incorporation of selected viral proteins in CsCl gradient-purified BAV.VIId$^-$ virions (Figure 6A), proteins from purified virions were separated by SDS-PAGE and analyzed by Western blotting using protein-specific anti-sera. As expected, no VII incorporation could be detected in purified BAV.VIId$^-$ virions (Figure 6B, panel VII). There appears to be no significant difference in the incorporation of viral protein IVa2 (panel IVa2) or protein VIII (panel VIII) in BAV304a or BAV.VIId$^-$ virions (Figure 6B). Interestingly, compared to BAV304a, protein VI could be detected as two bands of higher molecular weight in purified BAV.VIId$^-$ (Figure 6B, panel VI). The relative amount of protein in purified BAV304a or BAV.VIId$^-$ is plotted (Figure 6C).

3.8. Analysis of Mutant BAV-VIId$^-$ in GT23 (VII$^-$) Cells

To determine the growth of mutant BAV.VIId$^-$ in GT23 cells, a monolayer of the cells in a 24-well plate was infected with indicated viruses at a MOI of 2. The infected cells, harvested at 6, 12, 24, 36 and 48 h post infection, were lysed by freeze–thawing three times and titrated as fluorescent-forming-unit per ml (FFU/mL) in GT23 cells. As seen in Figure 6D, BAV304a grew to a titer $10^{8.5}$ FFU/mL. In contrast, there was no detectable increase in the titer of BAV.VIId$^-$ (Figure 6D).

To determine the expression of viral proteins, monolayers of GT23 were infected with BAV304a or BAV.VIId$^-$ at MOI of 2. At 48 h post infection, the cell lysates were analyzed by Western blotting using protein-specific anti-sera. As expected, early (DBP), intermediate (IVa2) and late (VII, VIII) protein-specific bands could be detected in BAV304a-infected GT23 cells (Figure 7A). In contrast, no such bands could be observed in GT23 cells infected with BAV.VIId$^-$ (Figure 7A).

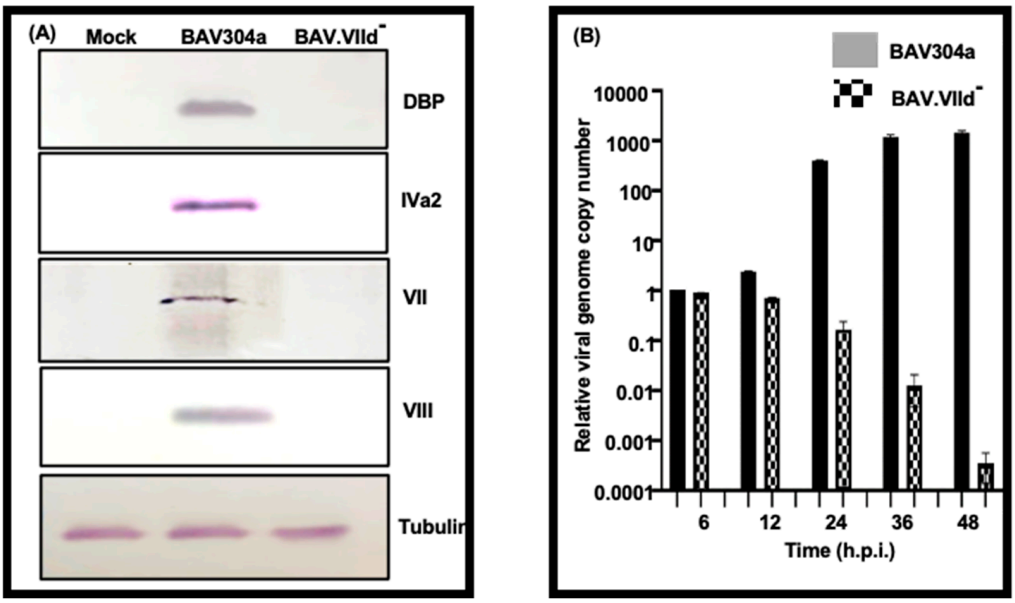

Figure 7. Analysis of BAV.VIId$^-$ in GT23 cells. (**A**). Western blot. The proteins from the lysates of indicated virus-infected GT23 cells were separated by SDS-PAGE, transferred to nitrocellulose and analyzed by Western blot using protein-specific antibodies. The names of the proteins are shown on the right of the panel. (**B**). Viral genome replication. The GT23 cells were infected with equivalent amounts of the indicated virus in triplicate. At indicated times post infection, the infected cells were collected, and genomic DNA was isolated. The viral genome copy number was determined by quantitative PCR as described in the legend of Figure 4. h.p.i (hours post infection).

Finally, we determined the BAV.VIId$^-$ virus DNA replication in GT23 cells using quantitative real time PCR (qPCR) as described earlier. GT23 cells were infected with indicated viruses at a MOI of 2. At 6, 12, 24, 36 and 48 h post-infection, the genomic DNA was extracted and analyzed by qPCR. As seen in Figure 7B, there was substantial increase in the viral genome copy number in BAV304a-infected GT23 cells. In contrast, there was no increase in the viral genome copy number in BAV.VIId$^-$-infected GT23 cells.

3.9. Analyses of BAV.VIId$^+$ by Transmission Electron Microscopy (TEM)

To investigate the effect of deletion of protein VII on the formation of viral particles, KT21 cells and GT23 cells were infected with BAV304a or BAV.VIId$^+$ at MOI of 2. At 48 h post-infection, the cells were processed and analyzed by TEM. As seen in Figure 8, a similar number of virus particles appeared to be produced in BAV304a (panels a, b) or BAV.VIId$^+$-infected KT21 cells (panels d,e). In contrast, fewer virus particles could be observed in BAV.VIId$^+$-infected GT23 cells (panels g,h).

Next, we examined CsCl-purified BAV304a, BAV.VIId$^+$ (grown in KT21 cells) and BAV.VIId$^+$ (grown in GT23 cells) by TEM. As seen in Figure 8, icosahedral particles with intact capsids could be observed in purified BAV304a (panel c) and BAV,VIId$^+$ grown in either KT21 cells (panel f) or grown in GT23 cells (panel i).

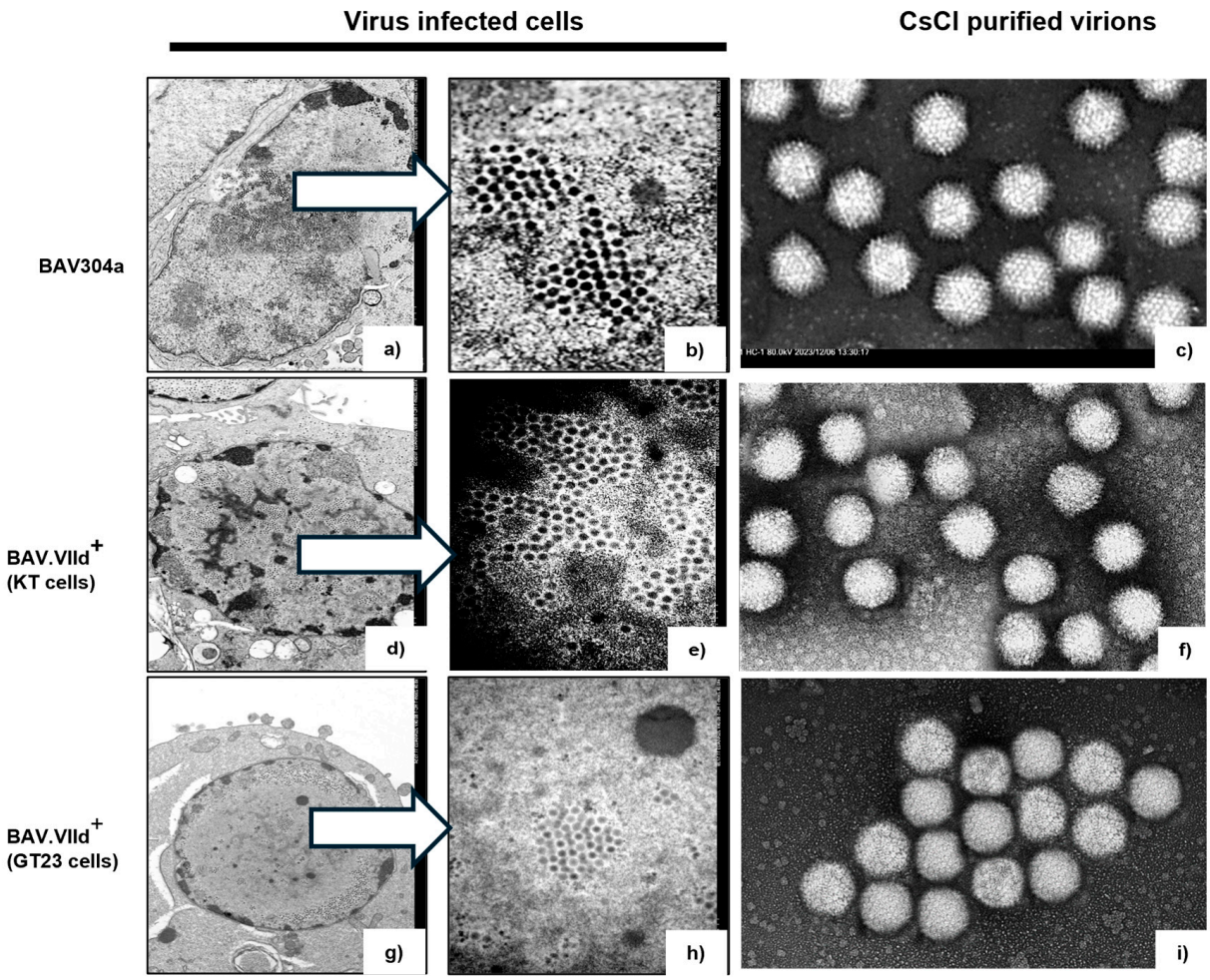

Figure 8. Analysis of mutant viruses by Electron microscopy. BAV304a-infected GT23 cells (panels **a**,**b**), BAV.VIId$^+$-infected KT21 (panels **d**,**e**) or GT23 (panels **g**,**h**). CsCl-purified BAV304a (panel **c**), BAV.VIId$^+$ grown in KT21 (panel **f**) or GT23 (panel **i**) cells.

3.10. Analyses of BAV.VIId by Transmission Electron Microscopy (TEM)

Since infection of GT23 cells with BAV.VIId$^-$-detected no viral protein expression or viral DNA replication (Figure 7), we determined the localization of BAV.VIId$^-$ in GT23 cells by TEM. The GT23 cells were infected with an equal amount of Cscl-purified BAV304a or BAV.VIId$^-$ virus particles for 1 h at 4 °C. The cells were further incubated at 37 °C for 1 h or 4 h before being analyzed by TEM. As seen in Figure 9A, protein VII-deleted BAV.VIId$^-$ virions (panels c,d) were significantly clustered in the cytoplasmic vesicles compared to BAV304a virus-infected (panels a,b) cells. The number of particles at plasma membrane, cytoplasm or vesicles/endosomes are depicted (Figure 9B).

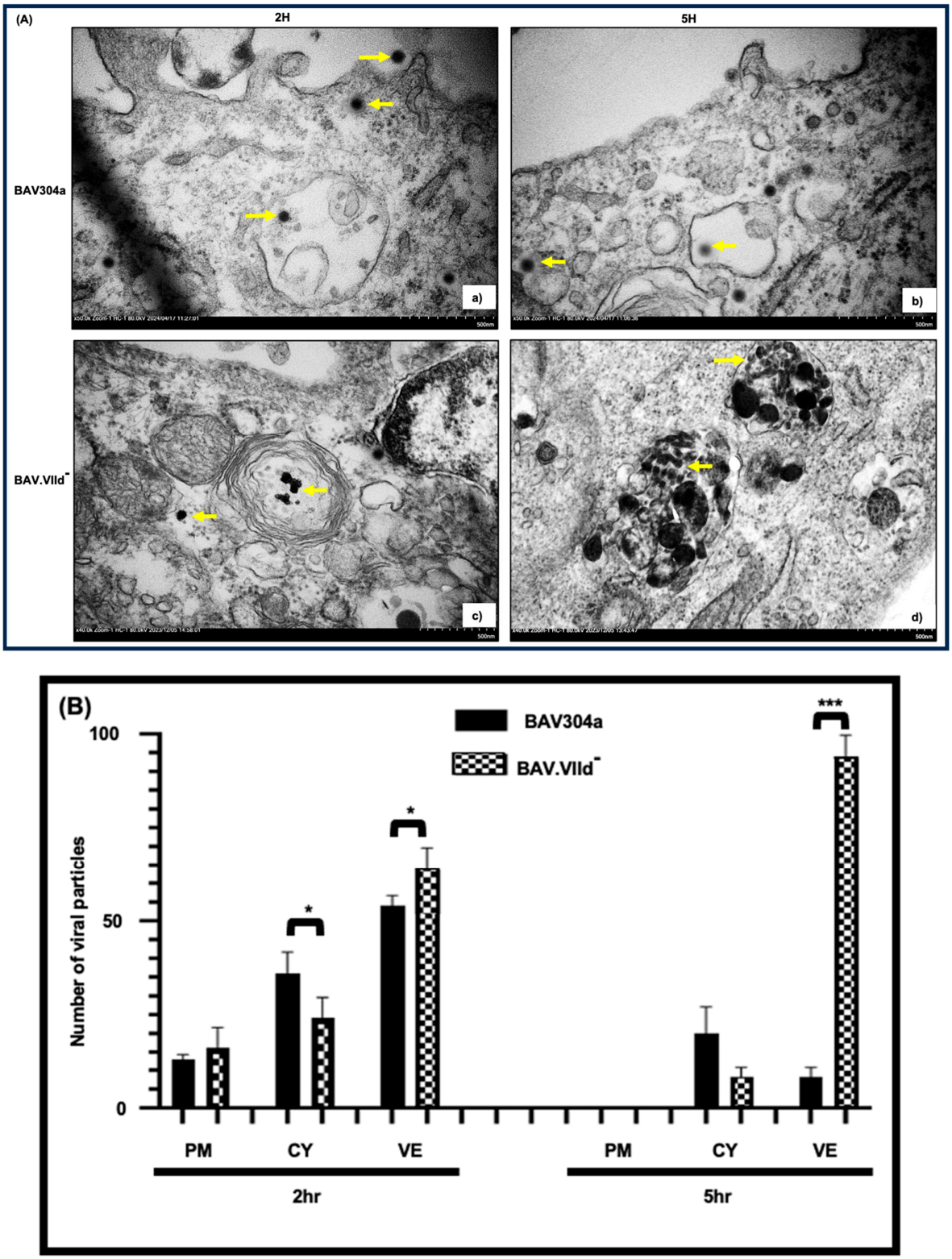

Figure 9. Subcellular localization of BAdV-3. (**A**). The monolayers of GT23 cells incubated with purified BAV304a (panels **a**,**b**) and BAV.VIId^{-} (panels **c**,**d**) virions for 1 h at 4 °C, followed by additional incubation for 1 h at 37 °C (panels **a**,**c**) or 4 h at 37 °C (panels **b**,**d**). Finally, the cells were processed and analyzed by transmission electron microscopy. (**B**). Quantification of virions at subcellular locations. Virus particles in plasma membrane, endosomes/vesicles and cytoplasm were counted at two different times in 10 randomly selected cells. Error bars indicate the SD of the means of three independent experiments. The statistical significance is depicted as follows; * $p < 0.05$; *** $p < 0.001$.

3.11. Proteolytic Cleavage of Adenovirus Proteins

To determine the effect of protein VII on proteolytic cleavage of adenovirus proteins, purified BAV304a, BAV.VIId^{+} or BAV.VIId^{-} were analyzed by Western blot using protein-specific antibodies. As seen in Figure 10, single protein 52 K could be detected for terminal protein in all purified virions. A 8 kDa band representing proteolytically cleaved VIII (VIIIc) could be detected in all purified virions. Similarly, N- and C-terminus proteolytically cleaved protein VI could be detected in purified BAV304a or BAV.VIId^{+}. In contrast, both precursor (preVI) and C-terminally cleaved protein VI (preVIn) could be detected in purified BAV.VIId^{-} virions.

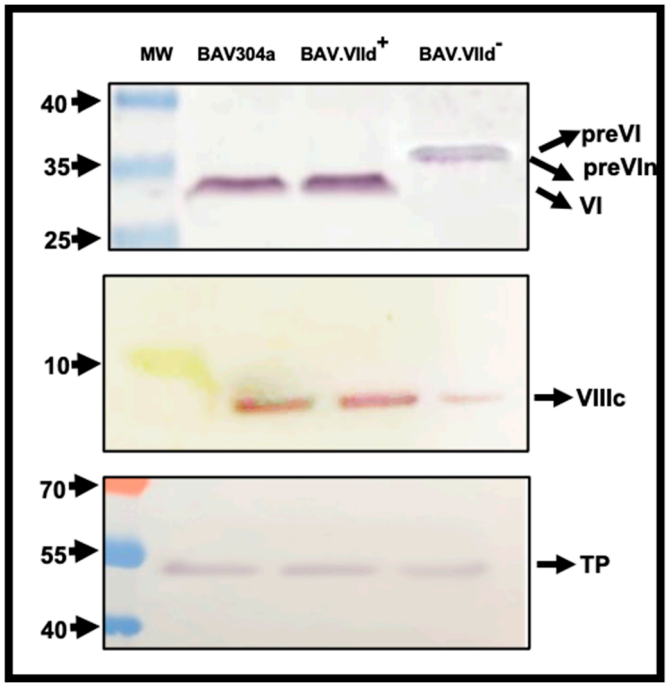

Figure 10. Protease cleavage of BAdV-3 proteins. The proteins from purified BAV304a, BAV.VIId^{+} and BAV.VIId^{-} were separated by SDS-PAGE gels, transferred to nitrocellulose and analyzed by Western blot using protein-specific sera. The molecular weight markers are shown on the left of the panel. The name of the proteins is shown on the right of the panel.

4. Discussion

All members of different genera of family Adenoviradae, including the *Mastadenovirus* genus, encode 16 proteins with conserved genome location [32]. Although the genome location appears conserved, recent reports have demonstrated significant differences in the structure, function and sub-cellular localization domains among positional homologues encoded by members of the *Mastadenovirus* genus [4,16–18,33].

Adenovirus assembly involves the interaction of viral proteins with other viral proteins, cellular proteins and viral DNA [34]. Adenovirus core proteins IVa2 and V appear essential for viral replication and are involved in DNA packaging [35] and maintaining the integrity of progeny virions by connecting viral core to capsid [29,36], respectively. Similarly, earlier reports suggested that core protein VII appeared to condense the adenovirus genome into nucleosome-like structures [15] and thus appears involved in virus assembly and DNA packaging. However, a recent report analyzing Ad5-VII-LoxP virus grown in Cre recombinase-expressing cells (VII^{-}) demonstrated that protein VII is dispensable for virus assembly, including the packaging of DNA into virions [11]. However, purified VII^{-} contains 1% of mature infectious VII^{+} virus, which can replicate viral DNA and expresses protein VII in infected cells [11].

Protein VII encoded by BAdV-3 shows 39–45% homology with homologues encoded by members of *Mastadenovirus* genus [25] and appears involved in inhibiting the loss of the mitochondrial membrane potential by increasing mitochondrial Ca($^{2+}$) and ATP production [4]. Unlike HAdV-5 [1], (a) BAdV-3 protein VII shares polyA signals/sites with 52 K, IIIA and III genes [1], and (b) the mutation of redundant nuclear localization signals abolished the nuclear/nucleolar localization of BAdV-3 protein VII in transfected cells [25]. Here, we sought to determine the function of protein VII in the BAdV-3 replication cycle by constructing and characterizing a mutant BAdV-3 containing a deletion of the protein VII gene.

The protein VII appears essential for the replication of BAdV-3, as viable infectious BAV.VIId^{+} could not be produced in non-complementing cells (GT23) but only by the transient expression of protein VII in the transfected cells or protein VII-complementing cell line (KT21). However, several lines of evidence suggest that BAdV-3 protein VII does not appear to be essential for the viral assembly/packaging of viral DNA. First, an analysis of BAV304a and BAV.VIId^{+} in GT23 cells showed a marginal difference in the expression of viral proteins and DNA replication. Second, there appears to be no significant difference in the incorporation of viral proteins in mutant viral capsids. Third, BAV304a and BAV.VIId^{+} produced loosely arranged and uniformly distributed viral particles in GT23 cells. Fourth, the progeny virus banded at the CsCl density is consistent with the formation of mature virions. Finally, the intact mature icosahedral viral particles could be observed by TEM analysis of CsCl-purified BAV304a, BAV.VIId^{+} (grown in KT21 cells) and BAV.VII^{-} (BAV.VIId^{+} grown in GT23 cells). The results confirm the earlier observation that protein VII encoded by HAdV-5 is not required for the virus assembly /packaging of viral DNA [11].

TEM analysis of the infected cells suggested that BAV304a appeared to produce more viral particles than BAV.VIId^{+} in GT23 cells. Moreover, compared to BAV304a, CsCl purification showed the thin band of mature BAV.VIId^{-}, suggesting that protein VII appears to be involved in efficient virus assembly/DNA packaging. Earlier, no difference in the mature virus band could be observed after the CsCl purification of HAdV-5 VII^{+} and HAdV-5 VII^{-} [11]. Since HAdV-5 protein VII expression and viral DNA replication appear to be detected in about 1% of CsCl-purified VII^{-}-infected cells [11], it is possible that the amount of expressed VII could enhance the viral assembly/DNA packaging.

An earlier report detected the expression of early viral genes and an increase in viral DNA replication in VII^{-} HAdV-5-infected cells [11]. This was attributed to the presence of a small proportion of HAdV-5 VII^{+} virions [11]. Though the CsCl purification of BAV.VIId^{-}-infected GT23 cells produced BAV.VIId^{-} virions banding at a density consistent with the formation of mature virions, we did not detect the expression of an early viral gene or an increase in DNA replication in BAV.VIId^{-}-infected GT23 cells, suggesting that BAV.VIId^{-} virions are not infectious.

Although adenovirus protease cleaves the precursors of protein VII, IIIa, TP, X, VIII and VI [34,37], the production of infectious adenovirus virions requires the cleavage of some adenoviral proteins by adenovirus protease [28,38]. Earlier, available reagents detected a proteolytically cleaved 8 kDa protein VIII in purified BAdV-3 virions [22]. A similar protein could be detected in purified BAV304a, BAV.VIId^{+} or BAV.VIId^{-}, suggesting that the processing of precursor protein VIII appears normal in BAV.VIId^{-} virions. Similarly, proteolytically cleaved protein IIIa could be detected in BAV304a, BAV.VIId^{+} and BAV.VIId^{-} (manuscript in preparation). However, although the C-terminal proteolytic cleavage of precursor protein VI appears normal, the N-terminal cleavage of precursor VI is not detected in BAV.VIId^{-} virions. Interestingly, the N-terminal processing of precursor protein VI(n) is also not detected in HAdV-5 VII^{-} virions [11]. These results have led to the speculation that protein VII may alter the adenovirus protease cleavage process by an unknown mechanism [11].

Although mutant BAdV-3-expressing protein VIII containing single protease cleavage site showed significant defects in the efficient production of progeny virions, TEM analysis detected no significant defect in the escape of virions from endosomes of infected cells [28].

An analysis of BAV.VIId$^-$-infected cells by TEM at 5 h post infection revealed a significant difference in the number of viral particles accumulated in endosomes compared to BAV304a-infected cells. Similar results have been reported for VII$^-$ HAdV-5 [11] and mutant AdV-2 ts1 [12], suggesting that protein VII encoded by members of the *Mastadenovirius* genus appears to be required for the release of virions from the endosomes. In the absence of protein VII, C-terminally processed protein VI remains glued to the hexon cavity in a virion, and is thus not able to interact with the endosome membrane [12].

In summary, although the unessential function of protein VII in viral assembly appears conserved in members of *Mastadenovirus* genus, protein VII appears to be required for the efficient virus assembly/DNA packaging of BAdV-3. The analysis of protein VII-deficient BAV.VIId$^-$ virions revealed (a) a defect in the N-terminus proteolytic cleavage of C-terminally cleaved protein VI and (b) the significant accumulation of virions in the endosomes.

Author Contributions: Conceptualization: S.K. and S.K.T.; methodology: S.K., F.D. and B.S.; lab experiments: S.K., F.D. and B.S.; data curation: S.K., F.D., B.S. and S.K.T.; writing—original draft: S.K., F.D. and B.S.; writing—review and editing: S.K. and S.K.T. All authors have read and agreed to the published version of the manuscript.

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References

1. Reddy, P.S.; Idamakanti, N.; Zakhartchouk, A.N.; Baxi, M.K.; Lee, J.B.; Pyne, C.; Babiuk, L.A.; Tikoo, S.K. Nucleotide sequence, genome organization, and transcription map of bovine adenovirus type 3. *J. Virol.* **1998**, *72*, 1394–1402. [CrossRef] [PubMed]
2. Russell, W.C. Adenoviruses: Update on structure and function. *J. Gen. Virol.* **2009**, *90*, 1–20. [CrossRef] [PubMed]
3. Davison, A.J.; Benko, M.; Harrach, B. Genetic content and evolution of adenoviruses. *J. Gen. Virol.* **2003**, *84*, 2895–2908. [CrossRef] [PubMed]
4. Anand, S.K.; Gaba, A.; Singh, J.; Tikoo, S.K. Bovine adenovirus 3 core protein precursor pVII localizes to mitochondria, and modulates ATP synthesis, mitochondrial Ca2+ and mitochondrial membrane potential. *J. Gen. Virol.* **2014**, *95*, 442–452. [CrossRef]
5. Lee, T.W.R.; Blair, G.E.; Matthews, D.A. Adenovirus core protein VII contains distinct sequences that mediate targeting to the nucleus and nucleolus, and colocalization with human chromosomes. *J. Gen. Virol.* **2003**, *84*, 3423–3428. [CrossRef] [PubMed]
6. Dai, X.; Wu, L.; Sun, R.; Zhou, Z.H. Atomic Structures of Minor Proteins VI and VII in Human Adenovirus. *J. Virol.* **2017**, *91*, e00850-17. [CrossRef]
7. Kulanayake, S.; Tikoo, S.K. Adenovirus Core Proteins: Structure and Function. *Viruses* **2021**, *13*, 388. [CrossRef]
8. Wodrich, H.; Cassany, A.; D'Angelo, M.A.; Guan, T.; Nemerow, G.; Gerace, L. Adenovirus core protein pVII is translocated into the nucleus by multiple import receptor pathways. *J. Virol.* **2006**, *80*, 9608–9618. [CrossRef]
9. Carlon-Andres, I.; Lagadec, F.; Pied, N.; Rayne, F.; Lafon, M.E.; Kehlenbach, R.H.; Wodrich, H. Nup358 and Transportin 1 Cooperate in Adenoviral Genome Import. *J. Virol.* **2020**, *94*, e00164-20. [CrossRef]

10. Avgousti, D.C.; Herrmann, C.; Kulej, K.; Pancholi, N.J.; Sekulic, N.; Petrescu, J.; Molden, R.C.; Blumenthal, D.; Paris, A.J.; Reyes, E.D.; et al. A core viral protein binds host nucleosomes to sequester immune danger signals. *Nature* **2016**, *535*, 173–177. [CrossRef]
11. Ostapchuk, P.; Suomalainen, M.; Zheng, Y.; Boucke, K.; Greber, U.F.; Hearing, P. The adenovirus major core protein VII is dispensable for virion assembly but is essential for lytic infection. *PLoS Pathog.* **2017**, *13*, e1006455. [CrossRef]
12. Hernando-Perez, M.; Martin-Gonzalez, N.; Perez-Illana, M.; Suomalainen, M.; Condezo, G.N.; Ostapchuk, P.; Gallardo, J.; Menendez, M.; Greber, U.F.; Hearing, P.; et al. Dynamic competition for hexon binding between core protein VII and lytic protein VI promotes adenovirus maturation and entry. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 13699–13707. [CrossRef] [PubMed]
13. Condezo, G.N.; San Martin, C. Localization of adenovirus morphogenesis players, together with visualization of assembly intermediates and failed products, favor a model where assembly and packaging occur concurrently at the periphery of the replication center. *PLoS Pathog.* **2017**, *13*, e1006320. [CrossRef]
14. Ostapchuk, P.; Hearing, P. Control of adenovirus packaging. *J. Cell Biochem.* **2005**, *96*, 25–35. [CrossRef]
15. Perez-Berna, A.J.; Marion, S.; Chichon, F.J.; Fernandez, J.J.; Winkler, D.C.; Carrascosa, J.L.; Steven, A.C.; Siber, A.; San Martin, C. Distribution of DNA-condensing protein complexes in the adenovirus core. *Nucleic Acids Res.* **2015**, *43*, 4274–4283. [CrossRef]
16. Kulshreshtha, V.; Babiuk, L.A.; Tikoo, S.K. Role of bovine adenovirus-3 33K protein in viral replication. *Virology* **2004**, *323*, 59–69. [CrossRef] [PubMed]
17. Makadiya, N.; Gaba, A.; Tikoo, S.K. 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. *J. Gen. Virol.* **2015**, *96*, 2749–2763. [CrossRef]
18. Zhao, X.; Tikoo, S.K. Nuclear and Nucleolar Localization of Bovine Adenovirus-3 Protein V. *Front. Microbiol.* **2020**, *11*, 579593. [CrossRef]
19. Papp, Z.; Middleton, D.M.; Mittal, S.K.; Babiuk, L.A.; Baca-Estrada, M.E. Mucosal immunization with recombinant adenoviruses: Induction of immunity and protection of cotton rats against respiratory bovine herpesvirus type 1 infection. *J. Gen. Virol.* **1997**, *78 Pt 11*, 2933–2943. [CrossRef] [PubMed]
20. Du, E.; Tikoo, S.K. Efficient replication and generation of recombinant bovine adenovirus-3 in nonbovine cotton rat lung cells expressing I-SceI endonuclease. *J. Gene Med.* **2010**, *12*, 840–847. [CrossRef]
21. Zhou, Y.; Pyne, C.; Tikoo, S.K. Determination of bovine adenovirus-3 titer based on immunohistochemical detection of DNA binding protein in infected cells. *J. Virol. Methods* **2001**, *94*, 147–153. [CrossRef] [PubMed]
22. Ayalew, L.E.; Gaba, A.; Kumar, P.; Tikoo, S.K. Conserved regions of bovine adenovirus-3 pVIII contain functional domains involved in nuclear localization and packaging in mature infectious virions. *J. Gen. Virol.* **2014**, *95*, 1743–1754. [CrossRef]
23. Woldemariam, T.; Wang, W.; Said, A.; Tikoo, S.K. Regions of bovine adenovirus-3 IVa2 involved in nuclear/nucleolar localization and interaction with pV. *Virology* **2020**, *546*, 25–37. [CrossRef]
24. Thanbichler, M.; Iniesta, A.A.; Shapiro, L. A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in Caulobacter crescentus. *Nucleic Acids Res.* **2007**, *35*, e137. [CrossRef] [PubMed]
25. Kulanayake, S.; Dar, F.; Tikoo, S.K. Regions of Bovine Adenovirus-3 Protein VII Involved in Interactions with Viral and Cellular Proteins. *Viruses* **2024**, *16*, 732. [CrossRef] [PubMed]
26. Sambrook, J.; Russell, D.W. *Molecular Cloning a Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, NY, USA, 2001.
27. Tollefson, A.E.; Kuppuswamy, M.; Shashkova, E.V.; Doronin, K.; Wold, W.S. Preparation and titration of CsCl-banded adenovirus stocks. *Methods Mol. Med.* **2007**, *130*, 223–235. [CrossRef]
28. Gaba, A.; Ayalew, L.; Makadiya, N.; Tikoo, S. Proteolytic Cleavage of Bovine Adenovirus 3-Encoded pVIII. *J. Virol.* **2017**, *91*, e00211-17. [CrossRef]
29. Zhao, X.; Tikoo, S.K. Deletion of pV affects integrity of capsid causing defect in the infectivity of bovine adenovirus-3. *J. Gen. Virol.* **2016**, *97*, 2657–2667. [CrossRef] [PubMed]
30. Chartier, C.; Degryse, E.; Dieterle, M.G.; Pavirani, A.; Mehtali, M. Efficient generation of recombinant adenovirus vectors by homolopgous recombination in *Escherichia coli*. *J. Virol.* **1996**, *70*, 4805–4810. [CrossRef]
31. Said, A.; Wang, W.; Woldermariam, T.; Tikoo, S.K. Domains of bovine adenovirus-3 protein 22K involved in interacting with viral protein 52K and cellular importins alpha-5/alpha-7. *Virology* **2018**, *522*, 209–219. [CrossRef]
32. Ayalew, L.E.; Kumar, P.; Gaba, A.; Makadiya, N.; Tikoo, S.K. Bovine adenovirus-3 as a vaccine delivery vehicle. *Vaccine* **2015**, *33*, 493–499. [CrossRef] [PubMed]
33. Blanchette, P.; Wimmer, P.; Dallaire, F.; Cheng, C.Y.; Branton, P.E. Aggresome formation by the adenoviral protein E1B55K is not conserved among adenovirus species and is not required for efficient degradation of nuclear substrates. *J. Virol.* **2013**, *87*, 4872–4881. [CrossRef] [PubMed]
34. Mangel, W.F.; San Martin, C. Structure, function and dynamics in adenovirus maturation. *Viruses* **2014**, *6*, 4536–4570. [CrossRef]
35. Zhang, W.; Imperiale, M.J. Requirement of the adenovirus IVa2 protein for virus assembly. *J. Virol.* **2003**, *77*, 3586–3594. [CrossRef] [PubMed]
36. Ugai, H.; Borovjagin, A.V.; Le, L.P.; Wang, M.; Curiel, D.T. Thermostability/infectivity defect caused by deletion of the core protein V gene in human adenovirus type 5 is rescued by thermo-selectable mutations in the core protein X precursor. *J. Mol. Biol.* **2007**, *366*, 1142–1160. [CrossRef]

37. Webster, A.; Russell, S.; Talbot, P.; Russell, W.C.; Kemp, G.D. Characterization of the adenovirus proteinase: Substrate specificity. *J. Gen. Virol.* **1989**, *70 Pt 12*, 3225–3234. [CrossRef]
38. Moyer, C.L.; Besser, E.S.; Nemerow, G.R. A single maturation cleavage site in adenovirus impacts cell entry and capsid assembly. *J. Virol.* **2016**, *90*, 521–532. [CrossRef]