Gutless helper-dependent and first generation HAdV5 vectors have similar mechanical properties and common transduction mechanisms

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Running title: Physical features and cell entry of HAdV5 vectors

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Abstract

Delivering vectorized information into cells with the help of viruses has been of high interest to fundamental and applied science, and bears significant therapeutic promise. Human adenoviruses (HAdVs) have been at the forefront of gene delivery for many years, and the subject of intensive development resulting in several generations of agents, including replication-competent, -defective or retargeted vectors, and recently also helper-dependent (HD), so-called gutless vectors lacking any viral protein coding information. While it is possible to produce HD-AdVs in significant amounts, physical properties of these virus-like particles and their efficiency of transduction have not been addressed. Here we used single-cell and single virus particle assays to probe the effect of genome length on HadV-C5 vector transduction. Our results demonstrate that first generation C5 vectors lacking the E1/E3 regions of the viral genome as well as HD-AdV-C5 particles with a wild type ~36 kbp or an undersized double-strand DNA genome are similar to HAdV-C5 wild-type regarding attachment to human lung epithelial cells, endocytic uptake, endosome penetration and dependency on the E3 RING ubiquitin ligase Mind Bomb 1 for DNA uncoating at the nuclear pore complex. Atomic force microscopy measurements of single virus particles indicated that small changes in the genome length from 94-103 percent of HAdV-C5 have no major impact on physical and mechanical features of AdV vectors. In contrast, an HD-AdV-C5 with ~30 kbp genome was slightly stiffer and less heat-resistant than the other particles, despite comparable entry and transduction efficiencies in tissue culture cell lines, including murine alveolar macrophage-like MPI-2 cells. Together, our in vitro studies reinforce the use of HD-AdV vectors for effective single round gene delivery. The results illustrate how physical properties and cell entry behaviour of single virus particles can provide functional information for anticipated therapeutic vector applications.
Introduction

Human adenoviruses (HAdVs) infect a wide range of cell types and tissues, including respiratory epithelia, endothelia, liver, kidney and the gastrointestinal tract, and persist in immune cells (1-3). In addition, documented instances of animal AdV crossing species barriers highlight the zoonotic potential of AdVs (4). The pathogenic nature of HAdVs can, however, be largely eliminated by genetic ablation of the immediate early viral transactivator protein E1A, which reduces viral replication, albeit incompletely, especially in cancer cells (5, 6), although mouse AdVs replicate well in absence of E1A (7).

The AdV vectors feature distinct advantages compared to other viral vectors, including their high titer and efficiency of in vivo gene delivery, large cargo capacity, and high targeting and gene expression in different tissue and cell types. This is due to powerful genetic and chemical engineering modalities, ongoing enhancements in recombineering and cost-effective production technologies, and last, but not least, a well-documented safety profile, notably under controlled conditions (8-16). Vectorization of HAdVs has been continuously enhanced for nearly three decades and reached animal models and increasingly human patients (8, 9, 17). Recent successes with AdV vectors include the approval of Nadofaragene fiadenovec (Adstiladrin), a human adenovirus C5 (HAdV-C5)-based non-replicating vector for local IFNα2b expression to treat bladder cancer (18, 19), or adenovirus-based vaccines against Coronavirus-induced disease (COVID-19 caused by SARS-CoV-2), including Chimpanzee ChAdOx1 nCov-19 and HAdV-D26.CO2V2.S (20, 21). Yet, the potential of animal AdVs for therapeutic applications in humans has not been fully explored. Together with the observation of rare and fortunately treatable AdV vector-induced thrombotic thrombocytopenia in COVID-19 vaccination, this highlights the need for further improvement of AdV vectors and their better understanding for applications in humans (22).

HAdVs are non-enveloped viruses with an outer icosahedral capsid structure and an inner core composed of the viral linear double-stranded DNA genome of about 36 kilobase pairs (kbp) and its associated proteins V, VII, X and terminal protein. For use as a vector, a given AdV-type can be left unmodified or genetically modified in the capsid proteins, or supplemented by addition of adaptors to control cell and tissue targeting (23-26).
Furthermore, liver targeting, and immune recognition can be minimized by the use of a protein shield (27, 28). The virus and vector genomes, furthermore, typically vary by gene deletions, insertions, promoter swaps or alterations of open reading frames to confer tissue-specific features of interest (29).

For the construction of AdV vectors two design principles have been used: the first generation vectors carry deletions of the viral E1 and E3 transcription units, whereas the newer helper-dependent “gutless vectors” retain only the inverted terminal repeat sequences and packaging signals, while all other viral sequences are typically deleted (15). The first generation vectors are defective in replication because proteins encoded in the E1 region include viral transcriptional activators required for stimulation of other viral transcription units (30). These vectors are produced in packaging cell lines that contain chromosomal copies of the E1 region (15). However, residual leaky viral protein expression from the first generation vectors induces host immune responses limiting the duration of vector transgene expression (31). This motivated the development of the gutless, helper-dependent (HD) AdV vectors with up to 37 kbp of cloning capacity and prolonged transgene expression (32). The gutless vectors are produced using a modified first-generation vector with an excisable packaging signal as a helper virus and a packaging cell line providing the E1 transcription unit. Genome size of the first generation and helper-dependent vectors can deviate from that of the corresponding wild type AdV, but there has been no systematic analysis whether the differences in genome size impact stability and/or cellular uptake mechanisms of the vectors.

Virus particles have distinct physical and mechanical properties, which can be studied at single particle resolution by atomic force microscopy (AFM) (33, 34). The physical and mechanical features of the particles are connected to the molecular mechanisms of virus entry into a host cell (35). For example, AFM studies have shown that changes in the protein composition of the AdV particle can affect the physical properties of virions, such as stiffness, brittleness and internal pressure (36-38), along with deficiencies in virus entry phenotypes (39-41). Mechanical fatigue exerted by an AFM tip probably mimics molecular impacts and/or power strokes by e.g. cellular motor proteins on the virus particle during entry (40, 42, 43). Yet, how the viral features observed in in vitro AFM studies relate to virus
navigation through the cellular environment during entry is still poorly understood. In particular, the impact of the genome length on the mechanical and physical features of AdV particles or particle entry and transduction efficiency has not been studied by AFM and correlated entry assays.

The AdV has a linear DNA genome embedded in an icosahedral particle with pseudo T=25 symmetry. The capsid limits the size of the encapsidated genome to about 75% - 105% of the wild type (wt) genome length and genomes outside of these limits are poorly packaged and/or unstable (44, 45). The size of the encapsidated genome also influences the heat stability of the AdV particles (46), thus suggesting consequences on the physical properties of the particle. In the present study, we used AFM to assess the impact of the genome size on the physical properties of AdV particles, in correlation to heat stability and entry phenotypes in tissue culture cells. Our results indicate that a gutless vector with a 30 kbp genome presented a stiffer capsid and was less heat-resistant than a wt virus, but had a cell entry efficiency comparable to that of wt virus. This suggests that although the genome length affects certain physical properties of the AdV particle, its impact on entry and transduction is limited.

**Material and Methods**

**Cells**

A549 human lung epithelial carcinoma cells and HeLa cells, both from the American Type Culture Collection (ATCC), as well as HeLa subline Ohio (kindly provided by Laurent Kaiser, University Hospitals of Geneva), KB cells (a subline of HeLa cells), human embryonic retinoblast 911 cells (HER-911) and HEK293 cells were maintained in DMEM (Sigma-Aldrich, D6429) supplemented with 7.5% fetal calf serum (FCS; Gibco/Thermo Fisher Scientific, 10270106) and 1% non-essential amino acids (Sigma-Aldrich, M7145). HeLa-Mib1 (Mind Bomb 1)-KO cells have been previously described (47) and were maintained in the same medium as parental HeLa cells. The MPI (Max-Planck-Institute) line 2 (MPI-2) mouse alveolar macrophage-like cells (48) were maintained in RPMI 1640 (Sigma-Aldrich, R8758) supplemented with 10% FCS, 1% non-essential amino acids and 10 ng/ml mouse GM-CSF (Miltenyi Biotech, 130-095-746).
Viruses

HAdV-C5_wt was grown in A549 cells. The penetration-deficient HAdV-C2_TS1 virus (49), which fails to package the viral protease and has unprocessed capsid proteins, was grown in KB cells at the restrictive temperature 39°C. The non-replicating, first generation HAdV-C5-deE1/dE3_GFP (FG34) vector (50, 51) was grown in HER-911 cells. FG34 is an E1/E3 deletion mutant virus expressing enhanced green fluorescent protein (GFP) from cytomegalovirus (CMV) major immediate early promoter transcription regulatory sequences. FG34_1 has the wild type INTETL sequence in the hexon hypervariable region (HVR) 7 changed to GNNSTY and is thus incapable of binding to factor X, whereas FG34_2 has a wild type hexon HVR7. All the above-mentioned viruses were purified on two consecutive CsCl gradients as previously described (52), and dialyzed against 10 mM Tris-HCl pH 8.1, 150 mM NaCl, 1 mM MgCl₂ for ~ 24 h with one change of buffer using Slide-A-Lyzer dialysis cassettes (10,000 MWCO, ThermoFisher Scientific 66383). Glycerol was added to a final concentration of 10% and the viruses were stored in aliquots at -80°C.

The first generation HAdV-C5 vector FG37 is derived from pmCherry-HVR7 described in (16) and was modified by an insertion of 5.4 kbp of lambda stuffer DNA via Gibson assembly. FG37 is deleted for E1 and E3 genes, contains a modified packaging signal flanked by LoxP sites, has the factor X-binding ablating point mutations in hexon HVR7 and a genome size of 37 kbp. FG37 contains the mCherry gene under the control of PGK1 (phosphoglycerate kinase) promoter. FG37 was used as a helper virus for the helper-dependent (gutless) HAdV-C5-derived viruses HD30 and HD36, which have genome sizes of 29834 bp and 35843 bp, respectively, and encode GFP from cytomegalovirus major immediate early promoter. The HD30 plasmid was created by inserting a CMV-GFP expression cassette into pC4HSU (purchased from Microbix, originally described in (53)) using Gibson assembly as described in (16). Human stuffer DNA with a length of 6.5 kbp (NCBI entry AF0118863) was inserted into the HD30 plasmid by Gibson cloning to generate the HD36 plasmid. Thus, the different genome sizes of HD30 and HD36 are due to different amounts of human stuffer DNA. For virus production, the FG37 plasmid was linearized with PmeI and transfected into HEK293 cells using Polyethylenimine (Sigma-Aldrich 408727). Adenovirus particles were amplified over four passages and purified from a lysate of 2x10⁸ cells by two consecutive CsCl gradient
centrifugations. To produce the gutless viruses, HD30 and HD36 plasmids were linearized with Pmel and transfected into human 116 cells (54). The FG37 helper virus was co-administered during all passages. The gutless virus particles were purified from 2x10^8 cells using optimized CsCl gradients as described in (16).

Atto565-labeled HAdV-C5_wt and vector viruses, as well as Alexa Fluor 488-labeled HAdV-C2_TS1 were prepared as previously described (41, 52, 55) using Atto565 NHS ester (Sigma 72464) and Alexa Fluor 488 TFP ester (ThermoFisher Scientific, A37570), respectively. Excess dye was removed with Zeba Spin Desalting 40K MWCO columns (ThermoFisher Scientific A57756).

**Infection assays**

For normalization of input virus amounts, different dilutions of viruses were incubated with A549 cells at 37°C for 60 min in DMEM medium supplemented with 0.2 % bovine serum albumin (BSA, Thermo Fisher Scientific, 11500496) and 1% penicillin-streptomycin (Sigma, P0781), after which unbound viruses were washed off with excess medium and incubation was continued for a further 15 min at 37°C in fresh medium. A similar experiment was carried out in MPI-2 cells except that in this case the medium was the normal growth medium of these cells. After the last 15-min incubation, cells were fixed and stained with mouse anti-hexon 9C12 antibodies (56) and secondary anti-mouse Alexa Fluor 488-conjugated antibodies (ThermoFisher Scientific, A-11029, final concentration 4 µg/ml) as described in (57). The 9C12 antibody, developed by Laurence Fayadat and Wiebe Olijve, was obtained from Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biology. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; final concentration 1 µg/ml, included into the secondary antibody staining solution) and the cell area with Alexa Fluor 647 succinimidyl ester (Thermo Fisher Scientific, A20006, diluted to a final concentration of 0.5 µg/ml in phosphate buffered saline (PBS) and incubated for 10 min at room temperature). The samples were imaged with a Leica SP5 confocal laser scanning microscope using a 63× objective (oil immersion, numerical aperture 1.4) and zoom factor 2 with 1 µm-interval for Z-stacks. The Leica SP5
microscope was maintained by the Center for Microscopy and Image Analysis core facility of University of Zurich. A custom-programmed MatLab (The Mathworks) script was used to produce maximum projections of the confocal stacks and the number of cell-associated virus particles at single-cell level was scored using custom-programmed CellProfiler (https://cellprofiler.org; version 3.1.9, (58)) pipelines. The resulting data were sorted using the Knime Analytics Platform (https://www.knime.com/knime-analytics-platform; version 3.7.2). Since virus counts per cell varied at the single-cell level, the median values for cell-associated virus counts were used as guidelines for similar input virus amounts in the infection assays.

For infection assays, A549 cells were seeded on 96-well imaging plates (Greiner Bio-One, 655090) so that cultures were at \( \sim 80\% \) confluency at the time of the experiment (e.g. 6000 cells/well for growth over two nights prior to conducting the experiment). FG34 in the infection assays refers to FG34_2. Cells were incubated with normalized input amounts of different viruses at 37°C for 60 min in DMEM medium supplemented with 0.2 % BSA and 1% penicillin-streptomycin (DMEM-BSA medium), after which unbound viruses were washed away with excess medium and incubation was continued in A549 growth medium supplemented with penicillin-streptomycin for further 23 h at 37°C. Cells were then fixed and nuclei were stained with DAPI as described in (59). For MPI-2 infection assays, 40000 cells/well were seeded on the imaging plates and grown over one night prior to start of the experiment. The infection was carried out as in A549 cells, except that the medium used throughout was the MPI-2 growth medium supplemented with penicillin-streptomycin. Cells were imaged with a Molecular Devices automated ImageX-press Micro XL widefield imaging system using 10 × Plan Fluor objective (numerical aperture 0.3) and a single focal plane for both GFP and DAPI channels corresponding to a middle section of nuclei. Images were analyzed using a custom-programmed CellProfiler pipeline in which nuclei were segmented with the DAPI image and mean nuclear GFP intensities over the DAPI masks were determined. JMP (JMP Statistical Discovery version 13 – version 15) was used to determine the threshold for an infected cell (99.5 % cut-off value from the non-infected control cells), and infection index, i.e. the number of infected cells over the total number of cells analyzed was calculated using the Knime Analytics Platform. GraphPad Prism 7 (GraphPad Software,
La Jolla, CA, USA) was used to create the graphs. Representative images were processed in Fiji (60), applying the same changes in brightness and contrast to all images in the series.

For comparing virus transduction in parental HeLa (ATCC) vs. HeLa-Mib1-KO cells, 6000 parental cells or 7000 Mib1-KO cells were seeded on a 96-well imaging plate and grown over night. Cells were infected and analyzed as described above for the infections of A549 cells.

**Endocytosis and protein VI exposure**

A549 cells were seeded on Alcian blue-coated coverslips (61) at 40000 cells/well in a 24-well dish and grown over two nights. Cells were incubated with Atto565-labeled viruses in RPMI 1640 medium (Sigma-Aldrich R7388) supplemented with 0.2% BSA and 1% penicillin-streptomycin (RPMI-H-BSA medium) for 60 min on ice at +4°C. FG34 in the entry assays refers to FG34_2. After removal of unbound viruses, fresh medium was added and cells were switched to a 37°C water bath for the indicated times. Afterwards, samples were returned to +4°C and intact cells were incubated for 60 min with the anti-hexon 9C12 antibody (diluted in the cold RPMI-H-BSA medium) to tag surface virus particles. Unbound antibodies were removed by washing twice with excess RPMI-H-BSA medium, once with PBS and cells were fixed with 3% paraformaldehyde/PBS and processed for immuno-staining with rabbit anti-protein VI antibodies and secondary Alexa Fluor 680-conjugated anti-mouse antibodies (Thermo Fisher Scientific, A-21057) and Alexa Fluor 488-conjugated anti-rabbit antibodies (ThermoFisher Scientific, A-11034). Samples were imaged with a Leica SP5 confocal laser scanning microscope using a 63× objective (oil immersion, numerical aperture 1.4) and a zoom factor 2 or a 40× objective (oil immersion, numerical aperture 1.4) and a zoom factor 3. Stacks were recorded for all channels at 1 µm-interval using between-frames switching mode and 3 × frame averaging for the virus channel. A custom-programmed MatLab script was used to produce maximum projections of the confocal stacks and the number of cell-associated virus particles, as well as the 9C12 and anti-protein VI signals on cell-associated virus particles were scored at single-cell, single-particle levels using a custom-programmed CellProfiler (version 4.2.1) pipeline. The threshold for a 9C12-positive particle was determined by placing a virus image on 9C12 images from noninfected cells and taking the 99.5% cutoff value from the 9C12 intensity values on cell-associated virus particles as the
threshold. Similarly, the mean anti-protein VI signal background value was determined by placing a virus image on anti-protein VI images from noninfected cells and taking the 99.5% cutoff value from the anti-protein VI intensity values on cell-associated virus particles as the threshold. KNime Analytics Platform custom-programmed pipelines were used to determine the fraction of surface-associated virus particles (i.e. 9C12-positive particles) per cell, as well as the per cell average anti-protein VI signal on cell-associated virus particles. For the latter, the mean anti-protein VI signal background value was first subtracted from anti-protein VI intensity values of each cell-associated virus particles prior to calculating the average virus particle-associated anti-protein VI intensity values per cell.

**Streptolysin O (SLO) assay to detect virus particles penetrated into the cytoplasm**

HeLa-Ohio cells were seeded on Alcian blue-coated coverslips at 40000 cells/well in a 24-well dish and grown over two nights. Cells were incubated with Atto565-labeled viruses or with Alexa Fluor 488-labeled penetration-deficient HAdV-C2_TS1 virus in RPMI-H-BSA medium for 60 min on ice at +4°C (FG34 in the entry assays refers to FG34_2). After removal of unbound viruses, fresh medium was added, and cells were switched to a 37°C water bath for 10 min or 30 min. Subsequently, cells were returned to ice and the plasma membrane was perforated with streptolysin O (SLO) as previously described (57) using pretitrated amounts of SLO that enabled efficient cell permeabilization (SLO was from Sigma-Aldrich, S5265-25KU). Permeabilized cells were incubated on ice at +4°C with mouse anti-hexon 9C12 (HAdV-C5) and rabbit anti-Giantin (Abcam ab80864, final concentration 0.8 µg/ml) antibodies or with rabbit anti-Alexa Fluor 488 (TS1 virus; ThermoFisher Scientific A-11094, final concentration 0.8 µg/ml) and mouse anti-Giantin (final concentration 67 µg/ml; antibody kindly provided by Hans-Peter Hauri, Biocenter of the University of Basel) antibodies in SLO-internalization buffer (57). Cells were then washed twice with excess SLO internalization buffer, fixed and stained with secondary anti-mouse Alexa Fluor 488- and anti-rabbit Alexa Fluor 680 (ThermoFisher Scientific A-21076)-conjugated antibodies (final concentration 4 µg/ml; HAdV-C5 sample) or with donkey anti-rabbit Alexa Fluor 594 (ThermoFisher Scientific A-21207) and goat anti-mouse Alexa Fluor 680 (ThermoFisher Scientific A-21057) antibodies (final concentration 4 µg/ml, TS1 sample). Nuclei were stained with DAPI. Control samples were fixed and permeabilized with Triton X-100 prior to antibody
stainings. Samples were imaged with a Leica SP5 confocal laser scanning microscope as described above using 2× frame averaging for the 9C12 channel. A custom-programmed MatLab script was used to produce maximum projections of the confocal stacks and the number of cell-associated virus particles, as well as the virus particle-associated 9C12/anti-Alexa Fluor 488 signals and cell-associated anti-giantin signals were scored at single-cell, single-particle levels using a custom-programmed CellProfiler (version 4.2.1) pipeline. The threshold for a 9C12/anti-Alexa Fluor 488-positive particle was determined by placing a virus image on 9C12/anti-Alexa Fluor 488 images from noninfected cells and taking the 99.5% cutoff value from antibody intensity values on cell-associated virus particles as the threshold. The threshold for an anti-giantin-positive cell was determined manually by comparing anti-giantin images to the cytoplasmic anti-giantin signals. Afterwards, proper classification of cells into anti-giantin-positive and –negative was verified manually, and only anti-giantin-positive cells with no staining artefacts were included into the data set. Knime Analytics Platform custom-programmed pipelines were used to determine the fraction of cytoplasmic (i.e. 9C12/anti-Alexa Fluor 488-positive) virus particles per cell. Statistical analyses were performed in GraphPad Prism using the Kolmogorov-Smirnov test. Representative images were processed in Fiji, applying the same changes in brightness and contrast to all images in the series.

Heat-resistance of viruses

Viruses were diluted in a buffer containing 25 mM Hepes-KOH pH 7.4, 150 mM NaCl and 1 mM MgCl₂ and incubated at 43.5°C, 46.6°C or 50.4°C (PCR machine) for 5 min. Controls were kept at room temperature. The FG34 virus used in this assay was FG34_2. The virus suspension was further diluted in A549 growth medium supplemented with penicillin-streptomycin and applied to A549 cells seeded on 96-well imaging plates the day before (seeding density was 15000 cells/well). The cells were fixed at 25 h post infection (pi), processed and analyzed as described above for the infection assays in A549 cells. Mean nuclear GFP intensity per well minus background (determined from noninfected controls) was used for scoring transduction efficiencies in the case of FG34_2, HD30 and HD36, whereas mean nuclear mCherry intensity per well minus background was used for FG37 and
immunostaining with rabbit anti-protein VI antibody (40) and secondary Alexa Fluor 488-conjugated anti-rabbit antibody for HAdV-C5_wt.

**AFM experiments**

Muscovite mica was used as the support substrate. A droplet of 20 µl of each sample was deposited onto freshly cleaved mica and left for 20 min for incubation before cleaning with the same buffer five times with the volume of the buffer being added and removed after each clean. A final volume of 200 µl was then used for virus measurement.

All experiments were performed using rectangular silicon nitride cantilevers from Olympus (RC800PSA) with nominal spring constants of 0.05N/m. The cantilevers used were calibrated using the Sader’s method [https://doi.org/10.1063/1.1150021]. Measurements were performed with an AFM (Nanotec Electrónica S.L., Madrid, Spain) in Jumping Plus Mode. In this mode the tip is moved in the Z-axis a certain distance (jump off), and then released form the surface and moved laterally to the next pixel to repeat the loop.

Nanoindentation assays were carried out in individual particles. The AFM tip deformed the viral capsids by performing single force curves (FZs) at a constant speed of 100 nm/s. The spring constant of the particles was obtained from the linear regime of the FZ curves. Critical indentation values were obtained from the point at which a major drop in the normal force was obtained, usually meaning a failure in the integrity of the capsid. Analysis of the images and the FZs was done using the WSxM software [https://doi.org/10.1063/1.2432410].

**Data availability**

The data including maximum projections of microscopy images and CellProfiler pipelines used to create the figures in this manuscript are deposited at Zenodo.org (https://doi.org/10.5281/zenodo.10591729).
Results

Mechanical properties of adenoviral vectors

We studied the following four different HAdV-C5-derived particles by AFM: a HAdV-C5_wt (genome length 36 kbp), a first generation FG34_1 vector with a 33.8 kbp genome, a modified first generation vector FG37 with a 37.1 kbp genome and a helper-dependent vector HD30 with a 29.8 kbp genome (Fig. 1A). In the vectors FG34_1, HD30 and FG37 the factor X binding site was ablated by mutations in the hexon hypervariable region (HVR) 7, where the sequence INTETL was changed to GNNSTY as described earlier (27). These HVR7 amino acid swaps (HVR7*) did not affect the entry of HAdV-C5 into murine macrophages (59). A silver stain gel of the virus preparations is shown in Fig. 1B. The viruses had similar protein profiles except that protein V in wt virus migrated slightly faster than protein V in the other particles. The capsid coding regions of the wt and the vectors are derived from different parental HAdV-C5 clones, and this most likely explains the differences in the protein V.

For the AFM experiments, viral particles were adsorbed to mica, resulting in a random distribution of individual particles on the surface. This was done under saline-buffered conditions, and the particles were then imaged as shown in Fig. 2. AFM topography images, which provided detailed views of individual protein capsomers, showed no significant differences between the four particle types (Fig. 2A). Height measurements of all particles examined were around 86 nm (Fig. 2B), consistent with the capsid diameter and indicating that none of the different particles exhibited measurable deformations due to substrate interactions. Topographical profiles were measured in the centre of the 3-fold symmetry axis of each viral particle resulting in $h_{\text{wt}} = 86.1 \pm 1.4$ nm (mean $\pm$ SD), $h_{\text{FG34}} = 85 \pm 1.7$ nm, $h_{\text{HD30}} = 86 \pm 1$ nm and $h_{\text{FG37}} = 84.9 \pm 1.3$ nm.

To explore the impact of the differences in genome size on the physical properties of particles, we conducted single nanoindentation assays on the FG34_1 (n=45), HD30 (n=45), FG37 (n=45) and wt particles (n=37). AFM indentation assays inform on the particle mechanical properties. We analysed the spring constant and critical strain of the particles to obtain stiffness and brittleness, respectively. The spring constants calculated from the
linear regime of the single indentations were: $k_{\text{wt}} = 0.57 \pm 0.03 \text{ N/m (mean \pm SEM)}$, $k_{\text{FG34}} = 0.62 \pm 0.04 \text{ N/m}$, $k_{\text{HD30}} = 0.67 \pm 0.03 \text{ N/m}$ and $k_{\text{FG37}} = 0.63 \pm 0.04 \text{ N/m}$ (Fig. 2C). These values are in agreement with previously reported findings for the HAdV-C5 particles (37, 38, 62). Kolmogorov-Smirnov test indicated no statistically significant differences between the populations at a confidence level of 0.025. The fact that we did not observe notable differences between the stiffness of the adenoviral particles may be attributed to the comparable structural composition of the capsid within the specimens.

Previous studies have shown that the difference in stiffness between proteolytically processed wt capsids and unprocessed empty ones is about 0.12 N/m (37). Likewise, the stiffness of unprocessed DNA-containing TS1 particles was about 0.08 N/m lower than wt particles (63). These data suggest that small modifications in genome size do not govern capsid stiffness, unlike proteolytic processing of capsid proteins. Interestingly, our averaged data show that HD30 particles are slightly stiffer than HAdV-C5_wt, suggesting that the differences in genome size >6 kbp start to be notable in the mechanics of particles with comparable capsid proteins. In addition, FG34_1, FG37, HD30 and wt had similar critical strains, as calculated from the ratio between the critical deformation ($\delta_{\text{critical}}$) and the height of the intact viral particles (right inset of Fig. 2B). The critical strain provides information about the brittleness of the particle, and was 15.3 ± 5.7 % (mean ± SD) for HAdV-C5_wt, 13 ± 5 % for FG34_1, 14.5 ± 4.6 % for HD30 and 15.7 ± 6.6 % for FG37 (Fig. 2D). One-way ANOVA tests indicated that population means were not significantly different within the compared particles.

**Thermal stability of the vector particles**

We also compared thermal stabilities of the vector particles by testing transduction efficiency after a short exposure to elevated temperature. The helper-dependent vector HD36 with a wt-like genome length (35.8 kbp) was included into the test because this vector was used in the cell entry assays described below, along with FG34_2. FG34_2 differed from the FG34_1 used in the AFM experiments by having a wild type hexon HVR7 sequence. HAdV-C5_wt and the vector particles were exposed to 43.5°C, 46.6°C and 50.4°C for 5 min, and the remaining transduction activity was assessed in A549 cells by scoring for GFP-positive cells in case of FG34_2, HD30 and HD36, for mCherry-positive cells in case of FG37,
while the transduction efficiency of HAdV-C5 wt was assessed by immunostaining for the late virus protein VI. Particles kept at room temperature were used as controls. As shown in Fig. 3, HD30 particles were less resistant to heat-treatment than HAdV-C5_wt, FG37, FG34_2 or HD36 particles: whereas other particles retained essentially full activity after 5-min incubation at 46.6°C, a drastic decrease in HD30 transduction was observed. The lower heat tolerance of HD30 is unlikely to be attributed to HD30 being a gutless virus, but more likely due to the size of its encapsidated genome, since the HD30 phenotype is consistent with the results previously obtained from AdV particles with similar-sized genomes (46).

**Genome size of HAdV vectors has no major impact on the early steps of the cell entry**

We used A549 and HeLa cells to map the entry efficiency of the vectors using quantitative single-cell, single-particle assays. FG34_2, HD30, HD36 and HAdV-C5_wt were included in these assays. HD30 and HD36 contained the factor X binding site mutations in hexon HVR7, but, as mentioned above, no impact of these mutations on virus cell entry in tissue culture cells has been observed in previous studies (59). After binding to the cell surface, HAdV-C5 particles are internalized by clathrin-mediated endocytosis (64-67), and this step can be probed by measuring accessibility of the fluorophore-tagged virus particles to antibodies in intact cells, where internalized particles are inaccessible to externally added antibodies (40, 57). In Fig. 4A, Atto565-tagged particles were bound to A549 cells at +4°C, and after removing unbound inoculum, samples were either retained at +4°C or switched to 37°C for 10 min or 30 min. After the 37°C incubation, cells were returned to +4°C and particles remaining at the cell surface were marked by a combination of anti-hexon and secondary fluorophore-conjugated antibodies, followed by confocal microscopy imaging. Efficient uptake of all vector types was evident by the decrease of surface signal already at 10 min, however with cell-to-cell variabilities in all samples. A clear majority of particles was internalized in all samples at 30 min post-warming. Although statistical analyses (Kolmogorov-Smirnov test) indicated significant differences for some of the samples at both internalization time points, the differences overall were relatively minor: for example, the mean values for fractions of surface particles at the 30-min time point were 0.12 for HAdV-C5_wt, 0.09 for FG34_2 and 0.08 for HD36.
Following uptake, HAdV-C5 particles penetrate into the cytoplasm by causing disruption of early endosomes (52, 64, 68-70). A prerequisite for this step is exposure of the membrane lytic protein VI from the capsid interior (40, 70-72). The protein VI exposure can be monitored by anti-VI antibody staining of cell-associated particles in fixed cells (40, 57). As shown in Fig. 4B, protein VI exposure in A549 cells occurred in all vector preparations, although statistically significant differences in the average VI signal on cell-associated particles were evident between HAdV-C5_wt control and HD30 or HD36 vectors at the 10-min time point, or between HAdV-C5_wt and HD36 at the 30-min time point. Since the majority of protein VI separates from the incoming particles post penetration, the observed differences in the particle-associated protein VI signal could be due to less efficient overall protein VI exposure in the gutless vectors or to slightly more rapid dissociation of VI from these particles.

We next probed the penetration efficiency of the vector particles by testing accessibility of internalized particles to exogenously applied anti-hexon antibodies following perforation of the plasma membrane with streptolysin O (viruses in endosomes being inaccessible to the antibodies; (57)). HeLa-Ohio cells were used in the assays because they are easily perforated by streptolysin O. Proper perforation of cells was assessed by accessibility of the C-terminally anchored Golgi-associated protein Giantin to anti-Giantin antibodies. As previously documented (39, 57, 59), the penetration occurred with variable efficiencies at the single-cell level (Fig. 4C), but the differences of mean vector penetration efficiencies in comparison to HAdV-C5_wt were minor despite being statistically significant: the mean value for fraction of antibody-positive particles for the HAdV-C5_wt was 0.70 and for the three vectors 0.76. Thus, the observed differences in Fig.4B in protein VI exposure did not translate into less efficient penetration of the HD vectors. Endosomes remained intact in this assay, as evidenced by the penetration-deficient TS1 mutant of HAdV-C2 (40, 41, 57, 64) being largely inaccessible to the exogenously added antibodies (Fig. 4D).

Quantitative studies of the last steps in the entry of AdV, i.e. nuclear targeting, capsid disassembly at the cytoplasmic side of the nuclear pore complex (NPC) and import of the viral genome into the nucleus through the NPC channel, are based on 5-ethynyl-2'-deoxycytidine (EdC)-labeled virus genomes and a click reaction for a fluorescent labeling of
the incoming genomes (e.g. (39, 47, 73)). Unfortunately, these assays cannot be used with first generation or gutless AdV vectors, because EdC is not metabolized in the cell lines used for vector production. Therefore, we turned to transduction assays to indirectly assess the last steps in entry, as well as to relate the results from the entry assays described above to overall transduction efficiency of the vectors with different genome sizes.

**Genome size of HAdV vectors has only a minor effect on transduction efficiencies in tissue culture cells**

Comparison of vector transduction efficiency requires that the same transgene is under similar transcription regulatory sequences and that the input of vector amounts is carefully controlled. FG34_2 and the gutless vectors HD30 and HD36 fulfill the first requirement because they all have the enhanced green fluorescent protein (GFP) gene under the cytomegalovirus major immediate early transcription regulatory sequences. The input vector amounts were normalized based on initial experiments, in which cell-associated virus particles were scored after incubation of A549 cells using different amounts of inoculum at 37°C for 60 min, and the resulting median values for cell-associated virus counts were used as guidelines for similar input vector amounts.

A representative transduction experiment in A549 cells is shown Fig. 5A. The cells were fixed at 24 h pi and the number of GFP-positive cells was scored by widefield microscopy. The transduced cells displayed variable GFP intensities. Overall, only relatively minor differences in the number of GFP-positive cells were observed between the three vectors, although we note that with the higher amount of vectors (v1), HD30 and HD36 yielded a slightly lower number of GFP-positive cells than the first generation vector FG34_2.

The transduction assay was repeated also in murine alveolar macrophage-like MPI-2 cells with three different input virus amounts. Whereas the transduction in A549 cells is mediated by surface receptors coxsackie adenovirus receptor (CAR) and integrins ανβ3 or ανβ5 (74-76), MPI-2 cells lack CAR. In these cells, HAdV-C5 uses the scavenger receptor SR-A6 as a primary receptor (59). As shown in Fig. 5B, transduction efficiency between the FG34_2 and HD30 was comparable, whereas HD36 transduction was moderately less efficient.
A critical late step in HAdV-C5 entry is the E3 ubiquitin ligase Mib1-controlled disassembly of the incoming particles at the nuclear pore complex (39, 47, 77). We analyzed the Mib1 dependency of vector transduction by scoring transduction efficiencies in Mib1-normal and Mib1-KO HeLa-ATCC cells (Fig. 5C). The normalized input virus amounts resulted in comparable transduction efficiencies for FG34_2, HD30 and HD36 in Mib1-normal HeLa-ATCC cells, whereas none of the vectors efficiently transduced the Mib1-KO cells.

**Discussion**

This study assessed the effect of the length of the packaged genome on physical features of HAdV-C5 vectors and vector entry and transduction efficiencies. Our results indicate that small changes in the genome length (~ 94% to 103% of length of wt genome) have no major impact on physical or mechanical features of AdV vectors as assessed by AFM or heat susceptibility. In contrast, the HD30 vector with a 29.8 kbp genome (~ 83% of wt genome) was slightly stiffer and less heat-resistant than the other particles, but had largely comparable entry and transduction efficiency akin to the first generation FG34 (33.7 kbp) and gutless HD36 (35.8 kbp) vectors in different tissue culture cell lines. These findings are in line with earlier reports showing that the composition of the core impacts on the mechanical properties of the AdV, as seen by low pH shift experiments decreasing the stiffness of HAdV-C5 virions (78), or with an HAdV-C5 mutant lacking protein VII and exhibiting increased capsid stiffness (37). The reduced thermostability of HD30 is consistent with data from the literature showing that AdVs with genomes less than ~80% of wt length rapidly disintegrate upon heat treatment (46, 79), whereas genomes larger than 105% or lower than 75% are difficult to propagate as they recombine to attain a normal size (44, 45, 79). In summary, our study emphasizes the versatility of HAdV vector design, but also illustrates that further experiments depicted here and in recent studies (36) are required to assess how the physical and mechanical features of virus particles impact on virus entry mechanisms.

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Conflict of interest

None

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References


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Fig. 1: HAdV-C5 vectors used in the study

A. Schematic representation of HAdV-C5-derived vectors used in the study (not to scale). FG34 and FG37 are first generation vectors, whereas HD30 and HD36 are gutless, helper-dependent vectors. FG34_1, HD30 and HD36 have point mutations in the hexon hyper variable region (HVR) 7 that ablate factor X binding to the particles. FG34_1, FG34_2, HD30
and HD36 contain a GFP expression cassette under the control of cytomegalovirus major immediate early promoter. L-ITR and R-ITR refer to inverted-terminal repeats found at the ends of the linear double-stranded genome of adenoviruses. These ITR-sequences are important for initiation of genome replication. ES refers to the encapsidation (packaging) sequence. FG37 has a modified encapsidation sequence, flanked by LoxP sites. B. Silver stain gel of HAdV-C5_wt, HD30, FG37 and FG34_1 particles. The particles have otherwise similar protein profiles, except that protein V of HAdV-C5_wt migrates slightly faster than protein V in the other particles. The difference is most likely due to HAdV-C5_wt and the vector viruses being derived from different parental HAdV-C5 clones.
Vectors FG34_1 (n=45), HD30 (n=45) and FG37 (n=45) were visualized and mechanically characterized by AFM and compared with HAdV-C5_wt (n=37). Mechanical properties such as stiffness and brittleness were extracted from this study. A. Examples of topographical images before and after mechanical disruption show the same symmetry adsorption (three-fold symmetry) and breaking pattern. B. Height distribution of the viral particles was analysed by tracing profiles in each viral particle (index, left). C. Spring constant analysis was performed by fitting the linear slope of a force vs indentation curve. D. Critical strain was calculated by estimating the ratio between the critical indentation (δ critical) and the height of each viral particle calculated in B (scheme index B right).
Fig. 3: HD30 loses infectivity at lower temperatures than HAdV-C5_wt, FG37, FG34 or HD36

Vectors were incubated for 5 min at indicated temperatures, whereas control viruses were kept at room temperature (RT). Transduction efficiencies after heat-treatment were assessed in human lung carcinoma A549 cells at 25 h pi by widefield microscopy and scored as mean nuclear intensities of GFP per well in the case of FG34_2 (FG34), HD30 and HD36, whereas mean nuclear intensities of mCherry were used for FG37 and immunostaining for the late protein VI for HAdV-C5_wt. Three different input virus amounts representing two-fold dilutions were used (input virus amounts were not normalized between vectors). The two technical replicates are shown separately. The right-hand y-axis shows the number of cells analyzed per sample.
Fig. 4: Analyses of vector uptake, protein VI exposure and particle penetration into the cytoplasm

A and B. Kinetics of vector uptake and protein VI exposure in A549 cells. HAdV-C5_wt particles were used as a control. Atto565-tagged particles were bound to cells at +4°C, and after removing unbound particles, samples were switched to 37°C for the indicated time points. Afterwards, cells were returned to +4°C and particles remaining at the cell surface were tagged by mouse anti-hexon 9C12 antibodies. After fixing and permeabilization of cells, the samples were incubated with rabbit anti-protein VI antibodies, followed by anti-mouse Alexa Fluor 680- and anti-rabbit Alexa Fluor 488-conjugated secondary antibodies. Samples were imaged by confocal microscopy, and images from maximum projections of confocal stacks were analyzed by CellProfiler to quantitate the fraction of cell surface viruses (A) and protein VI exposure efficiencies (B). The graphs show the fraction of particles per
cell remaining at the cell surface at the given time points (A), and the average VI signal on cell-associated viruses (B; AU=arbitrary units). One dot represents one cell. Since the samples of the different timepoints in (B) were analyzed in different imaging sessions, the 0 min and 10 min, or 10 min and 30 min HAdV-C5_wt samples were acquired in each imaging session to better visualize the time-dependent changes in particle-associated protein VI signals. The number of cells and virus particles analyzed for each sample is indicated. Error bars in A and B represent the means ± SEMs. Kolmogorov-Smirnov test was used for statistical analyses. FG34 refers to FG34_2. C. Penetration of incoming virus particles into the cytoplasm of HeLa-Ohio cells. Atto565-tagged virus particles were bound to cells at +4°C, and after removing unbound particles, samples were switched to 37°C for 30 min. Afterwards, samples were returned to +4°C, the plasma membrane was perforated by streptolysin O and cytosolic virus, as well as virus particles still remaining at the cell surface were tagged by mouse anti-hexon 9C12 antibodies. Rabbit anti-Giantin antibodies were used to identify properly perforated cells. After fixation, cells were incubated with secondary anti-mouse Alexa Fluor 488- and anti-rabbit Alexa Fluor 680-conjugated antibodies. The graph shows the fraction of 9C12-positive virus particles per Giantin-positive cells with one dot representing one cell. The 9C12-positive particles represent mainly cytoplasmic viruses, since only a minor fraction of particles remain at the plasma membrane at this time point (A). The number of cells and virus particles analyzed are indicated. Error bars represent means ± standard deviations. Kolmogorov-Smirnov test was used for statistical analyses. Representative images on the right-hand side show that virus particles only in properly permeabilized cells, ie. in Giantin-positive cells, are accessible to anti-hexon 9C12 antibodies. In the images virus particles and Giantin are pseudocolored red, whereas the hexon signal is shown in green. Nuclei were stained with DAPI and are shown in blue. Scale bar = 10 µm. D. The penetration-deficient HAdV-C2_TS1 virus is largely inaccessible to exogenously added antibodies in streptolysin O-permeabilized cells. The samples were analyzed at 10 min post warming as described in (C), except that the rabbit anti-Alexa Fluor 488 antibody was used for the Alexa Fluor 488-tagged HAdV-C2_TS1 and mouse anti-Giantin antibody was used to identify properly perforated cells.
A and B. Transduction efficiencies of vectors in A549 (A) and murine alveolar macrophage-like MPI-2 (B) cells. FG34 refers to FG34_2. Cells were incubated with normalized input vector amounts for 60 min, after which unbound particles were removed and incubation was continued for additional 23 h until cells were fixed and imaged by widefield microscopy. Transduction efficiencies are given as an infection index, i.e. the fraction of GFP-positive cells over total number of cells analyzed. V1, v2 and v3 refer to two-fold dilutions of input virus. The two technical replicates in A are shown separately. The number of cells analyzed is indicated on the right-hand y-axis. Representative images from the v1-input virus infection.
are shown on the right-hand side with GFP-positive cells shown in green and DAPI-stained nuclei in blue. Scale bar = 50 µm. C. Mib1 is required for HD30 and HD36 transduction, similar to FG34_2. Hela-ATCC parental cells and HeLa-ATCC cells deficient of Mib1 (Mib1-KO) were incubated with the vectors for 60 min, after which virus inocula were removed and incubation was continued for additional 23.5 h before fixation. Infection efficiency was scored by infection index. The two technical replicates are shown separately. V1 and v2 refer to two-fold dilutions of input virus.