1 **Molecular characterization of a lizard adenovirus reveals the first**

2 **atadenovirus with two fiber genes, and the first adenovirus with either one**

3 **short or three long fibers per penton**

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30

31   **Abstract**

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33   Although adenoviruses have been found in a wide variety of reptiles including numerous

34   squamate species, turtles and crocodiles, the number of reptilian adenovirus isolates is still

35   scarce. The only fully sequenced reptilian adenovirus, snake adenovirus 1 (SnAdV-1),

36   belongs to the *Atadenovirus* genus. Recently, two new atadenoviruses were isolated from a

37   captive Gila monster (*Heloderma suspectum*) and Mexican beaded lizards (*H. horridum*).

38   Here we report the full genomic and proteomic characterization of the latter, designated as

39   lizard adenovirus 2 (LAdV-2). The dsDNA genome of LAdV-2 is 32,965 bp long with an

40   average G+C content of 44.16%. The overall arrangement and gene content of the LAdV-2

41   genome was largely concordant with those in other atadenoviruses, except for four novel

42   ORFs at the right end of the genome. Phylogeny reconstructions and plesiomorphic traits,

43   shared with SnAdV-1, further supported the assignment of LAdV-2 to the *Atadenovirus*

44   genus. Surprisingly, two fiber genes were found for the first time in an atadenovirus. After

45   optimizing the production of LAdV-2 in cell culture, we determined the protein composition

46   of the virions. The two fiber genes produce two fiber proteins of different size that are

47   incorporated into the viral particles. Interestingly, the two different fiber proteins assemble as

48   either one short or three long fiber projections per vertex. Stoichiometry estimations indicate

49   that the long fiber triplet is present at only one or two vertices per virion. Neither triple fibers,

50   nor a mixed number of fibers per vertex, had previously been reported for adenoviruses, or

51   any other virus.

52   **IMPORTANCE**

53   Here we show that a lizard adenovirus, LAdV-2, has a penton architecture never observed

54   before. LAdV-2 expresses two fiber proteins, one short and one long. In the virion, most

55   vertices have one short fiber, but a few of them have three long fibers attached to the same

56   penton base. This observation raises new intriguing questions on virus structure. How can the

57   triple fiber attach to a pentameric vertex? What determines the number and location of each

58   vertex type in the icosahedral particle? Since fibers are responsible for primary attachment to

59   the host, this novel architecture also suggests a novel mode of cell entry for LAdV-2.

60   Adenoviruses have a recognized potential in nanobiomedicine, but only a few of the more

61   than 200 types found so far in nature have been characterized in detail. Exploring the

62 taxonomic wealth of adenoviruses should improve our chances to successfully use them as

63 therapeutic tools.

64

Introduction

Adenoviruses (AdVs) occur commonly in humans and in a large spectrum of animals representing every major class of vertebrates (1). Individual AdV types are generally restricted to a single host species, suggesting a long common evolutionary history. Nonetheless, numerous examples of hypothesized host switches have also been described (2). The family *Adenoviridae* is currently divided into five officially approved genera (1). The *Mastadenovirus* and *Aviadenovirus* genera include AdVs infecting only mammals and birds, respectively. The most recently approved genus *Ichtadenovirus* was established for the single known fish AdV isolated from white sturgeon (3). The two additional genera, *Atadenovirus* and *Siadenovirus*, contain viruses found in more divergent hosts, so their names reflect characteristic features other than a particular vertebrate class. The origin of siadenoviruses, named after the unique presence of a sialidase-like gene close to their left genome end, is yet to be clarified (4). Atadenoviruses are thought to represent the AdV lineage co-speciating with squamate reptiles (2, 5), although the first members of the genus were found in ruminants and birds. They were noted for the strikingly high A+T content of their DNA, hence the genus name (6-8). The genome of the first fully sequenced reptilian AdV (snake adenovirus 1, SnAdV-1) showed a characteristic atadenoviral gene arrangement (9), albeit with an equilibrated G+C content (5, 10). The analysis of short sequences from the DNA-dependent DNA polymerase (*pol*) gene of further, newly reported AdVs from a number of additional animals in the order Squamata, confirmed the balanced G+C content and the phylogenetic place in the *Atadenovirus* genus for all these snake and lizard viruses (11-13).

Members of the *Adenoviridae* family present linear dsDNA genomes ranging from 26 to 48 kb in size (1). Comparative analysis of genes across this family has identified conserved protein-encoding regions, classified into genus-common and genus-specific genes (14). Genus-common genes are centrally located in the genome, encoding proteins with functions involved in DNA replication (pol, pTP and DBP), DNA encapsidation (52K and IVa2) and architecture of the virion (pIIIa, penton base, pVII, pX, pVI, hexon, protease, 100K, 33K, pVIII and fiber). Genus-specific genes are mainly located near the ends of the genome, except for mastadenovirus protein V. Minor coat polypeptide IX and core polypeptide V are unique to mastadenoviruses. In atadenovirus virions, the genus-specific structural polypeptides are LH3 and p32K (15, 16). Another variable in AdV genome and virion architecture is the number of fiber proteins. Most mastadenoviruses sequenced so far possess a single gene

97 coding for fiber protein except members of the species *Human mastadenovirus F* and *G*

98 (HAdV-F and HAdV-G), as well as a number of unclassified simian adenoviruses, which

99 have two fiber genes. The products of these genes appear in the virions as a single fiber per

100 penton (17-20). Contrarily, in several poultry AdVs, classified into the *Aviadenovirus* genus,

101 two fibers per penton are often observed, regardless of the presence of one or two different

102 fiber genes in the genome (21-24).

103 The AdV fiber is a main determinant of viral tropism. All fiber proteins characterized so far

104 form trimers with three differentiated structural domains. Short N-terminal peptides (one per

105 fiber monomer) form the frayed end of the trimer that joins the fiber to the capsid, by

106 interacting with clefts between monomers of penton base, in a 3 to 5 symmetry mismatch (25,

107 26). The three fiber monomers then intertwine to form the shaft domain, made up by a

108 variable number of 15–20 residue pseudorepeats, which in turn result in a variable number of

109 structural repeats, and variable fiber lengths depending on the particular virus type (27, 28).

110 Fibers also vary in flexibility; occasional disruptions of the sequence repeat pattern result in

111 shaft kinks (29). Finally, the distal C-terminal head domain (knob) attaches to the primary

112 receptor at the host cell membrane (30), after which AdV is internalized by receptor-mediated

113 endocytosis. In the majority of AdVs characterized so far, internalization is mediated by

114 interaction of an RGD sequence in the penton base with integrins in the cell surface (31).

115 Fiber length and flexibility differences most likely reflect differences in viral attachment and

116 internalization mechanisms (32, 33).

117 The first AdV-like particles in lizards of different species were described based on

118 microscopy studies several decades ago (34-36). Although the frequent occurrence of AdV in

119 different reptilian specimens was well documented (37, 38), for a long time only a few cases

120 of successful isolation and *in vitro* propagation were known (39, 40). A consensus nested

121 PCR, targeting a conserved region of the DNA-dependent DNA polymerase gene (*pol*) of

122 AdVs, has been used successfully to detect novel atadenoviruses in lizards of six different

123 species (11). The isolation of the first three AdV strains originating from lizards was reported

124 more recently (12). Two of these strains seemed to be identical and were obtained from oral

125 swabs taken from apparently healthy Gila monsters (*Heloderma suspectum*) during a follow-

126 up study after a serious disease outbreak in a Danish zoo. Their *pol* sequence was identical

127 also with that of the helodermatid AdV described in the USA previously (11). A third strain,

128 with slightly divergent *pol* sequence, was isolated from the homogenate of internal organs

129 (intestines, liver, heart) of a Mexican beaded lizard (*H. horridum*) that had been kept in the

130   same enclosure with the Gila monsters and died with several other Mexican beaded lizards

131   during the disease outbreak. The isolates seemed to represent two types of a new AdV

132   species, and have been described as helodermatid adenovirus 1 and 2, from the Gila monsters

133   and the Mexican beaded lizard, respectively (12). More recently however, a closely related

134   *pol* sequence, showing 100% amino acid (aa) and 99% nucleotide (nt) identity with the

135   helodermatid AdV-2, has been obtained from a western bearded dragon (*Pogona minor*

136   *minor*) in Australia (13). This finding indicated that the helodermatid isolates might not be

137   strictly host specific. A less exclusive name, such as lizard adenovirus, seemed to be more

138   appropriate for viruses of a seemingly broader host spectrum. Extensive characterization of

139   these first lizard adenovirus (LAdV) isolates appeared intriguing.

140   In the present paper, molecular analyses including the full genome sequence and proteome

141   analysis of LAdV-2 are described. The presence of two fiber genes and an unexpected

142   architecture of the pentons consisting of either one short or three long fiber projections per

143   penton base were the most interesting findings.

144

Materials and methods

Virus propagation and purification. The LAdV-2 strain was isolated and initially propagated as previously described (12). For the sequencing experiments, virus purification and concentration was performed by ultracentrifuge pelleting (Beckman, Ti90 rotor). After a freeze and thaw cycle, cell-debris was first removed from the cell culture supernatants with low speed centrifugation (1,500×g, 10 min, 4°C), and viruses were then concentrated by ultracentrifugation (120,000×g, 3 hours, 4°C). Supernatants were decanted, and pellets were resuspended in PBS.

For large-scale virus propagation and purification, iguana heart epithelial cells (IgH-2, ATCC: CCL-108) (41) were cultured in Dulbecco's Modified Eagle's Medium, containing 10% fetal bovine serum, 10 U/ml penicillin, 10 µg/ml streptomycin and 1× Non-essential Amino Acid Solution (Sigma), and maintained at 28ºC in a humidified incubator with 5% CO_2. Cells were seeded in 10-cm tissue culture dishes and split at a ratio of 1:3. When the cell monolayers reached 70% confluence, virus infected supernatant was added. The infection was carried out at 28ºC. The cells were collected when substantial cytopathic effect was observed, from 3 to 5 days post-infection.

Virus purification was carried out following protocols similar to those used for HAdV. Infected IgH-2 cells from 200 p100 tissue culture plates were collected and centrifuged for 10 min at 800 rpm and 4ºC. The cells were then resuspended in 35 ml of 10 mM Tris-HCl pH 8.1 and lysed by four freeze-thaw cycles. Cell lysates were clarified to remove cellular debris by centrifugation in a Heraeus Megafuge 1.0R at 3000 rpm for 60 min at 4ºC. The supernatant was layered onto a discontinuous gradient of 1.2 g/ml, 1.45 g/ml CsCl in 10 mM Tris-HCl pH 8.1 and centrifuged at 20,000 rpm for 120 min at 4ºC in a Beckman SW28 swinging bucket rotor. The low and high density viral particle bands from each tube were separately collected, pooled, mixed with 10 mM Tris-HCl pH 8.1, layered on a second CsCl gradient, and centrifuged overnight at 20,000 rpm and 4°C in a Beckman SW40 Ti swinging bucket rotor. The virus band from each tube was collected and pooled. The final virus pool was loaded into BioRad Econo-Pac 10 DG disposable chromatography columns with 6,000 Dalton molecular weight cutoff for buffer exchange to 20 mM HEPES, 150 mM NaCl, pH 7.8. The virus

175   concentration (in viral particles/ml) was determined by absorbance as described (42). Purified

176   virus was stored at -80ºC after adding glycerol to 10% final concentration.

177

178   **PCR and molecular cloning.** Genomic DNA was extracted from LAdV-2 particles

179   concentrated by ultracentrifugation with the Qiagen© DNeasy™ Tissue Kit (Hilden,

180   Germany). Initially, random cloning of the HindIII and PstI (Fermentas, St Leon-Rot,

181   Germany) digested viral DNA into Phagemid pBluescript® II KS(+/-) (Stratagene Ltd., Santa

182   Clara, CA, USA) was performed. The cloned fragments were identified by sequence analysis.

183   The sequences of additional genome regions were obtained from the genes of the IVa2 and

184   penton base proteins after PCR amplification with degenerate consensus primers. A consensus

185   primer was also designed based on the known adenoviral ITR sequences. With this, the left

186   genome end encompassing a part of the IVa2 gene was amplified and sequenced. A specific

187   leftward primer was designed from the sequence of the putative p32K gene. The exact

188   sequence of the left ITR was determined after a unidirectional PCR with this p32K-specific

189   primer. With the use of the terminal transferase from the 5'/3' 2nd Generation RACE Kit

190   (Roche Applied Science, Penzberg, Germany), a polyA tail was added to the 3' end of the

191   single stranded PCR product. This molecule was then PCR-amplified using the p32K-specific

192   and the polyT primers. Additional specific PCR primers were designed to amplify regions

193   spanning between the genome fragments already sequenced. Amplification of the genome

194   part rightwards from the fiber genes was facilitated by the sequence of several smaller

195   fragments obtained from the random cloning. For PCR-amplification of fragments shorter

196   than 1000 bp, the DreamTaq Green DNA Polymerase (Thermo Fisher Scientific Baltics UAB,

197   Vilnius, Lithuania) was applied, whereas for larger fragments, the Phusion High-Fidelity

198   DNA Polymerase by Finnzymes (Thermo Fischer Scientific, Espoo, Finland) was used.

199   Annealing temperatures and elongation times were adjusted according to the primers used and

200   the expected length of the PCR products.

201

202   **Sequencing and sequence analysis.** To confirm their identity and homogeneity, each PCR

203   product was first sequenced using the respective PCR primers. The sequencing reactions were

204   set up with the use of the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied

205   Biosystems), and run on an ABI PRISM 3100 Genetic Analyzer by a commercial service. The

programs applied for bioinformatics analyses have been described in detail recently (43). Raw nucleotide sequences were handled with the BioEdit program. To detect homology and identity of the sequenced gene fragments, different BLAST algorithms at the NCBI server were used. Sequence editing and assembly were performed manually or with the Staden Sequence Analysis Package. Genome annotation was carried out with the CLC Main Workbench, version 6.9. Protein sequences were analyzed using SMART (http://smart.embl-heidelberg.de).

Phylogenetic tree reconstruction. Multiple aa alignments from the hexon and protease sequences were prepared with ClustalX 2.1. Several representatives from all the five officially approved AdV genera were included. Phylogenetic tree reconstructions were performed by maximum likelihood (ML) analysis (44). Model selection was carried out by ProtTest 2.4, based on the guide tree constructed by the protdist and fitch algorithms (JTT and global rearrangements) of the Phylip package (version 3.69). For the hexon tree, the LG+G model was used with an alpha value (gamma distribution shape parameter) of 0.61. For constructing the protease-based tree, LG+I+G was applied (proportion of invariant sites: 0.06, gamma distribution shape parameter: 1.13). ML with bootstrapping (100 samples) was performed by the phyml algorithm, provided at the Mobyle portal (http://mobyle.pasteur.fr). FigTree v1.3.1 was used for visualizing the phylogenetic trees.

Characterization of purified virus and disassembly products by protein electrophoresis and electron microscopy. For SDS-PAGE, samples were incubated at 95ºC in loading buffer containing 2% SDS, 1% β-mercaptoethanol, 10% glycerol and 50 mM Tris-HCl pH 6.8. and loaded into either 15% or 4-20% (BioRad Mini-PROTEAN TGX Precast gradient) acrylamide gels that were silver stained according to standard protocols. To estimate the relative amounts of fiber proteins, band intensities in silver stained gel images were measured with ImageJ (45). To avoid interference in the measured values for fiber1 due to the proximity of the intense LH3 band, only gels where a clear separation between the two bands was observed were used for quantitation. Moreover, only the area below the half peak away from the closest neighbor was measured, after correction for the background intensity.

236   For negative staining electron microscopy (EM), 5 μl sample drops were incubated on glow

237   discharged collodion/carbon coated grids for 5 minutes and stained with 2% uranyl acetate for

238   45 sec. Grids were air dried and examined in a JEOL 1011 transmission electron microscope

239   at 100 kV. Measurements of viral particle diameter and fiber length were carried out using

240   ImageJ, on micrographs digitized on a Zeiss SCAI scanner with a sampling step of 7 μm/px,

241   giving a sampling rate of 4.67 Å/px for the virus, and 3.5 Å/px for the fibers. Fiber shaft

242   length was measured between the edge of penton base and the broadening indicating the C-

243   terminal knob domain.

244

245   **Controlled disruption of virions.** A virus disruption protocol based on hypotonic dialysis

246   followed by centrifugation (46) was used for obtaining a sample enriched on viral vertex

247   components. Purified virus was dialyzed against 5 mM Tris-maleate pH 6.3, 1 mM EDTA for

248   24 hours at 4ºC, and centrifuged at 20,200×g for 60 minutes at 4ºC. The supernatant was

249   analyzed by SDS-PAGE and negative staining EM as described above.

250

251   **Mass spectrometry analyses**. The proteins present in purified LAdV-2 virions were

252   determined by in-solution digestion and LC-ESI MS/MS analysis, as follows. Samples were

253   dissolved in 8 M urea, 25 mM ammonium bicarbonate, reduced and alkylated with 50 mM

254   iodoacetamide. Urea concentration was reduced to 2 M with 25 mM ammonium bicarbonate.

255   Trypsin (Roche Diagnostics GmbH, Mannheim, Germany) was added in a 25:1 (w/w) ratio

256   and incubation proceeded overnight at 37ºC. Nano LC ESI-MS/MS analysis of the digested

257   samples was performed using an Eksigent 1D-nanoHPLC coupled *via* a nanospray source to a

258   5600 TripleTOF QTOF mass spectrometer (AB SCIEX, Framinghan, MA, USA). The

259   analytical column used was a silica-based reversed phase column Eksigent chromXP 75 μm ×

260   15 cm, 3 μm particle size and 120 Å pore size. The trap column was a chromXP, 3 μm

261   particle diameter, 120 Å pore size. The loading pump delivered a solution of 0.1%

262   trifluoroacetic acid in 98% water / 2% acetonitrile (LabScan, Gliwice, Poland) at 30 μl/min.

263   The nanopump provided a flow-rate of 300 nl/min, using 0.1% formic acid (Fluka, Buchs,

264   Switzerland) in water as mobile phase A, and 0.1% formic acid in 80% acetonitrile / 20%

265   water as mobile phase B. Gradient elution was performed as follows: isocratic conditions of

266   96% A: 4% B for five minutes, a linear increase to 40% B in 60 min, a linear increase to 95%

267   B in one minute, isocratic conditions of 95% B for seven minutes and return to initial

268   conditions in 10 min. Injection volume was 5 µl. Automatic data-dependent acquisition using

269   dynamic exclusion allowed the acquisition of both full scan (m/z 350-1250) MS spectra

270   followed by tandem MS CID spectra of the 25 most abundant ions. MS and MS/MS data were

271   used to search against a customized database containing all the LAdV-2 protein sequences

272   derived from the genome, using MASCOT v.2.2.04. Search parameters were:

273   carbamidomethyl cysteine as a fixed modification, and oxidized methionine as a variable one.

274   Peptide mass tolerance was set at 25 ppm and 0.6 Da for MS and MS/MS spectra,

275   respectively, and 1 missed cleavage was allowed.

276   For in-gel protein digestion and MALDI TOF/TOF protein band identification, silver- or

277   Coomassie-stained bands were excised, deposited in 96-well plates and processed

278   automatically in a Proteineer DP (Bruker Daltonics, Bremen, Germany). The digestion

279   protocol used was based on (47) with minor variations. After in-gel digestion, 20% of each

280   peptide mixture was deposited onto a 386-well OptiTOF^{TM} Plate (Applied Biosystems,

281   Framingham, MA, USA) and allowed to dry at room temperature. A 0.8 µl aliquot of matrix

282   solution (3 mg/ml α-Cyano-4-hydroxycinnamic acid in MALDI solution) was then added, and

283   after drying at room temperature samples were automatically analyzed in an ABI 4800

284   MALDI TOF/TOF mass spectrometer (AB SCIEX, Framingham, MA, USA) working in

285   positive ion reflector mode (ion acceleration voltage 25 kV for MS acquisition and 1 kV for

286   MSMS). Peptide mass fingerprinting and MS/MS fragment ion spectra were smoothed and

287   corrected to zero baseline using routines embedded in the ABI 4000 Series Explorer Software

288   v3.6. Internal and external calibration allowed to reach a typical mass measurement accuracy

289   of <25 ppm. To submit the combined peptide mass fingerprinting and MS/MS data to

290   MASCOT software v.2.1 (Matrix Science, London, UK), GPS Explorer v4.9 was used,

291   searching in the customized protein database containing all the LAdV-2 protein sequences

292   derived from the genome.

293   **Database sequence deposition.** The full sequence was deposited in GenBank and assigned

294   accession number KJ156523.

295

Results and Discussion

General features of the genome. The genome of LAdV-2 consists of 32,965 base pairs (bp) with an average G+C content of 44.2%. The genome is flanked by inverted terminal repeats (ITRs) of 127 bp, the longest known to date in the *Atadenovirus* genus. **Fig. 1A** shows a schematic of the LAdV-2 genetic map, with 34 putative genes. Of these, 30 were identified based on homology with adenoviral genes described earlier. In the highly variable right end of the genome, four additional ORFs (ORF2, 3, 4 and 5) capable of coding for proteins of a minimum of 50 aa were found. The average distance between two genes was short, generally less than 50 bp with only four exceptions. On the other hand, 11 gene overlaps were detected. The proportion of coding region is 95.5%.

The first gene at the left end of the genome was the p32K gene on the *l* (leftward transcribed) strand. This protein is a unique characteristic of the *Atadenovirus* genus. Rightwards, the homologues of three LH (left hand) genes, another special attribute of atadenoviruses (48), were identified. The orientation and size of these ORFs were similar to their homologues in SnAdV-1 (5).

The organization of the highly conserved central part of the LAdV-2 genome is largely concordant with that in all AdVs. On the *l* strand, the general AdV pattern of the E2A and E2B regions was found, with homologues for the genes of DNA-binding protein (DBP), terminal protein precursor (pTP), pol, and IVa2, as well as a putative homologue of the atadenoviral U-exon (5, 14). On the *r* strand, 14 conserved genes were identified. Unexpectedly, at the right end of this (supposedly late) transcription unit, two fiber genes of different size were found in tandem (**Fig. 1A**). The first fiber gene (*fiber1*), highly similar to the fiber of SnAdV-1 , was shorter (996 bp), whereas the second one (*fiber2*) encompassed 1302 bp. In accordance with previous findings (14), neither gene homologues for the mastadenoviral structural proteins V and IX, nor a region corresponding to the mastadenoviral E3 were found.

At the right-hand side of the LAdV-2 genome, a typical atadenoviral E4 region was identified, with homologues for all three E4 genes (E4.1 to E4.3) of SnAdV-1 in the same position, as well as a homologue of the SnAdV-1 URF1 on the *l* strand (named ORF6 in **Fig. 1A**). Also as in SnAdV-1, a single RH gene was identified, followed by a putative gene (ORF5) capable of

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coding for a protein of 416 aa with no detectable homology to any known proteins. Further rightwards on the *r* strand, an ORF with homology to the unique ORF1 of duck adenovirus 1 (DAdV-1) was found, and consequently also named ORF1. Another putative gene (ORF2), predicted to code for a protein of 97 aa of unknown function, was found as the last ORF on the *r* strand. On the *l* strand, a homologue of the 105R gene was found. This hypothetical gene was first described in the tree shrew AdV-1 (49), then a homologue was identified in the SnAdV-1 genome (50). The last two short ORFs (ORF3 and ORF4 in **Fig. 1A**) on the *l* strand closest to the right ITR showed no detectable homology to any known genes in GenBank. None of these ORFs (ORF1 to 6) have been experimentally confirmed to be functional genes.

In all phylogeny reconstructions performed, LAdV-2 clearly clustered among members of the *Atadenovirus* genus. The ML trees based on the hexon and protease protein sequences are presented in **Fig. 1B**. LAdV-2 always appeared as a sister branch to SnAdV-1. There was no significant difference in the topology of trees resulting from the different genes. Branching of the five genera was supported with maximal or high bootstrap values.

Large-scale propagation and purification of LAdV-2. Initial seeds consisted of supernatants from IgH-2 cells infected with LAdV-2 isolated from a Mexican beaded lizard, *Heloderma horridum* (12), containing approximately 10^5 vp/ml. Serial amplification was used to achieve enough infective supernatant for large-scale virus production. After four rounds of amplification, a viral concentration of 1.5×10^{12} vp/ml was obtained, indicating that LAdV-2 can be propagated and purified by a double CsCl gradient from cell culture with yields similar to those of other well characterized AdVs, such as HAdV-5. EM analyses showed the expected morphology for an atadenovirus (**Fig. 2A**), with particles of 84 ± 6 nm (N = 50) in diameter, and an icosahedral but less faceted shape than HAdV (16).

Molecular composition of purified LAdV-2. After full sequencing of the LAdV-2 genome, we sought experimental confirmation of the expression and incorporation into the virion of the predicted structural proteins. **Table 1** summarizes the proteins identified when samples of purified LAdV-2 were subject to Nano LC-ESI MS/MS analysis. Expected virion components, by analogy with human AdV, were detected: hexon, penton base, IIIa, IVa2, VI, VII, VIII, terminal protein, protease. The product of the 52K gene was also detected in non-negligible amounts. In HAdV-5, the equivalent protein L1 52/55K is removed from the capsid during packaging and maturation (51, 52). Therefore, the detection of 52K in LAdV-2 points to the presence of a minor population of immature viral particles (young virions) in the CsCl

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359   gradient heavy band. In addition, the specific gene products from *Atadenovirus* LH3 and

360   p32K were found. Small traces of 33K and 100K proteins were also present in the samples.

361   The LAdV-2 genome contained two different genes for fiber, with predicted products of 35

362   kDa (fiber1) and 46 kDa (fiber2). The MS/MS analysis revealed that both fiber gene products

363   are expressed and incorporated into the virions. This is the first case reported of an

364   atadenovirus with two different fibers. **Fig. 2B** shows the SDS-PAGE characterization of

365   purified virions. Bands for hexon (102 kDa), IIIa (67 kDa), penton base (51 kDa) and fiber2

366   were observed at the positions expected for their molecular weight. Bands in the 35-40 kDa

367   range were excised and analyzed by MS/MS. Interestingly, the band identified as containing

368   the 35 kDa fiber1 protein had a slower electrophoretic mobility than LH3 (42 kDa) and p32K

369   (40 kDa). This anomalous electrophoretic mobility may indicate post-translational

370   modifications in LAdV-2 fiber1. Protein p32K is considerably larger in reptilian AdVs than

371   its homologue in the prototype atadenovirus OAdV-7 (32 kDa) (9), and is highly basic (pI =

372   11, **Table 1**), suggesting its ability to bind to the viral genome and play a role similar to that

373   of mastadenovirus-specific polypeptide V (16). We interpret a ~30 kDa band as the precursor

374   of polypeptide VIII (pVIII), probably coming from the immature particles present in the

375   purification. The next band, running at ~25 kDa, was assigned to polypeptide VI based on

376   both its molecular weight and MS/MS identification (see below). A band running close to the

377   20 kDa marker was assigned to polypeptide VII on the basis of its abundance, although the

378   protein molecular weight is much lower (15 kDa). The 13 kDa OAdV-7 polypeptide VII has

379   also been reported to have a lower electrophoretic mobility than expected (9, 15). Finally, two

380   bands in the 15-18 kDa range could correspond to the maturation products of polypeptide

381   VIII, as previously reported for OAdV-7 (15). If we consider the consensus cleavage patterns

382   for the HAdV-2 protease (53), a possible cleavage site at position 121-122 (LHGGA) in

383   LAdV-2 polypeptide VIII would lead to a 14 and a 17 kDa fragment consistent with the two

384   observed bands.

385   **Penton architecture in LAdV-2.** Previously reported AdVs with two fiber genes present two

386   different types of penton architecture: either the two fibers bind to different penton bases as in

387   HAdV-40 and HAdV-41 (17, 19, 20), or both fibers bind to the same penton base, as in

388   FAdV-1 (CELO virus, species *Fowl adenovirus A*) (23), FAdV-4 and FAdV-10 (*Fowl*

389   *adenovirus C*) (24, 54). Fibers are difficult to visualize in negatively stained EM images of

390   complete virions, due to their large difference in size with the capsid. Therefore, to ascertain

391   which of the two arrangements was present in LAdV-2, we subjected the purified virus to

controlled disruption based on a protocol previously shown to cause penton and peripentonal hexon release in HAdV (46). In this way, a preparation enriched in LAdV-2 vertex components was obtained. SDS-PAGE analysis together with MS protein identification of the gel bands (**Fig. 3A**) showed the two fiber proteins, as well as penton base, hexon, and protein VI, consistent with the preparation containing pentons and peripentonal hexons together with associated polypeptide VI.

When imaged at the EM, some of the vertex complexes showed a single fiber (**Fig. 3B, C**), while others, surprisingly, presented three longer fibers attached to a single penton base (**Fig. 3D, E**). Long fiber triplets could also be discerned occasionally on negatively stained purified virus (**Fig. 3F**). Measurements on the negative staining EM images of vertices indicated that the short fiber shaft is 180 ± 30 Å long (N = 110), while the long fiber shafts measure 260 ± 30 Å (N = 237 fibers; 79 penton complexes). The presence of the three domains, namely the N-terminal tail, the shaft and the C-terminal knob of the LAdV-2 fiber proteins was predicted by manual alignment of the aa sequence with the model proposed by van Raaij *et al.* (1999) as shown in **Fig. 3G, H**. The tail region is longer for fiber1 than for fiber2 (38 and 30 residues respectively), while the head domains contain 123 (fiber1) and 117 (fiber2) residues, predicting smaller knobs than those found in mastadenoviruses (~180 aa in HAdV-2) or aviadenoviruses (over 200 aa for both fiber knobs of FAdV-1) (28, 55, 56). Both fibers include a conserved penton base binding motif in their N-terminal tails (**Fig. 3G, H**) (25). The LAdV-2 fiber1 shaft is predicted to consist of 10 repeats, while fiber2 would have 15. Given the fiber length measured from the EM images, the repeats would be spaced by 18 Å in fiber1, and 17 Å in fiber2, slightly larger in both cases than the spacing observed in the structure of the HAdV-2 fiber shaft (13 Å) (28). Disruptions of the pattern sequence suggest possible sites for shaft kinks in the third repeat of fiber1 (counting from the head), and in the 5th, 7th and 11th repeat in fiber2 (black arrows in **Fig. 3G, H**). In agreement with these predictions, EM images of penton complexes often showed long fibers with sharp kinks occurring at different distances from the knob, while short fibers appeared more rigid, with an occasional kink close to the head domain (arrowheads in **Fig. 3B, D**). All together, these observations indicate that in LAdV-2 there are two different kinds of vertices (**Fig. 3C, E**): some with a triplet of the long fiber (fiber2), and some with a single, short fiber coded by the *fiber1* gene. There is no previous evidence of any AdV with three fibers per penton, or with two different fiber/penton ratios in the same virion.

424   The presence of a triple fiber raises a new question regarding the interaction with penton base.
425   In vertices with single fibers, each one of the three N-terminal fiber tails can bind to each one
426   of the five penton base clefts, adopting a 3 to 5 symmetry mismatched arrangement (25). By
427   comparison with all other characterized AdV fibers, the LAdV-2 fiber2 triplet should have 9
428   N-terminal tails, all with the same ability to bind to only 5 sites in the penton base oligomer.
429   How can this 9 to 5 symmetry mismatch be solved? One possibility is that the five binding
430   sites in penton base are filled by fiber tails randomly, that is: one fiber would occupy three
431   sites and the other two only one each; or two fibers would occupy two binding sites and the
432   third one the last one. This binding pattern would seem too prone to instability and fiber loss.
433   Another possibility is that the fibers interact with each other independently of penton base.
434   This possibility is supported by the occasional observation of groups of three fibers without
435   any associated penton base in heat disrupted LAdV-2 preparations (**Fig. 4A**). These images
436   suggest a structural arrangement in which fibers interact with each other by their N-terminal
437   tails. **Fig. 4B** shows the two alternative ways in which a fiber triplet, assembled as an
438   independent complex, could interact with penton base maintaining a 3-to-5 symmetry
439   mismatch equivalent to that of a single fiber. One possibility is that for each fiber trimer, two
440   of the three N-terminal tails are engaged in interactions with the other fiber molecules
441   forming a dimeric tail, while the third one is free to bind to penton base. Alternatively, all N-
442   terminal tails might be interacting among themselves forming three trimeric tails, each one
443   interacting with a penton base cleft.

444   **Stoichiometry of fibers.** The emPAI (**e**xponentially **m**odified **P**rotein **A**bundance **I**ndex)
445   parameter obtained in LC-MS/MS analyses is linearly related to the relative abundance of
446   each protein in the sample (57). For each protein in the sample, the ratio between the number
447   of observed and observable peptides (called PAI) is calculated, and the emPAI is given by
448    $emPAI = 10^{PAI}-1$. In the proteome analysis of purified virions (**Table 1**), emPAI values were
449   2.37 for fiber1, and 1.53 for fiber2, suggesting that fiber1 is more abundant that fiber2, with
450   an approximate 1.5 fiber1:fiber2 ratio. A repetition of the LC-MS/MS with a different viral
451   preparation gave emPAI values of 1.60 (fiber1) and 0.59 (fiber2), that is, an estimated
452   fiber1:fiber2 ratio of 2.7. The intensity of fiber1 bands in SDS-PAGE of either vertex
453   preparations (**Fig. 3A**) or purified virus (**Fig. 2B**) also appeared slightly stronger than that of
454   fiber2. Gel band densitometry in conditions where silver staining was not saturated (**Fig. 4C**)
455   gave a 2.0 ± 1.0 (N = 3) fiber1:fiber2 ratio. The exact fiber stoichiometry cannot be derived
456   from either of the two estimations described above. However, the fact that they all indicate a

457   higher copy number for fiber1 than for fiber2, together with the constraints imposed by

458   icosahedral geometry, provide a narrow set of possibilities for the arrangement of the two

459   different kinds of vertices in the LAdV-2 virion.

460   Let us call the number of vertices with a single fiber1 projection *V1*, and the number of

461   vertices with a triplet of fiber2 *V2*. Since all previously described AdV fibers form trimers, we

462   assume that both fiber1 and fiber2 form trimers also. Therefore, the total number of fiber1

463   molecules in the virion will be $f1 = 3*V1$, and the total number of fiber2 molecules will be $f2$

464   $= 3*3*V2$. From the LC-MS/MS emPAI and the gel band densitometry estimations, we know

465   that $f1 > f2$, and therefore $V1 > 3*V2$. On the other hand, an icosahedron has a total of 12

466   vertices, therefore $V1+V2 = 12$. It is clear that these two conditions can only be fulfilled for

467   *V2* values lower than 3. Therefore, our results indicate that each LAdV-2 virion has only one

468   or two vertices harboring a triplet of long fibers. The first possibility, *V2* = 1, would give an

469   *f1:f2* ratio of 11:3 = 3.67, while the second one, *V2* = 2, that would give *f1:f2* = 10:6 = 1.67.

470   The LC-MS/MS and gel band densitometry estimations give a range of *f1:f2* values around 2,

471   favoring the solution where *V2* = 2. . A model for this peculiar vertex arrangement is shown

472   in **Fig. 4D**. However, it must be considered that the data presented do not rule out the

473   possibility that the actual vertex distribution varies between viral particles. Interestingly, a

474   similar situation may be present in the enteric HAdV-41, for which it has been reported that

475   the short fiber is approximately 6 times more abundant than the long one in virions (19).

476   Therefore, to fulfill the icosahedral geometry, the long fiber should be present in only two

477   vertices per HAdV-41 virion.

478   **Biological significance of the unusual LAdV-2 penton architecture.** The LAdV-2 genome

479   differs from that of all previously characterized members of the *Atadenovirus* genus in that it

480   contains two different genes coding for fiber proteins. LAdV-2 not only has two fibers with

481   different lengths and flexibility, but surprisingly, we observe that one of the fibers associates

482   in triplets with a single penton base, in only a few vertices per virion. Neither triple fibers, nor

483   a mixed number of fibers per vertex, had previously been described for any AdV. These first

484   observations on the architecture of LAdV-2 open intriguing questions for future structural

485   studies. At this point, it is not known what the spatial distribution of the different vertices in

486   the virion is; or if this distribution is the same in all particles; or what its determinant factors

487   are. For HAdV-41, it has been reported that different expression levels for the two fiber

488   proteins correlate with their stoichiometry in the capsid (Song *et al*., 2012). In LAdV-2, the

489　ability of fiber2 to assemble in triplets in the absence of penton base introduces a new

490　variable.

491　AdV fiber proteins are responsible for receptor attachment, thus determining tissue tropism.

492　Most of the well characterized AdVs use cell surface protein CAR as a primary receptor.

493　CD46, DSG-2, or sialic acid are also known receptors for HAdVs (58). Regarding the AdVs

494　with more than one fiber gene, it is known that the long fiber of species HAdV-F members

495　can bind CAR (59). The FAdV-1 long fiber, which is dispensable for infection in chicken

496　cells, is required for infection of CAR-expressing mammalian cells, although direct binding

497　has not been proved (55, 60). No receptor has been identified for the short fibers in either

498　HAdV-40, 41 or FAdV-1. Apart from the receptor binding properties of the knob, the length

499　and flexibility of the shaft also play a role during entry (33). For example, the fiber of HAdV-

500　37 (species HAdV-D) has a CAR binding knob, but its short (8 repeats), rigid shaft hinders

501　efficient entry using CAR (32). On the other extreme, engineering an extra-long fiber (32

502　repeats, 10 more than in the native fiber) reduced CAR-dependent infectivity in HAdV-5 (61).

503　These studies suggest that fiber length and flexibility modulate the virus-cell surface distance

504　upon attachment to its primary receptor (*e.g.* CAR), to ensure the correct molecular

505　interaction between other capsid proteins (*e.g.* penton base) and a second cellular receptor

506　(integrin), triggering virus endocytosis.

507　What, then, is the role of the two different fibers in LAdV-2? Since the LAdV-2 penton base

508　lacks an integrin-binding RGD loop, it is possible that the two different fiber heads are needed

509　for binding two different receptors, one for attachment and one for internalization.

510　Remarkably, HAdV-40 and 41 (species HAdV-F), which also have two different fibers, are

511　the only known HAdVs lacking an RGD motif in their penton base. The peculiar triple fiber,

512　unique so far among all described AdVs, might be involved in clustering cell membrane

513　factors required for viral entry. Alternatively, the presence of two different fiber heads may

514　expand the viral tropism, allowing propagation in two different types of cells or tissue. In

515　mastadenoviruses, fiber knobs binding sialic acid tend to have high isoelectric points (beyond

516　8 for canine AdV-2, HAdV-19, and HAdV-37). The two predicted LAdV-2 fiber heads (**Fig.**

517　**3G, H**) have lower pIs (4.69 for fiber1 and 6.76 for fiber2), closer to those of viruses using

518　CAR (e.g. HAdV-2, pI ~ 6), or CD46 (pI ~ 5 or even lower for HAdV-11, 21 and 35) as

519　receptors. A LAdV-2-like virus, with a single nucleotide difference in the sequence of the

520　PCR-amplified *pol* fragment, has been described in the sample of a Western bearded dragon

521　recently (Hyndman and Shilton, 2011). Thus, the provenance of LAdV-2 concerning its

522 original host remains unclear. In the seemingly relaxed host specificity of LAdV-2, the
523 presence of the two types of fiber genes and different penton architectures certainly play a
524 crucial role that deserves further scrutiny. It would also be interesting to sequence and
525 compare the genome of LAdV-1, whereas targeted surveys may help find out if lizards of any
526 additional species can be infected by LAdV-2.

527 Recombinant HAdVs are widely used as vehicles for gene transfer, oncolysis and vaccination
528 (62-64). However, their successful use in humans requires surmounting a series of problems,
529 among them the need to reprogram the natural tropism of the vector. A whole field of AdV
530 retargeting by modification of outer capsid proteins is devoted to solve this problem (65).
531 Further characterization of the LAdV-2 receptor binding properties may open the possibility
532 to target tissues and cell types inaccessible at present to existing vectors.

533

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Figure legends

FIGURE 1. Genomic characterization of LAdV-2. **(A)** Schematics of the LAdV-2 genetic map. Shading of the arrows marks the specificity of the genes. The G+C content of the genomic DNA is shown under the genome map. **(B)** Phylogeny reconstructions based on the hexon and protease amino acid sequences. Unrooted calculations with ML method.

FIGURE 2. Molecular composition of purified LAdV-2. **(A)** Negative staining EM image showing the general morphology of the LAdV-2 capsid. The bar represents 100 nm. **(B)** SDS-PAGE analysis of purified virions in a 4-20% gradient gel. Labels at the left hand side indicate the position of standard molecular weight markers. Labels at the right indicate the position of virion proteins. Stars (*) denote bands where protein identification was carried out by MS/MS.

FIGURE 3. LAdV-2 penton architecture. **(A)** SDS-PAGE analysis of the vertex-enriched preparation obtained after mild disruption using hypotonic dialysis and centrifugation. Stars (*) indicate protein identification by MS/MS of excised gel bands. **(B)** Gallery of negative staining EM images showing examples of a single fiber bound to one penton base, as represented by the cartoon in **(C)**. **(D)** Gallery of pentons with three fibers attached to a single penton base, as illustrated by the cartoon in **(E)**. In (B) and (D), the scale bar represents 20 nm, and white arrowheads point to kinks in the fiber shafts. **(F)** Examples of negatively stained viral particles showing a fiber triplet. In the top row, arrows indicate the fiber knobs, while the trajectory of the shafts is highlighted with white curves in the bottom row. The bar represents 50 nm. **(G)** Prediction of structural domains in fiber1, and **(H)** in fiber2. The shaft pseudo-repeats are aligned, and those with the largest departures from the repeating pattern that could originate kinks are highlighted with a gray box. The putative penton base binding peptide is underlined. At the right hand side of each panel, a cartoon shows the predicted number of structural repeats in the fiber shafts, and arrows indicate the location of the predicted kinks.

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756 **FIGURE 4.** Symmetry mismatches in the LAdV-2 penton organization. **(A)** Gallery of
757 negative staining EM images showing examples of triple fibers forming a complex in the
758 absence of penton base, observed in purified LAdV-2 preparations after heating at 50ºC. The
759 bar represents 20 nm. **(B)** Cartoons depicting different ways to fulfill the fiber-penton base
760 symmetry mismatches. For a single fiber, each of the three N-terminal tails binds to one of the
761 five equivalent interfaces between penton base monomers. For the LAdV-2 triple fibers, two
762 possibilities are envisaged. First, each fiber uses two N-terminal tails to bind to its two
763 partners and the third to bind to penton base in a similar way as for the single fiber.
764 Alternatively, all N-terminal tails associate as triplets and each triplet binds to penton base.
765 Zigzag lines (continuous, dashed or dotted) represent the three N-terminal tails of each
766 trimeric fiber. Short transversal lines between zigzags indicate interactions between N-
767 terminal tails of different fibers. The penton base pentamer is represented as a pentagon. **(C)**
768 Example of purified LAdV-2 protein bands in a 10% acrylamide gel used for estimation of the
769 fiber1:fiber2 ratio by densitometry. **(D)** A model for the distribution of the two different types
770 of vertices in the LAdV-2 virion with a fiber1:fiber2 ratio of 1.6.

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774 **Tables**

775 **TABLE 1.** Proteome of purified LAdV-2 virions.[1]

Protein name	Predicted MW (kDa) for the immature protein[2]	Calculated isoelectric point	Peptide total (significant) matches	Sequence coverage (%)	MASCOT score (66)	emPAI
hexon	102040	5.44	404 (399)	73	21650	24.08
pIIIa	66941	5.89	140 (138)	62	7919	15.25
pVI	24075	9.73	90 (87)	83	5186	16.43
penton base	50713	5.75	71 (69)	69	3666	7.19
LH3	41838	6.59	77 (75)	61	3516	6.63
pVIII	30750	5.78	51 (51)	71	2993	7.65
pVII	15239	12.23	47 (46)	55	1848	11.31
fiber1	34826	5.15	29 (28)	44	1465	2.37
p32K	40025	11.02	32 (32)	36	1327	3.90
protease	23245	9.32	28 (28)	50	1150	5.08
IVa2	48186	8.53	23 (22)	46	1128	1.42
fiber2	45930	5.55	22 (21)	40	776	1.53
52K	37765	5.56	14 (14)	27	648	1.84
pTP	69780	7.71	10 (10)	15	448	0.42
pX	10080	12.8	11 (10)	17	233	1.34
100K	77178	6.07	3 (3)	8	125	0.13
33K	20321	6.36	5 (5)	17	71	0.80

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[1] Identified proteins are sorted by MASCOT score (64).
[2] The cleavage specificity of the atadenovirus protease is not well defined yet.