Stepwise loss of fluorescent core protein V from human adenovirus during entry into cells

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Running head: Stepwise uncoating of a fluorescent DNA-core tagged virus

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Abstract

Human adenoviruses (HAdVs, short Ad) replicate and assemble particles in the nucleus. They organise a linear double-strand DNA-genome into a condensed core with about 180 nucleosomes by the viral protein VII (pVII), pX, and pV attaching the DNA to the capsid. Using reverse genetics we generated a novel, non-conditionally replicating Ad reporter by inserting green fluorescent protein (GFP) at the amino-terminus of pV. Purified Ad2-GFP-pV virions had an oversized complete genome, and incorporated about 38 GFP-pV molecules per virion, about 25% of Ad2 pV. GFP-pV cofractionated with the DNA-core like pV, and newly synthesized GFP-pV had a subcellular localization indistinguishable from pV, indicating that GFP-pV is a valid reporter for pV. Ad2-GFP-pV completed the replication cycle, although at lower yields than Ad2. Incoming GFP-pV (or pV) was not imported into the nucleus. Virions lost GFP-pV at two points during the infection process, entry into the cytosol, and at the nuclear pore complex, where capsids disassemble. Disassembled capsids, positive for the conformation specific anti-hexon antibody R70, were devoid of GFP-pV. The loss of GFP-pV was reduced by the macrolide antibiotic leptomycin B (LMB), which blocks nuclear export and adenovirus attachment to the nuclear pore complex. LMB inhibited the appearance of R70 epitopes on Ad2 and Ad2-GFP-pV, indicating that the loss of GFP-pV from Ad2-GFP-pV is an authentic step in the adenovirus uncoating program. Ad2-GFP-pV is genetically complete, and hence enables detailed analyses of infection and spreading dynamics in cells and model organisms, or assessment of oncolytic adenoviral potential.
**Introduction**

DNA-viruses and retroviruses maintain and replicate their genomes in host cell nuclei using histone-based nucleosomes, similar to chromatin, or they encode their own DNA-binding and organizing proteins (35, 46, 48). They assemble and maintain their genomes in different chromatin states by packaging the nucleic acids into proteinaceous capsids and sometimes lipid envelopes, and thereby traffic their genome within and transmit it between cells (8, 42). The SV40 polyomavirus, for example, packages its virion DNA with cellular core histones and uses histones to replicate in infected nuclei (20). Herpesviruses, on the other hand, condense their double-strand DNA in particles with the help of polyamines, and use histones during latent residence within infected nuclei, or use irregularly spaced nucleosomes during productive phases of infection (46).

Adenoviruses replicate and assemble particles in the nucleus. They encode their own histone-like proteins to condense a linear double-strand DNA genome of about 36 kbp into a proteinaceous DNA-core. Although it is unknown how the viral DNA is precisely organised in the virion, isolated cores of species C human adenovirus serotypes 2 or 5 (HAdV2/5, short Ad2/5) contain six viral proteins, the basic proteins V, VII and X (pV, pVII, pX), the terminal protein covalently attached to the 5’ ends of the DNA, and a small number of protein IVα2 and L3/p23 protease, which are involved in DNA encapsidation and transcriptional regulation, or virion processing (reviewed in 4, 49). Proteins V, VII and X are tightly interconnected as indicated by chemical cross-linking experiments (12). Digestion of virion cores with staphylococcal nuclease combined with electron microscopy (EM) analyses and stoichiometric calculations suggested a model where the viral DNA is organised into approximately 180 nucleosome-like units by three subunits of dimeric pVII interspersed with one copy of pV (5, 11, 14, 41, 57, 64). This model predicts 1080 copies of pVII and 180 copies of pV, which is 10-20% higher than the experimentally determined amounts of pVII and pV in isolated virion DNA (34, 63), suggesting that there are stretches in the viral genome lacking pVII and pV. pV not only binds the viral DNA in a sequence independent manner, it also bridges the DNA-core and the capsid by interacting with pVI on the inner side of the major capsid protein hexon (12,
Interestingly, a pV-deleted Ad5 gave rise to low levels of viral particles, suggesting that pV was involved in the assembly of infectious virions (61). Mutations in the gene encoding the precursor of pX could compensate for the lack of pV suggesting redundancy for core organisation by molecular adaptation. This is also supported by the notion that pV is specific for Mastadenoviruses, which exclusively infect mammals (http://www.vmri.hu/~harrach/ADENOSEQ.HTM).

Viral infections start with entry, which delivers subviral particles to the cytosol. Invariably, the condensed viral genomes have to be uncoated for infection to proceed (23). The uncoating process of Ad2/5 starts at the plasma membrane, when virion fibers bind their primary receptor, the coxsackievirus Ad receptor CAR, and alpha v integrin coreceptors bind to penton base, which anchors fiber to the capsid (3, 8, 39, 67). Ad2/5 release their fibers prior to or during endocytosis although the underlying mechanism is unknown (27, 43). An early step of Ad2/5 uncoating is sensitive to defensins, which bind virions and preclude viral escape from endosomes to the cytosol (45), phenotypically mimicking the fate of the uncoating-impaired and escape-defective Ad2 mutant Ad2-ts1 (22, 31). The cytosolic Ad2/5 capsids traffic on microtubules to the nuclear pore complex (NPC), where they bind to the CAN/Nup214 receptor, and release their genome into the nucleus (25). This process is sensitive to the macrolide antibiotic leptomycin B (LMB), which inhibits nuclear protein export and prevents the attachment of incoming virions to the nuclear pore complex (56). The precise composition of the imported DNA is unknown (reviewed in 48). It is known, however, that pVII remains with the incoming viral genome in the nucleus during the early infection phase (65, 68). pVII together with pV then assembles newly synthesised viral DNA into core structures that are packaged into virions and released upon nuclear disintegration (reviewed in 4). Here we report the generation and initial characterization of a novel adenovirus, which expresses the GFP-pV fusion protein instead of pV under the endogenous viral promotor. This is the first small DNA-tumor virus with a fluorescent full length core.
Materials

Construction of Ad2-GFP-pV

Ad2_BAC53 is derived from Ad2 reference strain "adenoid 6" (31). The Ad2-GFP-pV insertion mutant was obtained using exponon mutagenesis as described (50) (see Fig. 1). Briefly, in the first step a mini-Tn cassette (transprimer-1) was amplified from pGPS1.1 and inserted by ET recombination into BAC53 containing the full length wild type genome of Ad2 (31). Synthetic oligonucleotide primers for insertion of transprimer-1 contained at their 5' end homologies to position 16491-16538 (upstream of pV) and 16539-16586 (downstream) relative to the adenoviral genome. The priming sequences for the transprimer-1 cassette of pGPS1.1 were as published (50). Successful insertion of transprimer-1 was examined by restriction analysis and partial sequencing of the insertion sites. To replace the transprimer-1 element with eGFP coding sequences recombinant BAC-DNA was amplified from two independent clones and transprimer-1 sequences were excised in vitro using TnsABC (New England Biolabs) in the presence of the acceptor plasmid pST76T. The insertion fragment containing the ORF of eGFP was obtained as follows. The eGFP gene including a small linker portion was PCR amplified from pEGFP-C1 (Clontech). Priming regions were designed such that the fragment contained position 613-1380 of the plasmid. The 5' extensions contain the recognition site for SapI, one random base plus 3 bases identical to the upstream or downstream region of the insertion site. Primer sequences were as follows: upstream primer (5'-GTC AGC TCT TCC GCT ATG GTG AGC AAG GGC GAG-3'), downstream primer (5'-CAA GGC TCT TCT CAT GGT ACC GTC GAC TGC AGA ATT C-3'). The SapI excised insertion fragment was ligated into the TnsABC* linearised BAC and the recombinants were recovered by transformation into competent bacteria. Successful replacement of pV by GFP-pV was confirmed by restriction analysis and partial sequencing of the insertion sites. Four independent colonies were chosen and amplified for reconstitution of virus by calcium phosphate transfection of 80% confluent HER-911 cells in a 10 cm cell culture dish with 10 μg SnaBI linearised BAC DNA. 48 h post-transfection cells were split 1 to 4, and viruses harvested from cells and supernatant at onset of cytopathic effects (passage 1, p1), serially passaged in four 10 cm dishes of A549 cells (p2), and then in twenty 10 cm dishes (p3), followed by virus purification from cells. The
supernatants were used for further amplification rounds until p7. Viruses (termed Ad2-GFP-pV) from passages lower than 8 had normal DNA patterns of restriction enzyme digests.

**Cells, transfection and antibodies**

HeLa-ATCC and human lung carcinoma A549 cells were purchased from American Type Culture Collection, Rockville, MD. Ad5-E1 transfected human embryonic retinoblast 911 cells (19) were obtained from Dr. S. Hemmi (University of Zurich, Switzerland). Cells were grown as monolayers in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO-BRL) on alcian blue-coated glass coverslips (58) or cell culture dishes. Plasmid POM121-mCherry (obtained from Dr. Daniel Gerlich, ETH Zurich) was transfected into 50% confluent cells using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacture’s protocol. Mouse anti-GFP was purchased from Roche (Roche Diagnostics (Schweiz) AG, Rotkreuz, Switzerland). Rabbit anti-hexon R70 antiserum was obtained from the late Marshall Horwitz (2). Rabbit anti-protein V serum was obtained from W.C. Russell (University of St Andrews, UK, 37) and rabbit anti-protein VII antibody from L. Gerace (The Scripps Research Institute, La Jolla, USA). Guinea Pig anti-DBP (DNA Binding Protein) was from W. Deppert (University of Hamburg, Germany). Purified rabbit anti-pV C-term antibody was manufactured by immunising rabbits with the synthetic peptide RRVAREGGRTLVLPTAR, position 347-363 of Ad2 pV. Serum was harvested and an enriched IgG fraction was affinity purified on immobilised peptide (Davids Biotechnologie, Regensburg, Germany). All animal work was done in accordance with NIH standards for animal welfare under the animal welfare number A5646-01, and approved by the Animal Care and Use Committee, Office for Environment, Nature and Consumer Protection (Regensburg, Germany).

**Amplification, purification and labeling of viruses**

Ad2 and Ad2-GFP-pV were grown, isolated and labelled with Atto647 and Atto565 dyes (Atto-tec, Germany) as described (26, 44).
Virus quantification

Virus titers were determined using either tissue culture infective dose 50 (TCID\textsubscript{50}) or a modified fluorescent focus assay (FFA). In both assays virus was titrated on HER-911 cells plated on 96-well plates. In case of TCID\textsubscript{50} infection was allowed to proceed for 6 days while in the FFA cells were fixed after 4 days. For the calculation of fluorescent focus forming units per ml (fffu/ml) cells were fixed, quenched, immunostained for expression of protein V and fluorescent focus units counted using a fluorescence microscope. A fluorescent focus was defined as 3 or more adjacent cells expressing protein V or GFP-pV. FFA titers were calculated using the formula of Spearman and Kaerber (54). Protein concentrations of purified viruses were measured using the Micro BCATM Protein Assay Reagent Kit (Pierce, Thermo Fisher Scientific, Switzerland).

E1A measurement

50% confluent A549 cells in 96-well plates were infected with serial dilutions of 1 µg Ad2 or Ad2-GFP-pV in growth medium for 12, 16 or 20 h. Cells were fixed, immunostained with mouse anti-E1A (28) and goat anti-mouse Alexa 594 (1/500), DAPI stained and recorded with the ZEISS Axiovert 40 CFL fluorescence microscope using suitable filters. The ratio between E1A expressing cells and DAPI positive nuclei was calculated and plotted against the virus dilution. Around 150 cells were counted per time point. The experiment was repeated 3 times and standard errors of the mean (SEM) were calculated.

One step growth curves

About 70% confluent A549 cells in 35 mm cell culture dishes were inoculated with 5 fffu of either Ad2 or Ad2-GFP-pV in a volume of 1 ml RPMI 0.2% BSA at 4°C on a shaker for 1 h. Cells were washed twice with PBS and incubated in 2 ml of growth medium consisting of DMEM/10% FBS/NEA (nonessential amino acids) /PS (penicillin/streptomycin) at 37°C and 5% CO\textsubscript{2} for 0, 3, 12, 24, 48 or 72 h. Supernatant was collected in 2 ml tubes and frozen while cells were broken up in 1 ml medium by freeze-thaw 3 times, transferred to 2 ml tubes and supplemented with another ml of
growth medium. Supernatants and cell lysates were centrifuged and viral titers determined on A549 cells.

**Preparation of pyridine cores**

To test if GFP-pV fractionated with the viral core, double CsCl gradient-purified Ad2-GFP-pV (or Ad2) virions were dialysed against 5 mM Tris pH 8.1, and 50 μg of each virus supplemented with 16 μl pyridine to 8% (v/v), similar to a previously described protocol (17). Samples were incubated at 37°C for 2 h, followed by centrifugation on a 10-30% sucrose gradient at 111000 x g, 4°C for 2 h. 14 160 μl fractions were collected, TCA precipitated and dissolved in 20 μl sample buffer. Fractions 1-4, 5-8, 9-12 were combined as well as fraction 13 and 14 with the pellet (collectively the pellet fractions), and analysed on a Coomassie stained 12% SDS-PAGE. From the pellet fractions of Ad2, a Western blot was prepared and immunostained with affinity-purified anti-pV antibody (1:200), and for Ad2-GFP-pV with anti-GFP antibody (1:1000).

**Thermostability assay**

Accessibility of viral DNA from double CsCl gradient purified Ad2 or Ad2-GFP-pV for the DNA intercalating fluorescent dye TOTO-3 iodide (1 mM solution in DMSO, Invitrogen) with 642/660nm excitation/emission was measured after heat-shock treatment at different temperatures. Specifically, samples containing 5 μg virus diluted in buffer containing 10 mM Tris pH 8.1, 150 mM NaCl, 1 mM MgCl and 1 μM TOTO-3 in a total volume of 50 μl were prepared in 1.5 ml Eppendorf tubes on ice, heated at indicated temperatures in a heating block for 3 min and rapidly chilled on ice. The samples were transferred to a 96-well plate and fluorescence was measured at 642±8 nm excitation and 660 ± 9 nm emission with the Tecan SAFIRE II microplate reader.

**SDS-PAGE, Western blots, fluorography and protein quantification**

SDS-PAGE gels were run with a Hoefer minigel device according to the manufacturer’s instructions, and stained with Coomassie Brilliant Blue. Pictures were recorded either directly with the Syngene G:BOX gel documentation system (BIOLABO Scientific
Instruments SA, Chatel-St-Denis, Switzerland) under white-light conditions or scanned after drying on filter paper. A431 human epithelial carcinoma cell lysate was purchased from BD Transduction Laboratories (Lexington, KY, USA). For Western Blots, SDS-PAGE gels were electro-transferred in semi-dry mode onto Millipore Immobilon-P SQ 0.2 μm PVDF transfer membranes (Milian SA, Geneve, Switzerland), and developed with the Amersham ECL Plus Western Blotting Detection kit (GE Healthcare Life Sciences, Glattbrugg, Switzerland). Chemiluminescence was recorded either with the Kodak Digital Science Image Station 440CF or by exposing the membranes onto Amersham Hyperfilm ECL films (GE Healthcare Life Sciences, Glattbrugg, Switzerland). Fluorography of Atto647-Ad2-GFP-pV after 12% SDS-PAGE was recorded with the Amersham Typhoon 9400 gel scanner using the 633 nm Helium-Neon laser.

Protein bands from Ad2 and Ad2-GFP-pV (5 µg each) on Coomassie-stained SDS-gel were quantified with the MacBiophotonics ImageJ open source software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2010). Briefly, a region of interest (ROI) was drawn around the protein band of interest and the integrated density of the selection calculated. A background ROI with the same dimensions near the band was chosen and the integrated density subtracted from the protein band ROI. The protein amount was calculated relative to a BSA standard curve using the background subtracted total intensity of each bands. The number of molecules was determined by dividing the protein amount by the theoretical molecular mass of the protein. The copy number of an individual protein per virion was calculated relative to hexon (720 copies per virion). Calculated copy numbers were the mean of three independent experiments including SEM. Optical densities shown were derived from the same representative experiment. Molecular weights given in Dalton and the pl values were calculated with the “Compute pl/Mw tool” (http://www.expasy.ch/tools/pi_tool.html) from the Swiss Institute of Bioinformatics ExPASy Proteomics Server. Sequences used were from the UniProtKB/Swiss-Prot database: P03277 (pII), P03276 (pIII), P03267 (pV), P03274 (pVI) and P68950 (pVII). In case of precursor proteins the mature form of the corresponding proteins was used for calculation. Values for copy numbers (structural) and copy numbers (35S metabolic labeling) were obtained from the literature (63).
Single particle fluorescence analysis of Atto647-Ad2-GFP-pV

Atto647-Ad2-GFP-pV was adsorbed to the bottom of 96-well clear bottom plates at 20°C for 15 min, washed with PBS, and overlaid with 100 μl fresh PBS. Images were recorded with the automated ImageXpress\textsuperscript{MICRO} fluorescence microscope (Molecular Devices) using settings for Atto647 and GFP. Images were processed as described in supplemental methods. Briefly, images were corrected for background and aberration and single virus particles were scored. Average fluorescence intensities within the selected particle areas in the Atto647 and GFP channels were measured. For determination of background threshold 10 random ROIs which contained no virus particles were chosen using MacBiophotonics ImageJ and the average fluorescence intensity in the Atto647 or GFP channels calculated. The background thresholds are represented as blue lines in Fig. 3D. Population statistics were performed with Microsoft Office Excel software. The average intensity per particle (GFP and Atto647) and calculated ratios of average GFP/Atto647 intensities were plotted against the number of particles.

Kinetics of GFP-pV release and pVII epitope exposure of Atto647-Ad2-GFP-pV particles during infection

HER-911 cells were seeded to 60% confluency onto alician blue coated coverslips in 24-well cell culture dishes overnight. Growth medium was replaced by serum-free medium, and cells were starved overnight to reduce cell background. Cells were infected with 0.1 μg/ml Atto647-Ad2-GFP-pV at 37°C 5% CO\textsubscript{2} for 10 min, washed briefly and supplemented with 0.5 ml serum free medium. 0 min samples were immediately fixed with 3% PFA-PBS for 15 minutes, washed and quenched for 10 min with 25 mM NH\textsubscript{4}Cl in PBS and permeabilised with 0.5% Triton X-100 in PBS for 10 min. Other samples were allowed to proceed for 30, 90, 150 or 240 min post-addition of virus. Cells were blocked with 20% goat serum and stained against pVII with preadsorbed anti-pVII/Alexa594 goat anti-rabbit and DAPI. Images were recorded on the same day with an inverted Leica SP5 single-point confocal microscope (Leica Microsystems, Switzerland) equipped with a 63x (oil immersion, NA 1.4) objective. Excitations were at 405 nm (DAPI), 488 nm
(GFP), 594 nm (pVII) and 633 nm (Atto647). Individual stacks were recorded with 0.21 μm intervals using 10x accumulation and 4x averaging. Maximum projections were generated with MacBiophotonics ImageJ. A Matlab-based routine (for details see supplementary information) was used to score individual viral particles and measure average fluorescence intensities in the particle areas from the Atto647 (capsid), GFP (GFP-pV) or Alexa594 (pVII) channels. Threshold values for fluorescence intensities were obtained by subtracting the average intensity of the entire image calculated using MacBiophotonics ImageJ from individual average intensities of single virus particles. Statistics were performed with Microsoft Office Excel. The total numbers of scored particles and average fluorescence intensities in the Atto647 channel (capsid) were plotted against time. Average GFP fluorescence intensities in scored (Atto647) particles were plotted against time as well as GFP/Atto647 average fluorescence ratios of single particles. The population of particles with pVII intensities over threshold (Atto647 and pVII positive Ad2) was determined and the ratio of this population relative to the total amount of scored (Atto647) particles plotted against time. From the same population average pVII fluorescence intensities were calculated and plotted against time, or average GFP fluorescence were calculated and plotted against time.

**GFP-pV dissociation from Ad2-GFP-pV**

HER-911 cells were plated on 96-well clear bottom plates to 30% confluency in 75 μl DMEM/10% FBS/1% NEA/1% PS plus 25 μl Optimem and incubated for 3 days at 37°C and 5% CO₂. For background controls medium was added to wells containing no cells. Cells were supplemented with 10 μl LMB dissolved in DMEM/0.2% BSA/1% PS to a final concentration of 20 nM or were MOCK treated 1 h prior to infection. LMB was present during the whole experiment. Atto647-labelled Ad2-GFP-pV (0.125 μg, centrifuged for 10 min at 10000 rpm in an Eppendorf 5415 R centrifuge at 4°C prior use) was added in 10 μl DMEM/0.2% BSA/1% PS for 15 min. Medium was removed and fresh DMEM/0.2% BSA/1% PS was added. 30 or 150 min p.i. 25 μl 16% PFA in PBS was added for 10 min, cells were washed 2 times with PBS and quenched with 25 mM NH₄Cl in PBS for 10 minutes followed by another washing step and treatment with 0.5% TX-100 in PBS containing 0.5 μg/ml DAPI for 10 min. Cells were then washed extensively and 100 μl PBS-N₃ was added prior to recording.
Fluorescence was recorded with the automated ImageXpress \textsuperscript{MICRO} fluorescence microscope (Molecular Devices) using a Nikon 40x air objective NA 0.95, Semrock BrightLine\textsuperscript{®} filters (GFP-3035B-NTE-ZERO, Cy5-4040A-NTE-ZERO and DAPI-5060B-NTE-ZERO) and the Molecular Devices MetaXpress 2 software. 9 individual regions per well were recorded as stacks comprised of 9 sections with a z-distance of 1 µm and images were saved as 16 bit TIFFs. Illumination times were 6 seconds for GFP, 8 seconds for Atto647 and 5 ms for DAPI without binning. Images were processed as described in supplementary information. Briefly, images were corrected for background and aberration. Maximum projections were generated and single viral particles were scored in the Atto647 channel. Average fluorescence intensities in selected particle areas in the GFP and Atto647 channels were determined and GFP/Atto647 intensity ratios calculated for all scored particles individually. Average GFP/Atto647 intensities were plotted against time, and statistics performed with Microsoft Office Excel, including SEM calculated with n = 9.

**Localisation of GFP-pV and pV relative to DBP and characterisation of the anti-pV antibody**

HeLa-ATCC cells were infected with Ad2 or Ad2-GFP-pV at moi 2 for 20 h or left non-infected. They were fixed, quenched, Triton X-100 treated and blocked for 1 h with 20% goat serum in PBS. DNA binding protein (DBP) was immunostained with a guinea pig anti-DBP antibody, pV with an affinity purified rabbit anti-pV antibody and the cell nucleus with DAPI. Purified Ad2 or Ad2-ts1 and cell lysates from Ad2 infected cells (3, 18 and 30 h) or non-infected HeLa-ATCC cells grown on cell culture dishes were fractionated by 12% SDS-PAGE, blotted and stained with affinity purified rabbit anti-pV antibody followed by goat anti-rabbit conjugated horse radish peroxidase staining.
Results

Genetic construction and reconstitution of Ad2-GFP-pV

We generated recombinant Ad2 genomic DNA with an N-terminally eGFP-tagged core protein V (GFP-pV) using exposon mutagenesis of Ad2_BAC53, yielding Ad2-GFP-pV (Fig. 1A, B). The resulting bacterial artificial chromosome (BAC) DNA had the expected in silico predicted restriction fragments and correct sequence between GFP and pV (Fig. 1C, not shown). Linearised BAC_Ad2-GFP-pV DNA was transfected into HER-911 cells, and infectious particles were serially expanded in A549 cells through about 5 passages, and purified by double CsCl density gradient as described (27). XhoI restriction enzyme digests of DNA from these particles yielded the expected fragments (Fig. 1B). This suggested that Ad2-GFP-pV was stable for at least 5 passages. At passages higher than 10, loss of GFP-pV expression by Ad2-GFP-pV was, however, observed and this coincided with genetic alterations in the pV locus (not shown).

Ad2-GFP-pV particles contain GFP-pV and are infectious

We next analysed the protein composition of CsCl-gradient purified Ad2-GFP-pV. SDS-PAGE and Coomassie blue analyses indicated that Ad2-GFP-pV had a protein band of about 75 kDa, which was absent in Ad2, and lacked a prominent band at about 48 kDa (Fig. 2A). Both of these bands were immunostained by an anti-pV antibody in Western blots, but only the 75 kDa band of Ad2-GFP-pV also reacted with an anti-GFP antibody (Fig. 2B). This corresponded well with the calculated mass for this fusion protein, which is 70.303 kDa (Fig. 2C). The other major structural proteins of Ad2-GFP-pV and Ad2 were normal in SDS-PAGE (Fig. 2A). The relative abundance of the viral structural proteins was estimated by densitometry using Coomassie blue stained protein II (hexon, pl = 5.1) as an internal reference and bovine serum albumin (BSA, pl = 5.8, http://expasy.org/tools/) as a calibration standard (Fig. 2C, D). Each virion contains 720 copies of hexon, as indicated by structural and metabolic labeling analyses (55, 63). In our Coomassie blue analyses we found 63 +/-5 copies of pIII (penton base, pl = 5.3, expected copy number 60), and 316 +/- 92 copies of pVI (pl = 9.6, expected copy number 360) per virion, which agrees well with the expected values. The copy numbers
of pVII and pV had been estimated by biochemical assays or reversed phase high performance liquid chromatography to be 833 ± 33 or 633 ± 59 for pVII, and 157 ± 1 or 170 ± 15 for pV, respectively (34, 63). The basic proteins pV (pI = 10.3) and pVII (pI = 12.3, expected copy number 833) were overestimated by a factor of 1.5 and 3.3, respectively, compared to metabolic labeling (63). This was expected from the fact that Coomassie blue attaches better to highly positively charged proteins than to neutral proteins (59). Coomassie blue analyses indicated 38 ± 6 copies of GFP-pV (pI = 9.5) per virion, which is about 25% of the pV levels in Ad2. Importantly, both GFP-pV and Ad2 pV segregated with the viral DNA core in pyridine-disrupted virions fractionated in sucrose density gradients, although we noticed some proteolysis in both of the pelleted core fractions (Fig. 2E, F).

Since pV links the viral DNA to the inside wall of the capsid (37), we determined the thermostability of Ad2-GFP-pV particles by spectrofluorometric measurements of the binding of the DNA-intercalating dye TOTO-3 to isolated virions (Fig. 2G). TOTO-3 binding to both Ad2-GFP-pV and Ad2 was constant in the range of 25° to 47°C, and sharply increased from 47 to 51°C where it reached a plateau at about 60°C. However, about 10-15% of the Ad2-GFP-pV particles bound the dye at 25°C, indicative of defective particles in the preparation, which could in part explain that 15-20% of the Ad2-GFP-pV particles bound to cells but did not endocytose (see Fig. 4A).

**Ad2-GFP-pV enters cells but is attenuated at particle production**

To track the incoming GFP-pV, we isolated Ad2-GFP-pV particles and labeled the capsids with the fluorophore Atto647 according to previously established protocols (58). The Atto647 dye was found to be specifically incorporated into the major capid protein hexon, as indicated by fluorography of SDS-PAGE fractionated virions (Fig. 3A). Purified Ad2-GFP-pV virions labeled with red Atto fluorophores retained their full infectivity compared to the non-labeled virions (not shown). Both, Atto-647-Ad2-GFP-pV and Ad2 were monodisperse as shown by negative-stain EM (Fig. 3B). Dual color fluorescence microscopy indicated that most of the Atto-647-Ad2-GFP-pV particles were positive for both colors (Fig. 3C, arrows) with Gaussian distributions for both colors, indicating the
presence of a single species of dual-colored particles (Fig. 3D). Non-labeled Ad2 or Ad2-GFP-pV gave no signals in the red and green, or red channels, respectively, indicating specificity of detection (data not shown). Importantly, the Atto-647-Ad2-GFP-pV particles were stable in PBS at 37°C up to 150 min, indicating that these particles were suitable for analyses of entry into cells (Fig. 3E, F). Interestingly, Atto-647-Ad2-GFP-pV particles lost about 50% of GFP-pV upon short incubation at 53°C, which was, however, not due to thermal GFP denaturation as shown before (1). The loss of GFP-pV correlated with a sharp increase in DNA accessibility at 51°C, and represented rupture of particles (Fig. 2G).

Entry of Ad2-GFP-pV particles into HeLa-ATCC cells was analysed by transmission EM. Viral particle counts at the plasma membrane, endosomes and the cytosol indicated that Ad2-GFP-pV endocytosis occurred with similar kinetics as wild type Ad2, although 15-20% of the Ad2-GFP-pV particles were not endocytosis-competent (Fig. 4A). At 90 min pi, about 50% of the Ad2-GFP-pV particles were in the cytosol, 30% in endosomes and 20% at the plasma membrane. Endosomal escape of Ad2-GFP-pV was confirmed by fluid phase marker dextran-FITC (10 kDa) uptake showing that Ad2-GFP-pV infection not only triggered dextran uptake but also release to the cytosol and the nucleoplasm, similar to Ad2 (data not shown, 38).

Ad2-GFP-pV was delayed at expressing the immediate early transactivator E1A compared to Ad2 (Fig. 4B). This was more pronounced at 12 h than 20 h pi, suggesting that Ad2-GFP-pV could compensate a defect with time, consistent with the notion that Ad2-GFP-pV had a lower infectious particle to particle ratio than Ad2. In lung epithelial A549 cells, Ad2-GFP-pV gave 10 to 100-fold lower yields of cell-associated infectious particles compared to Ad2, although the amounts of cell-free infectious units were similar to Ad2 at 72 h pi (Fig. 4C). These results were confirmed by measurements of tissue culture infectious dose 50 (TCID50) and fluorescent focus forming units (fffu) (Fig. 4D). We speculate that Ad2-GFP-pV is slightly impaired at particle assembly. Importantly, both newly synthesised GFP-pV and pV were found in similar subnuclear structures
lacking the viral DNA binding protein (DBP) (Fig. 5A), as indicated by an anti-pV polyclonal antibody (Fig. 5B, C).

**GFP-pV dissociates from Ad2-GFP-pV particles during entry**

We next analysed entry of Ad2-GFP-pV and the major core protein pVII into cells by confocal microscopy in live and fixed cells. Spinning disc confocal microscopy indicated progressive attachment of dual-color Atto565-labeled Ad2-GFP-pV to the periphery of HER-911 cells (Fig. S1, and Suppl. Mov. 1). The number and average intensity of the Atto647-labeled capsid (representing hexon, see Fig. 3A) remained approximately constant from 0 until 240 min pi, indicating that incoming virions were neither degraded nor released from the cells, consistent with earlier findings (Fig. 5A, B, 26, 60).

In contrast to the Atto647 fluorescence of the main capsid protein hexon, the GFP-pV fluorescence of Atto647-labeled virions dropped in a two step process. A rapid loss to about 35% of the original value was seen at 30 min pi, and the remaining GFP-pV disappeared at a slower rate to almost background levels at 90 min pi (Fig. 6A-C, column 2). Individual GFP-pV puncta distinct from capsids were occasionally found at 30 min pi but not at 0 min pi, suggesting that GFP-pV separated or was degraded from capsids during cell-binding, endocytosis or escape from endosomes. The cytoplasmic GFP-pV puncta were not stained with an anti-pVII antibody (Fig. 6A), although this antibody detected a small percentage of cell-bound Ad2-GFP-pV at 0 min pi (Fig. 6D), suggesting that these surface-bound capsids, but not the intracellular GFP-pV puncta contained viral DNA. We speculate that the pVII-positive particles at 0 min pi (Fig. 6A) belong to the 10-15% DNA-dye positive Ad2-GFP-pV particles detected in the virus inoculum (Fig. 2G). The pVII-positive particles were faintly positive for GFP-pV at 0 min but not 30 min pi, suggesting that GFP-pV disappeared from the broken particles (Fig. 6E).

In addition, the average pVII fluorescence of the Atto647-capsid population steadily decreased from 0 to 240 min pi, suggesting that pVII is lost in a single phase (Fig. 6D), in contrast to GFP-pV, which dissociates in two phases. Remarkably, the number of pVII-
positive Atto647-capsids transiently increased to about 11% at 90 min and decreased again after 150 min pi (Fig. 6D), consistent with ongoing nuclear import of viral genomes. Many of the strongly pVII-positive puncta at the late time points (90-240 min pi) were in the nuclear area, often lacking Atto-647 fluorescence (Fig. 6A, column 4), suggesting that these pVII puncta represented uncoated genomes (24). Since incoming pVII but not capsids are delivered into the nucleus 1 to 3 h pi (24, 56, 68), and pVII is released from the viral DNA in the nucleus by ongoing transcription (13), our data suggest that the nuclear pVII puncta represent infectious incoming genomes, in agreement with recent analyses (65). Remarkably, we did not find GFP-pV on pVII-positive puncta in the nucleus, suggesting that it was lost prior to or during nuclear import of the viral genome.

The second step of GFP-pV dissociation is revealed by treatment of cells with leptomycin B

We noticed that the loss of GFP-pV from capsids correlated with increased capsid staining by the polyclonal anti-hexon antibody R70 from 0 to 150 min pi as indicated by confocal microscopy, and quantifications of R70-positive particles (Fig. 7A, B, E). The R70-positive particles had significantly lower amounts of GFP-pV than the R70-negative particles (Fig. 7C), while the overall number of particles remained fairly constant (Fig. 7D), indicating that loss of GFP-pV correlated with increased R70 epitopes. R70 preferentially labels the hexon protein of disassembled virus particles (60). This was confirmed by Western blotting, where R70 reacted with newly synthesized SDS-denatured monomeric hexon, and monomeric and aggregated hexon from Ad2 and Ad5 particles (Fig. 8A). By immunofluorescence analyses, R70 recognized hexon from Ad2 infected control HeLa cells but not Ad2-infected cells treated with the nuclear export inhibitor leptomycin B (LMB) (see Fig. 8B,). LMB confines the incoming Ad2/5 particles to the cytoplasm of HeLa cells or blocks them at the microtubule-organizing center in other cell types, such as human embryonic retinoblast (HER) 911 cells, and prevents Ad2/5 attachment to the nuclear pore complex, and nuclear import of pVII (Fig. 8B, 56). This suggested that R70 epitopes, and hence the second step of GFP-pV dissociation depend on virus contact with the nuclear pore complex, which is the site where capsids disassemble and release the DNA (60).
This notion was confirmed by confocal microscopy of LMB-treated HER-911 cells infected with Atto647 labeled Ad2-GFP-pV (Fig. 9). As expected, the particles readily enriched at a perinuclear spot, which was identified as the MTOC by gamma-tubulin staining (not shown, 56), from 15 min up to 150 min pi. These perinuclear viruses were strongly positive for GFP-pV, but did not contain R70 epitopes, indicating that they were not disassembled but contained DNA-cores. Likewise, LMB treatment of HeLa cells blocked the dissociation of GFP-pV as indicated by ratiometric analyses of GFP-pV / Atto647 fluorescence in image stacks (Fig. 10). These images were recorded by automated fluorescence microscopy at constant illumination and with chromatic aberrations corrections (Fig. S2). The ratiometric analyses showed that GFP-pV / Atto647 was 0.105 at 30 min pi, and dropped to 0.085 at 150 min pi, close to background levels (Fig. 10A, B, and not shown). At 0 min pi it was approximately 0.2, which closely matched the ratio of GFP-pV / Atto647 determined by single slice confocal microscopy (see Fig. 6C, insert). The GFP-pV / Atto647 ratios at 150 min pi but not 30 min pi were largely and significantly increased by LMB (Fig. 10B), showing that the second step of GFP-pV loss is affected by LMB. LMB blocks virus attachment at the nuclear pore complex (56), which suggests that the second step of GFP-pV loss requires virus contact with the nuclear pore complex. This notion was corroborated by the detection of Atto647-Ad2-GFP-pV particles in single slice confocal micrographs in close proximity of POM121-mCherry-labeled nuclear pore complexes (16) (Fig. 11, white arrows). Together, these results show that the early detachment of GFP-pV occurs before or shortly after virus penetration into the cytosol, while the second step takes place at the nuclear membrane, presumably the nuclear pore complex. The Ad2-GFP-pV is hence a novel tool to analyse distinct steps of capsid uncoating.

**Discussion**

Imaging the subcellular localizations of viral DNA, RNA, proteins or particles at high resolution has greatly advanced concepts in molecular virology and cell biology. It has
been significantly enhanced by labeling of viruses with chemical fluorophores or fluorescent proteins (reviewed in, 6, 8, 25). For adenoviruses, GFP had been fused to the capsid-stabilizing protein pIX (240 copies per virion, 52), and purified virions were observed at the plasma membrane and the cytoplasm (40). In addition, an adenovirus encoding pV-GFP, pre-pVII-GFP and a dual color Ad5 with pIX-RFP and pV-GFP were also reported (33, 62). The utility of these viruses was, however, limited. Both pre-pVII-GFP and pV-GFP were expressed from an artificial promoter in the deleted E3 region of the genome, while endogenous pre-pVII and pV were also expressed, which resulted in low incorporation of pV-GFP in virus particles (62). In addition, the pV-GFP expressing Ad5 stocks contained significant amounts of disrupted virions (33). The pIX-RFP / pV-GFP labeled virions on the other hand were heterogeneous with respect to green and red fluorescence, and it remained unknown how these viruses enter or exit cells.

Our detailed entry studies here revealed two new phases of virus uncoating. The first phase was the loss of a large fraction of GFP-pV from the particles at 30 min pi, when the majority of endocytosis-competent Ad2-GFP-pV has arrived in the cytosol (Fig. 6C). We suggest that this early loss of GFP-pV coincides with structural changes of the capsid, such as the loss of fibers and pentons, or capsid-stabilizing proteins IIIa and VIII (18, 27, 43, 51). We assume that these disassembly steps occur in most of the entry-competent Ad2-GFP-pV, as shown for Ad2 before (27). Biochemical experiments and crosslinking studies have suggested that pV attaches the viral DNA to the capsid possibly via pVI (12, 37). pVI has recently been assigned to hexon cavities inside the capsid (47, 53), but precisely how the 360 copies of pVI are arranged within the capsid is unknown. Intriguingly, unassigned structures around the 5-fold symmetry of the vertices projecting towards the DNA-core have been detected in cryo-EM studies, and these structures have been tentatively assigned to pVI (18, 55). Since dimers of pVI bind pV in biochemical assays, it is possible that pV acts as a DNA-organizer near the vertices. pV could be released when penton base or fibers detach from the capsid, either together or separately from pVI and penton base.
The second novel phase for adenovirus uncoating identified here was that about 30% of GFP-pV dissociates from the cytoplasmic particles in the absence of LMB, because LMB blocked the release of 30% of the GFP-pV from cytoplasmic particles (Fig. 6). LMB blocks the attachment of incoming Ad2/5 to the nuclear pore complex and inhibits capsid disintegration (56), consistent with a key role of the nuclear pore complex in the final step of capsid disassembly (60). This suggests that, in addition to linking the viral DNA to the capsid, pV functions in core assembly and organisation, in close contact with the virion DNA, as supported by UV-crosslinking and fractionation studies (7, 10, 17).

Remarkably, the nucleus did not contain GFP-pV (Fig. 6), as reported for wild type pV (30, 33). The absence of pV or GFP-pV on the viral cores in the nucleus is not surprising, since earlier studies showed that the viral DNA and pVII alone can be a template for replication and transcription without pV (9, 29, 68). Possibly, post-translational modifications of the viral core proteins pV and pVII, such as ADP-ribosylation, phosphorylation or acetylation facilitate a relaxation of the core, and thereby aid the detachment of DNA from the capsid (15, 21, 66).

In summary, Ad2-GFP-pV is the first fluorescent DNA-core labeled small DNA tumor virus carrying a full length genome. It has an estimated number of 38 GFP-pV copies per virion compared to 157-170 copies of pV in wild type Ad2 (34, 63), and Ad2-GFP-pV is monodisperse. This allows to use Ad2-GFP-pV as a point source of defined fluorescence for quantitative analyses of GFP in live cell microscopy experiments. Ad2-GFP-pV is also a novel tool to measure viral DNA uncoating, and in future experiments can provide insights into viral spreading dynamics within organisms in disease and therapy.

**Competing interests**

There are no competing interests.
Authors’ contributions

DP carried out the reverse genetics experiments, the biochemical and virological characterizations, and the fluorescence microscopy, ME wrote the MatLab code for single particle analyses and helped with data analyses, ZR helped with design of the reverse genetics experiments, CW performed the experiment in Fig. 8A, SS the immunofluorescence experiment in Fig. 8B, and UFG designed and coordinated the study and wrote the manuscript. All authors read and approved the final manuscript.

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References


Figure Legends

Fig. 1: Construction of Ad2-GFP-pV

A. Schematic overview of the pV and pVII loci of Ad2 (left) and Ad2-GFP-pV (right) with boxed open reading frames for pVII and pV, and nucleotide numbers of the starting and terminating nucleotides.

B. Xhol restriction enzyme analysis of genomic DNA isolated from purified Ad2 and Ad2 GFP-pV with an in silico predicted restriction pattern (right) and molecular weight markers (M) in kbp.

C. DNA sequence analysis and derived amino acid sequence at the GFP-pV junction from Ad2-GFP-pV virion DNA with nucleotide numbers. Dots (…) indicate places where the sequence is not shown.

Fig. 2: Biochemical characterization of Ad2-GFP-pV

A. 12% SDS-PAGE stained with Coomassie brilliant blue, including protein marker (M) with relative mass in kDa. Roman numbers denote viral structural proteins.

B. Anti-pV (left panel) or anti-GFP (right panel) stained Western blot of purified Ad2 and Ad2-GFP-pV, and recombinant GFP with a nuclear export sequence (NES). pII indicates hexon, pIII penton base and pIV fiber.

C. Estimation of copy numbers of viral structural proteins by densitometric analysis of Coomassie blue stained SDS-PAGE. The table shows calculated molecular weight (MW in dalton) values for indicated proteins, the isoelectric point (pI), the copy numbers based on the virus structure (69), the copy numbers estimated by metabolic labeling (63), the measured optical density (OD), the amount of protein based on the BSA standard curve (panel D, linear regression value $R^2$ 0.9924) and the estimated copy numbers. * indicates the theoretical mass (kDa), ** the copy number based on structural studies (55), *** the copy number based on metabolic labeling studies (63).
E, F. Sucrose density gradient-fractionated pyridine-extracted Ad2 or Ad2-GFP-pV, including a pellet (loaded as pellet plus fractions fr 13+14), molecular weight markers (M) and input sample. Gels were stained with Coomassie blue (left), or Western blotted against pV (right). Note that both intact and partially degraded pV and GFP-pV fractionate with the core protein pVII.

G. Purified Ad2 (open bars) and Ad2-GFP-pV (black bars) were heated in the presence of the DNA-intercalating dye TOTO-3 and fluorescence of the DNA-bound dye analysed by the Tecan SAFIRE II microplate reader at 642 ± 8 nm excitation and 660 ± 9 nm emission. One from three typical independent experiments is shown.

**Fig. 3: Ad2-GFP-pV particles contain GFP-pV**

A. Fluorogram of Atto647-labeled Ad2-GFP-pV separated by 12% SDS-PAGE and excited by by 633 nm light. Positions of pII (hexon), pIII and pVI are indicated.

B. Transmission-EM analysis of heavy metal (dark precipitates) stained Ad2 (left) and Ad2-GFP-pV show monodispersed intact particles.

C. Fluorescence analysis of Atto647-Ad2-GFP-pV at 96-well clear bottom imaging plates by ImageXpress Micro microscope using 600-640 nm (Atto647 channel, top left) and 450-480 nm excitation (GFP channel, top right), including merged pseudo-colored images (lower left). The particles scored by Matlab routine are shown in green and the size-threshold rejected background puncta in red. Arrows indicate examples of double labeled particles, and arrow heads rare examples of single labeled Atto647-particles. The arrow head on the left points to a particle which contains GFP-pV but subdetection levels of Atto647. The arrow head on the right depicts a particle, which did not incorporate enough GFP-pV to be detected, but was labeled with Atto647. The abundance of such particles is shown in the histograms of Fig. 3D.

D. Frequency profiles of Ad2-GFP-pV fluorescence showing Atto647 (top), GFP-pV (middle) and merged ratiometric colors (bottom), including background thresholds (blue lines).

E, F. Atto647-Ad2-GFP-pV particles are stable at 37°C. Ratiometric fluorescence analysis of Atto647 and GFP-pV, including absolute values for Atto647 (red line), was
conducted as described in C, albeit with different illumination settings. Representative images are shown for both channels (E) including the merged images, and plots in panel F (number of experiments = 2).

**Fig. 4: Growth of Ad2-GFP-pV**

A. Quantitative EM analysis of Ad2 and Ad2-GFP-pV entry into HeLa-ATCC cells, scoring virus particles at the plasma membrane (left), endosomes (middle) and the cytosol (right) was performed as published (32). Green: Ad2-GFP-pV, light brown: Ad2. Note that 15-20% of the Ad2-GFP-pV particles are not internalized at 90 min pi. Data points show the means of virus particle numbers from the indicated number of cells, including SEM (see table).

B. E1A expression immunofluorescence analysis of 1.5 x 10^4 A549 cells per condition infected with serial dilutions of 1 µg of Ad2 or Ad2-GFP-pV (1 µg is approximately 3 x 10^9 particles). Each point represents the mean of three independent measurements including SEM.

C. Multi-round growth curves of Ad2 and Ad2-GFP-pV in A549 cells infected with 5 fluorescent focus forming units (fffu) per cell and fffu titers of cell-associated or supernatant virus shown at indicated time points as the mean of three experiments with corresponding SEM.

D. End point titers of Ad2 and Ad2-GFP-pV measured as tissue culture infectious dose 50 (TCID50) units or fffu in HER-911 cells with SEM from four independent experiments.

**Fig. 5: Anti-pV antibody detects similar subcellular localization of GFP-pV from Ad2-GFP-pV as pV from Ad2**

A. Hela-ATCC cells were infected or not infected with Ad2 or Ad2-GFP-pV at moi 2 for 20 h, fixed and stained for the viral DNA binding protein (DBP) and the nucleus (DAPI), or with an affinity-purified rabbit anti-pV antibody. Note that GFP-pV and pV are both excluded from DBP positive areas.

B. Purified Ad2 or Ad2-ts1 and cell lysates from Ad2 infected or not infected (not inf.) cells were fractionated by 12% SDS-PAGE, blotted and stained with affinity purified rabbit
anti-pV antibody followed by staining with goat anti-rabbit conjugated horse radish peroxidase. The specific anti-pV band is highlighted with an arrow. The other bands on this blot are unrelated to pV and due to cross reactivity of the secondary antibody.

C. Hela-ATCC cells were infected with Ad2-GFP-pV for 20 h, stained with anti-pV antibody and Alexa594 goat anti-rabbit (rab594) or only with secondary Alexa 594 goat anti-rabbit antibody, and with DAPI. Note the extensive overlap of the GFP-pV signal with the anti-pV antibody stain.

**Fig. 6: Fluorescence microscopy reveals progressive loss of GFP-pV from incoming Ad2-GFP-pV before nuclear import of pVII**

A. Total projections of entire stacks from confocal fluorescence microscopy images of Atto647-Ad2-GFP-pV infected HER-911 cells depict progressive loss of GFP-pV from capsids of quadruple merged channels and stippled outlines of the nuclei (n) and the cytoplasm (c). The yellow frames indicate the enlarged area in the fourth column. Yellow arrows indicate Atto647/GFP-pV double positive particles, red arrows Atto647-positive particles, green arrows GFP-pV particles and blue arrows Atto647/pVII double positive particles. Note that the arrows at 0 min pi denote Atto647 plus pV (yellow) and pVII (blue) signals outside of the cell. Due to the green fluorescent background of the cell, the GFP-pV signal was thresholded (see materials), which reduced its apparent intensity. The DAPI inserts in column 3 (overviews) represent the entire fields shown in columns 1 to 3. Number of experiments = 2.

B. Quantification of the total number of scored particles (light red) and the average Atto647 fluorescence per particle (dark red).

C. Average GFP fluorescence per scored particle (green) and ratio of average GFP/Atto647 fluorescence. The ratio of GFP/Atto647 average fluorescence is shown in the inset.

D. Quantification of pVII positive Atto647-particles (blue), and average fluorescence intensity of pVII in Atto647-particles (violet).

Fig. 7: R70 disassembly marker-positive capsids lack GFP-pV

A. Atto647 labelled Ad2-GFP-pV particles were internalized into HER-911 cells for indicated times, fixed and stained with the rabbit anti-hexon R70 antibody and secondary anti-rabbit-Alexa594 antibody to detect preferentially disassembled capsids. The images in each row are from the same corresponding field. Downsized differential interference and DAPI images are shown in columns 2 and 3, respectively. Scale bar = 10 μm.

B, C, D. Quantification of R70 positive capsids and overall R70 fluorescence, GFP-pV in R70 positive and negative capsids, and Atto647 intensity as a function of infection time.

E. GFP-pV / Atto647 fluorescence ratios at different times of infection demonstrate progressive loss of GFP-pV from the capsids.

Fig. 8: Characterization of the anti-hexon R70 antibody

A. Western blot from denaturing SDS-polyacrylamide gels containing boiled and disulfide-reduced lysates of noninfected cells, Ad2 or Ad5 infected cells 40 h pi, and purified Ad2 and Ad5 particles stained with the rabbit polyclonal antibody R70 (1:200), followed by goat anti-rabbit-horse raddish peroxidase (HRP) and ECL detection of HRP (left panel), with the corresponding Coomassie blue stained gel including molecular weight markers indicated as relative molecular weight (Mr) in kD.

B. Periphery-localized Ad2-TR particles in LMB-treated cells are negative for R70 staining. HeLa cells treated or not-treated with 20 nM LMB were infected with Ad2-TR (texas red) for 150 min, fixed and stained for disassembled capsids with the R70 anti-hexon antibody (green), including DAPI signal for cell nuclei and differential interference contrast (DIC). Total projection of confocal stacks are shown for virus and R70 channels.

Fig. 9: LMB blocks nuclear targeting of Ad2-GFP-pV, and reduces R70 epitopes and loss of GFP-pV

The experiment was carried out as described in Fig. 6, except that the HER-911 cells were pretreated with 20 nM LMB 30 min prior to and during infection with Ad2-GFP-pV. Note the clustering of GFP-pV positive particles at a distinct perinuclear site, the MTOC (white arrows), identified by anti-gamma-tubulin staining (not shown).
Fig. 10: LMB blocks the second phase of GFP-pV loss from capsids

A. HER-911 cells on 96-well plates were infected with Atto647-Ad2-GFP-pV and analysed by fluorescence imaging using the automated ImageXpress^{MICRO} fluorescence microscope for GFP-pV, Atto647-fluorescence and the merged pseudocolored images, including the periphery of the nuclei (n) determined by DAPI staining and the cytoplasm (c) (white lines, and not shown).

B. GFP-pV quantification of the scored Atto647-particles, including total numbers of analysed viral particles, cells and numbers of experiments (exps) with error bars representing SEM and p-values from Student’s t-tests.

Fig. 11: Localisation of Atto647-Ad2-GFP-pV capsids near nuclear pore complexes

POM121-mCherry transfected HER-911 cells were infected with Atto647-Ad2-GFP-pV and live imaged with spinning disc confocal microscopy with focus near the bottom of the nucleus at 60 min pi. The left panel shows images recorded in the Atto647, GFP and cherry channels. Merged images are shown in pseudocolors in the right panel, where red represents Atto647 labeled Ad2, green GFP-pV and blue POM121-mCherry. White arrows point to triple positive particles (red, green, blue), yellow arrows to red and green double-positive particles, and magenta arrows to red and blue double-positive particles.
**F4**

**A**
- Plasma membrane
- Endosomes
- Cytosol

**B**
- Ad2 20h
- Ad2 16h
- Ad2 12h

**C**
- Ad2-GFP-pV 20h
- Ad2-GFP-pV 16h

**D**
- TCID50
- fflu

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A

HeLa infection
n.i. 40 h purified
Ad2 Ad5 Ad2 Ad5
aggregated hexon
monomeric hexon

anti-hexon (R70)

B

no drug

150' pi

LMB (20 nM)
total projections, HER-911 cells, 20 nM LMB
single slice spinning disc confocal images
Supplemental material

Materials and methods

Live cell microscopy

For spinning disc confocal microscopy (SDCM), images were recorded with an N.A. 1.35 UplanApo 100x objective on an Olympus IX81 inverted microscope (Olympus Schweiz) equipped with a temperature controlled incubator box (Life Imaging Services), a Yokogawa scanning head QLC100 (VisiTech International) with a triple bandpass excitation filter (488 nm/565 nm/647 nm) (Chroma) and an Innova 70C mixed gas laser (Coherent). For simultaneous dual color imaging (GFP/Atto565) a Dualview MultiSpec-MicroImager (Optical Insights, USA) was used. Images were recorded onto a back-illuminated monochrome Cascade 512 EM-CCD camera (Photometrics) containing a 512x512 pixel chip. Microscope and camera were controlled with the Metamorph software package (Molecular Devices). HER-911 cells were grown on cover slips, serum starved over night and maintained in DMEM with HEPES modification (SIGMA, D6171) containing 0.2% BSA during acquisition. Recording was started and Atto565-Ad2-GFP-pV was added to 0.2 μg/ml.

HER-911 cells were transfected with a POM121-mCherry expressing plasmid using FuGENE 6 (Roche) according to the manufacturer’s instructions. Cells were serum starved overnight and warm infected with 0.2 μg/ml Atto647-Ad2-GFP-pV for 15 min, medium removed and incubated for another 45 min at 37°C 5% CO₂. Cells were recorded with the spinning disc confocal microscope without beamsplitter.

Fluorescence crosstalk control experiments

Ad2, Ad2-GFP-pV, Atto565-Ad2 or Atto565-Ad2-GFP-pV were attached to coverslips, mounted with DAKO and recorded by spinning disc confocal fluorescence microscopy in beamsplitter mode (GFP and Atto565) using identical illumination settings. Ad2, Atto647-Ad2, Ad2-GFP-pV or Atto647-Ad2-GFP-pV were adsorbed to the bottom of 96-well clear
bottom plates at 20°C for 15 min. Wells were washed with PBS, medium removed and 100 µl fresh PBS was added. Virus particles were recorded with the MetaXpress-Micro automated microscope focusing on the bottom of the plate using identical illumination settings (GFP and Atto647) for all viruses.

**Image processing, particle scoring and quantification of GFP-pV in Ad2 particles**

Images were recorded with an automated ImageXpress\textsuperscript{Micro} fluorescence microscope (Molecular Devices) controlled by the Molecular Devices MetaXpress 2 software as described earlier. Image stacks comprising 9 serial sections separated by a z-distance of 1 µm were recorded in the DAPI, GFP, and Atto647 channels and saved as 16 bit TIFF stacks. To correct for uneven illumination across the field of view, a well containing no cells was recorded using identical settings (background stack). From each slice in the sample stack the pixel intensity of the corresponding slice in the background stack was subtracted.

Lens aberrations caused a shift of the virus position in the GFP channel relative to its position in the Atto647 channel. This was corrected with a custom written Matlab script. A factor for the radial and linear increasing position shift was determined manually. The pixel intensity value associated with each element in the image matrix was distributed into elements of a new matrix relative to the calculated shift. This resulted in a new image matrix where intensity values were shifted to the corrected image locations.

A Matlab based algorithm was used for particle scoring. In brief, Otsu's method (1) was used for thresholding of maximum intensity projections generated from background and aberration corrected images acquired in the Atto647 channel. A manually set area threshold was used to attribute contiguous pixel over the grey threshold to virus particles in an image mask. Particles that met the area threshold were displayed in green whereas particles that did not fulfil the threshold criteria were depicted in red (Figure S6C). This image mask was subsequently employed to measure average fluorescent intensities over the particle areas in both average intensity projections from GFP channel.
images and Atto565 channel images. The following particle parameters were extracted and used for analysis: Particle size, average GFP fluorescence intensity, average Atto565 fluorescence intensity, and the ratio (GFP/Atto647) of the two aforementioned intensities. Statistical analysis was performed with Microsoft Office Excel. The source code of all Matlab routines is available from the authors upon request.

Supplemental figures

Fig. S1: Rapid cell binding of Atto565-Ad2-GFP-pV

A. Attachment of dual color Atto565-Ad2-GFP-pV (0.2 µg/ml) to HER-911 cells was monitored by live cell spinning disc confocal microscopy with a beamsplitter focusing on the lower parts of the cells. The left part of each picture shows the GFP channel and the right part the Atto565 channel. Time stamps are in min and s.

B. The GFP image at 7 min 30 s was pseudo-colored in green and the Atto565 image in red. Processed images were merged, and the DIC image from the same time point is also shown. Arrows indicate a two of the many color puncta.

Fig. S2: Schematic representation for the cell-based GFP-pV dissociation assay of incoming Ad2-GFP-pV

A. Image processing procedure. Image stacks from the GFP, Atto647 and DAPI channels were background subtracted, and based on the Atto647 channel also aberration corrected individually, merged using maximum intensity projection algorithm and analysed for GFP-pV and Atto647 colocalization.

B. Illustration of the aberration correction procedure. Multi fluorescent Tetraspec beads were bound to the bottom of a 96-well clear bottom plate and recorded with the MetaXpress Micro microscope in the GFP and Atto647 channels. The mismatch between the Atto647 channel and the GFP channel was corrected with a Matlab (MathWorks, Inc. Natick, MA,USA) routine post image acquisition, as shown with the
GFP-pV/Atto647 merged original picture (upper left), and the shift-corrected image (upper right). Red arrows originate from the fixpoint used in the correction routine and define the direction of the correction displacement. The accuracy of the procedure is illustrated by the two lower images showing enlarged regions of the boxed areas in the two upper images.

C. Particle identification procedure in representative images taken with the MetaXpress Micro microscope of background and aberration corrected maximum projections from the Atto647, GFP and DAPI channels 30 min pi in the absence of drugs. The Matlab routine shown in the second panel identifies single particulate puncta in the Atto647 channel (green) and rejects nonhomogeneous signals (red) according to a size-threshold.

Movie S1: Rapid cell binding of Atto565-Ad2-GFP-pV and dynamic colocalization of GFP-pV with Atto565 labeled capsids

HER-911 cells were infected with Atto565-Ad2-GFP-pV and live imaged by spinning disc confocal fluorescence microscopy in beam splitter mode, allowing simultaneous recordings of GFP-pV and Atto565 signals. Total recording time was 15 min at 12 frames per min. Note that red and green signals were simultaneously detected in both channels at similar locations relative to the cell, indicating that at the time of cell binding, a large fraction of viral particles were double positive for Atto565 and GFP-pV.

Supplemental reference

Sup_F1
Sup_F2

A

Background Stack → Corrected Stack

GFP → Shift correction → Maximum projection

Subtraction → Merge → Image analysis

B

Original image → Corrected image

C

Atto647 → scored particles → GFP → DAPI

10 μm