Structures of Adenovirus Incomplete Particles Clarify Capsid Architecture and Show Maturation Changes of Packaging Protein L1 52/55k

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ABSTRACT

Adenovirus is one of the most complex icosahedral, nonenveloped viruses. Even after its structure was solved at near-atomic resolution by both cryo-electron microscopy and X-ray crystallography, the location of minor coat proteins is still a subject of debate. The elaborated capsid architecture is the product of a correspondingly complex assembly process, about which many aspects remain unknown. Genome encapsidation involves the concerted action of five virus proteins, and proteolytic processing by the virus protease is needed to prime the virion for sequential uncoating. Protein L1 52/55k is required for packaging, and multiple cleavages by the maturation protease facilitate its release from the nascent virion. Light-density particles are routinely produced in adenovirus infections and are thought to represent assembly intermediates. Here, we present the molecular and structural characterization of two different types of human adenovirus light particles produced by a mutant with delayed packaging. We show that these particles lack core polypeptide V but do not lack the density corresponding to this protein in the X-ray structure, thereby adding support to the adenovirus cryo-electron microscopy model. The two types of light particles present different degrees of proteolytic processing. Their structures provide the first glimpse of the organization of L1 52/55k protein inside the capsid shell and of how this organization changes upon partial maturation. Immature, full-length L1 52/55k is poised beneath the vertices to engage the virus genome. Upon proteolytic processing, L1 52/55k disengages from the capsid shell, facilitating genome release during uncoating.

IMPORTANCE

Adenoviruses have been extensively characterized as experimental systems in molecular biology, as human pathogens, and as therapeutic vectors. However, a clear picture of many aspects of their basic biology is still lacking. Two of these aspects are the location of minor coat proteins in the capsid and the molecular details of capsid assembly. Here, we provide evidence supporting one of the two current models for capsid architecture. We also show for the first time the location of the packaging protein L1 52/55k in particles lacking the virus genome and how this location changes during maturation. Our results contribute to clarifying standing questions in adenovirus capsid architecture and provide new details on the role of L1 52/55k protein in assembly.

Adenoviruses (AdVs) (1) are among the most complex nonenveloped, icosahedral viruses. The AdV capsid is an icosahedron with a ~950-Å maximum diameter and triangulation number pseudo-T=25. Each capsid facet has 12 trimers of the major coat protein, hexon. A pentamer of penton base protein sits at each vertex, in complex with a trimer of the projecting fiber. In addition, correct assembly requires four different minor coat proteins: IIIa, VI, VIII, and IX (2). The icosahedral shell encloses a nonicosahedral core with a linear, double-stranded DNA (dsDNA) genome (35 kbp in human AdV type 5 [HAdV-C5]), tightly packed in association with a variety of DNA binding, virus-encoded proteins: core polypeptides V, VII and μ ; the terminal protein (TP); and the maturation protease (adenovirus protease [AVP]) (3).

AdV assembles in the cell nucleus, where structural proteins are transported and associate to form capsids into which the virus genome is packaged, producing the so-called “young virions” (immature particles). These contain precursor versions of several capsid and core proteins (pIIIa, pVI, pVIII, pVII, pre- μ , and pTP), which have to undergo various degrees of cleavage by AVP to produce the final, infectious particle (4, 5). AVP is a DNA-depen-

dent protease which uses a unique one-dimensional chemistry mechanism to slide along the virus genome and reach all its substrates (5–8).

Although AdVs have been studied for over 50 years and are extensively used as experimental or therapeutic vectors, many aspects of their basic biology remain unclear. To begin with, there is still debate regarding the localization of minor coat proteins in the virion. The structure of the icosahedral HAdV-C5 capsid was

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FIG 1 Schematics illustrating the two alternative models for HAdV-C5 minor coat protein localization. One facet in the icosahedral particle is depicted, with the minor coat proteins placed according to either the cryo-EM (A) or the X-ray crystallography (B) high-resolution studies (9, 11). No density was assigned to polypeptide V in the cryo-EM study.

solved at high resolution (~ 3.5 Å) by both X-ray crystallography and cryo-electron microscopy (cryo-EM) (9, 10). HAdV-C5 is the largest structure solved by either of the two techniques. However, even after this tour de force, the location of some of the minor coat proteins is still a subject of debate (11–13). One issue is the location of polypeptide IIIa. This uncertainty is of particular relevance because the two different models (X-ray and cryo-EM) place this protein in widely different locations regarding its accessibility on the outer capsid surface, which directly impinges on its possible use as a platform for vector modification.

In the cryo-EM study (Fig. 1A), polypeptide IIIa is assigned to a pinwheel feature located beneath the vertices, in association with polypeptide VIII (9). An internal location for IIIa is also supported by other structural studies (2, 14–16), by biochemical evidence indicating that IIIa interacts with the genome-bound AVP and the genome itself (17–19), and by the observation that IIIa is released together with other internal components in the early stages of virus entry (20). In the X-ray study, however, polypeptide IIIa is assigned to a four-helix bundle located on the outer surface of the capsid, at the icosahedral edges (11) (Fig. 1B). This feature had been assigned to the C-terminal domain of polypeptide IX in the cryo-EM high-resolution analysis (9) and previous peptide mapping studies (21). In turn, according to the latest X-ray model, the pinwheel feature under the vertices would be composed by shell proteins VI and VIII and core polypeptide V (11).

Another yet unclear aspect of AdV biology is the genome encapsidation mechanism. A sequential process by which the genome is packaged into preformed empty capsids, similar to the

process for bacteriophages, has been proposed. This model is based on the existence of a putative packaging motor, protein IVa2 (22–26), and on the generation of large quantities of light-density particles in AdV infections. Light particles are considered assembly intermediates because in pulse-chase experiments they appear earlier than full virions (27), contain protein precursors (4, 28), and contain neither genome nor its associated core proteins, among them polypeptide V (28–31). However, assays at long periods of chase indicate that light particles are very stable structures, that their quantity is constant, and that they do not become mature virions (32). These observations suggest that light particles are not assembly intermediates but defective assembly products.

AdV genome packaging starts from the left end of the genome (30) and requires the coordinated interaction of virus proteins IIIa, L1 52/55k, L4 33k, L4 22k, and IVa2 among themselves and with the virus DNA packaging sequence, Ψ (17, 22, 26, 33–38). One of these proteins, L1 52/55k, is present in empty capsids (50 to 100 copies) but must be released upon genome entry as it is absent from the final virion. For this reason, it is considered a putative assembly scaffold (39). L1 52/55k can form homo-oligomers and interacts with both shell and core components (17, 37, 40–42). In particular, it binds to polypeptide IIIa and Ψ *in vivo*, suggesting that it might act as a tether between genome and capsid shell during encapsidation (17, 37). L1 52/55k is also a substrate for AVP. Proteolytic processing of L1 52/55k disrupts its interactions with other virion components, providing a mechanism for its removal during maturation (40).

Although various AdV mutants that produce only incomplete, light particles have been described (22, 33, 35, 43–46), the structure of these particles and the conformational changes that happen during packaging remain undetermined. Only recently, Cheng et al. (47) reported cryo-EM maps at 4.5- to 5-Å resolution of two types of bovine AdV light particles. In these maps, no density was attributed to protein L1 52/55k, a characteristic component of AdV light particles.

Here, we use an HAdV-C5 delayed-packaging mutant (Ad5/FC31) to study the composition and structure of two kinds of light particles. Ad5/FC31 carries two exogenous sequences flanking Ψ (Fig. 2A) (48). This mutant produces a negligible amount of mature virions compared to the control virus amount at 36 h postinfection (hpi) but reaches the same virus production yield as the control at 56 hpi. Replication studies showed that genome production levels are similar in Ad5/FC31 and control virus. However, only 1 to 5% of the Ad5/FC31 genome was encapsidated at 36 hpi, indicating that low virus production levels at this time of infection were due to packaging problems (48). Electrophoretic mobility shift assays (EMSA) suggested that one or several nuclear proteins were binding to the exogenous sequences and interfering with correct interaction of packaging proteins and Ψ (49). In Ad5/FC31 purifications, at 36 hpi the only visible band in a first CsCl gradient is the light band, while at 56 hpi both light and heavy bands are visible, but the light band is still more abundant (Fig. 2B). In a second gradient, the light band further separates into three bands (49).

Since the Ad5/FC31 light particles we analyze do not contain core protein V, their comparison with the two alternative models for minor coat protein organization provides evidence helping to ascertain which of the two models is correct. Also, our results show for the first time the localization of L1 52/55k in the empty capsids and its changes associated with maturation. This study

FIG 2 Purification of Ad5/FC31 light particles by double CsCl gradient centrifugation. (A) Schematics showing the Ad5/FC31 genome with the exogenous sequences *attB* and *attP* flanking the adenovirus packaging signal Ψ and a green fluorescent protein (GFP) cassette (48). (B) The result of the first gradient is shown for the wild-type (wt) control virus and the Ad5/FC31 mutant, as indicated. The control virus purified at 36 hpi; Ad5/FC31 purified at 56 hpi. L, light-density band; H, heavy-density band. (C) Result of the second CsCl gradient centrifugation for the Ad5/FC31 heavy (H) and light bands. The light band from the first gradient separates into three bands (L1 to L3) in the second one.

helps to clarify AdV architecture and provides new data about the function of L1 52/55k in assembly and maturation.

MATERIALS AND METHODS

Virus propagation and purification. Control mature, wild-type virus was the E1-deleted HAdV-C5 variant Ad5GL (50); for control immature, fully packaged viral particles, we used the HAdV-C2 mutant *ts1* produced at the nonpermissive temperature (39°C) (51). Ad5GL and FC31 were propagated at 37°C in HEK293 cells and harvested at 36 and 56 h postinfection, respectively. The *ts1* mutant was propagated in HeLa cells. All viruses were purified by centrifugation at 18°C in a 1.25- to 1.40-g/ml CsCl step gradient (90 min), followed by a 1.31-g/ml isopycnic one (18 h) at $217,485 \times g$. CsCl solutions were prepared in TD1X buffer (137 mM NaCl, 5.1 mM KCl, 0.7 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 25 mM Tris-HCl, pH 7.4). Bands extracted from the gradients were desalted on a Bio-Rad 10 DC column, and stored in HBS buffer (20 mM HEPES, pH 7.8, 150 mM NaCl) supplemented with 10% glycerol at -80°C until further use. Particle concentrations for all samples used are shown in Table 1. Capsid protein concentration was quantified using the hexon fluorescence emission spectra obtained in a Hitachi Model F-2500 FL spectrophotometer. Sample volumes of 0.150 ml were examined in sealed quartz cuvettes. The sample was excited at 285 nm, and the emission was monitored from 310 to 375 nm using excitation and emission slit widths of 10 nm. The spectra were corrected by subtraction of the buffer spectrum. The maximum emission intensity for each spectrum was found at 333 nm and recorded. The concentration was determined from a calibration curve calculated from a sample with a known concentration.

Protein electrophoresis and Western blotting. Samples were boiled and subjected to electrophoresis under denaturing conditions (SDS-PAGE) in 4% to 20% acrylamide gradient gels (Mini-Protein TGX; Bio-Rad). Similar amounts of particles were loaded in each lane, as estimated by the intensity of the hexon band in silver-stained gels. For Western blotting, proteins resolved by SDS-PAGE were transferred to a nitrocellulose membrane and probed with the required antibodies. The following antibodies were used: mouse monoclonal anti-V (52), rabbit anti-VI serum (53), rabbit anti-L1 52/55k serum (36), and mouse anti-IVa2 hybridoma supernatants (55). Primary antibodies were detected with the appropriate horseradish peroxidase-conjugated secondary antibody. The signal was detected using LiteAblo (Gentaur).

DNA extraction and Southern blotting. DNA was extracted from 1.5×10^{11} (Ad5/FC31 light particles of band L2 or L3) or 3.6×10^{10} (Ad5/FC31 heavy) particles (contained in 200 μl of HBS buffer) and purified according to the protocol described by Sambrook and Russell (56), with some modifications. Virus samples were heated for 10 min at 70°C before the addition of 200 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, and 0.5% SDS). The incubation with proteinase K (100 $\mu\text{g}/\text{ml}$) was overnight. DNA was precipitated overnight at -20°C using 0.4 volumes of 5 M NaCl and 2 volumes of ethanol and resuspended in 50 μl of Milli-Q water. All centrifugations in the protocol were carried out at room temperature and $20,000 \times g$ for 10 min, except for the final pelleting, which was run at 4°C . DNA concentration estimated by the absorbance at 260 nm indicated a total mass of extracted DNA of 110 ng for Ad5/FC31 L2, 500 ng for Ad5/FC31 L3, and 985 ng for Ad5/FC31 heavy particles. However, 260/280 absorbance ratios (0.91 for L2, 1.38 for L3, and 1.7 for heavy particles) suggested that the real amount of DNA extracted from the light particles was lower. This aspect was confirmed by electrophoresis of the purified DNA in 0.8% agarose gels (see Fig. 4C). Volumes loaded were 25 μl for L2 and L3 (the maximum well capacity) and 7.9 μl for heavy particles. Although these volumes contained nominal DNA amounts of 55, 250, and 150 ng for L2, L3, and heavy particles, respectively, no detectable bands were observed for L2, and only a weak band was observed for L3. To investigate if the isolated DNA was part of the viral genome, it was subjected to Southern blotting according to the protocol recommended by GE Healthcare for use with its Amersham Hybond-N+ product (positively charged nylon membrane), specifically, the protocol for capillary blotting. DraIII-digested HAdV-C2 *ts1* DNA was used as a probe after labeling with alkaline phosphatase according to the protocol of the Amersham Gene Image AlkPhos direct labeling and detection system (GE Healthcare Life Sciences), followed by hybridization, posthybridization, signal generation, and detection with CDP-Star (GE Healthcare Life Sciences).

TABLE 1 Concentration of samples used in this study

Virus and particle type ^a	Concn of sample (vp/ml) ^b
Ad5/FC31	
L2	1.53×10^{11}
L3	5.2×10^{11}
H	8.05×10^{10}
Wild type	
L	1.4×10^{11}
H	1.5×10^{12}
<i>ts1</i> mutant	
L	2×10^{12}
H	1×10^{13}

^a Wild type, E1-deleted HAdV-C5 Ad5GL variant (see Materials and Methods); *ts1* mutant, HAdV-C2 *ts1* mutant propagated at the nonpermissive temperature (39°C); L, light particles; H, heavy particles.

^b Virus particle (vp) concentration was estimated using the hexon fluorescence emission as described in Materials and Methods.

Conventional electron microscopy of purified viral particles and infected cells. Purified particles were imaged after negative staining with 2% uranyl acetate. HEK293 cells were grown in a p100 culture plate to 70% confluence and then infected at a multiplicity of infection (MOI) of 5 with the appropriate virus seed. At the desired time postinfection, the medium was removed, and the cells were fixed with 2% glutaraldehyde and 1% tannic acid in 0.4 M HEPES, pH 7.2, for 1.5 h at room temperature. Embedding in Epon resin was carried out as previously described (57). Ultrathin sections (~70 nm) were collected on Formvar-coated nickel grids, stained with saturated uranyl acetate and lead citrate as described previously (57), and examined in a JEOL JEM 1230 transmission electron microscope at 100 kV. The electron density of viral particles in sections was analyzed using Xmipp (58). Image frames (50 by 50 pixels) containing single viral particles were extracted from micrographs and normalized. The average electron density was calculated within a mask of a radius of 25 pixels, corresponding to the size of the viral particle.

Cryo-electron microscopy and image processing. Purified Ad5/FC31 light particles (L2 and L3) were dialyzed against HBS buffer for 1 h at 4°C to remove the storage glycerol and then concentrated (9 times for L3 and 10 times for L2) by spinning at 4°C in a 100,000-molecular-weight-cutoff (MWCO) Amicon Ultra centrifugal filter (Millipore). Samples were applied to Quantifoil R2/4 300-mesh Cu/Rh glow-discharged grids and vitrified in liquid ethane using a Leica CPC plunger. Low-dose cryo-EM images were acquired on an FEI Tecnai FEG200 electron microscope operated at 200 kV using a 4,000- by 4,000-pixel Eagle charge-coupled-device (CCD) camera, at a nominal magnification of $\times 50,000$ and a defocus range of 0.5 to 4 μm (as estimated using Xmipp) (58), with a nominal sampling rate of 2.16 Å/pixel in the sample.

Preprocessing was carried out using Xmipp as follows. Micrographs (889 for L2 and 580 for L3) were screened for minimal drift and astigmatism and corrected for the phase oscillations of the contrast transfer function (CTF; phase flip). Only complete particles were manually picked, extracted into box frames of 516 pixels, and normalized. Particle images (11,344 for L2; 11,962 for L3) were scaled down to 256-pixel frames (4.35 Å/pixel) for computational efficiency. All two-dimensional (2D) and three-dimensional (3D) classifications and refinements were performed using RELION (59). 2D classification was used to discard low-quality particles and run for 25 iterations, with 100 classes, angular sampling of 10°, and a regularization parameter of $T=2$. Classification in 3D was run for 50 iterations, with four classes, starting with an angular sampling of 3.7° and sequentially decreasing to 0.2°, and a regularization parameter of $T=4$. The initial reference for 3D classifications and refinements was an HAdV-C5 density map created from a cryo-EM high-resolution map (PDB accession number 3IYN [9]) using the program pdb2mrc (60) and low-pass filtered to 60-Å resolution. The class giving the best resolution (containing 6,743 particles for L2 and 6,679 particles for L3) was individually refined and used for further analyses. Map resolutions (12.5 Å for L3 and 12.3 Å for L2) (see Fig. 6B) were estimated according to the gold-standard criterion, with a Fourier shell correlation (FSC) threshold of 0.143, as implemented in RELION auto-refine and postprocess routines (61, 62). The gold-standard resolution calculation compares the Fourier transforms of two maps by resolution shells (FSC), with the particularity that the two maps are obtained by refining two random halves of the data set independently. This procedure has been designed to prevent artifactual estimations due to overfitting during the projection orientation search.

For comparison with the HAdV-C2 ts1 map, a previously published data set (63) at 2.8 Å/pixel was reprocessed with RELION auto-refine and postprocess procedures. The final 10,210-particle map (at 7.7-Å gold-standard resolution) was low-pass filtered at 12.3 Å for comparison with the L2 and L3 maps.

Map analysis. Reference density maps for comparison with the two alternative high-resolution models for mature HAdV-C5 virus were calculated from the corresponding deposited structures (PDB accession number 3IYN [9] and 4CWU [11]) using the program pdb2mrc and

low-pass filtered to the same resolution as the Ad5/FC31 maps. Atomic models were fitted to the experimental maps using UCSF (University of California, San Francisco) Chimera (64) after the scale of the experimental map was refined by cross-correlation with each reference map. Difference maps were calculated by subtracting the HAdV-C5 map from maps of the Ad5/FC31 light particles after all were filtered to the same resolution, refining the scale of the experimental map as described above, and normalizing the gray values. Subtractions were calculated, and the corresponding figures were obtained, with UCSF Chimera, using the HideDust tool for clarity when required.

Protein structure accession numbers. The Ad5/FC31 L2 and L3 cryo-EM maps were deposited in the Electron Microscopy Data Bank (EMDB [http://www.ebi.ac.uk/pdbe/emdb]) under accession numbers EMD-3003 and EMD-3004.

RESULTS AND DISCUSSION

Molecular characterization of light particles produced by Ad5/FC31. As indicated by previous studies, Ad5/FC31 produced three light-density bands (L1 to L3, in order of increasing density) when purified in a double CsCl gradient at 56 hpi (49) (Fig. 2). However, band L1 was found to have little reproducibility as its composition varied between purifications, and it was not further analyzed. To examine the possibility that the viral particles in bands L2 (buoyant density, 1.26 g/ml) and L3 (1.28 g/ml) were artifacts produced by degradation during purification, we obtained ultrathin sections of infected cells and measured the average electron density of viral particles present in the nuclei at 48 hpi (Fig. 3). This analysis indicated that capsids with electron-transparent interiors exist in both control and Ad5/FC31-infected cells, but the proportion of electron-transparent particles is higher in Ad5/FC31. That is, a larger amount of viral particles lacking the genome is already present in the Ad5/FC31-infected cell, and therefore L2 and L3 particles are genuine assembly products and not purification artifacts.

Denaturing protein electrophoresis and Western blot assays (Fig. 4A and B) showed that L2 and L3 particles contained the packaging protein L1 52/55k in two different stages of proteolytic maturation. In both cases, bands corresponding to the full-length protein and some proteolytic fragments were present. However, in L2 the full-length species was the most abundant, while in L3 up to three different proteolysis products were observed in larger amounts than the precursor protein. Similarly, L2 contained only the precursor version of polypeptide VI (pVI), while L3 contained both the mature version (VI) and the maturation intermediate, iVI, generated when pVI is cleaved at only one of two possible sites (65). These results indicate that L2 and L3 represent two different stages in the AdV proteolytic maturation, with L3 being further ahead than L2 in the process.

Both L2 and L3 contained the putative packaging ATPase IVa2 (Fig. 4B), but they lacked core protein V and carried no detectable DNA (L2) or only minimal amounts of a small, ~2-kbp fragment of viral origin (L3) (Fig. 4C). The AdV maturation protease is inactive in the absence of DNA (6–8, 18, 65). Therefore, the fact that L2 and L3 particles have undergone part of the maturation cleavages but are devoid of genome indicates that packaging must have started but not succeeded. It is possible that L2 and L3 particles have lost their partially encapsidated genomes due to inefficient tethering of the DNA to the packaging proteins, caused by the exogenous sequences flanking Ψ in Ad5/FC31. Since L3 particles have undergone more extensive proteolytic processing than L2 (Fig. 4A and B), it follows that they retained their genomes for a longer time (more efficiently) than the L2 particles. Therefore,

FIG 3 Analysis of particle electron density in infected cells indicates that Ad5/FC31 L2 and L3 particles are not purification artifacts. Viral particle arrays in ultrathin sections of Epon-embedded HEK293 cells infected with wild-type (A) or Ad5/FC31 (B) virus at 36 and 56 hpi, respectively. The proportion of DNA-containing, electron-dense (dark) particles is larger in panel A than in panel B. Scale bar, 250 nm. (C) Histogram quantifying the percentage of viral particles versus the electron density level at 48 hpi. Stronger electron density (darker) corresponds to more negative values. While in the wild-type (wt) strain there is a clearly dominant electron-dense population, for Ad5/FC31 the histogram shows more populated electron-transparent (larger average density) bins corresponding to light, genome-lacking particles.

Ad5/FC31 L2 and L3 particles are not packaging intermediates but abortive assembly products in which packaging and maturation were truncated at different stages of the process.

Structure of Ad5/FC31 L2 and L3 particles. When examined by negative-staining electron microscopy, L2 particles presented the typical aspect of AdV light particles (22, 35, 44), with a dark center indicating that the staining agent penetrates the protein shell and fills the absent core space (Fig. 4D). L3 particles, on the other hand, were indistinguishable from heavy particles, meaning that, in spite of lacking DNA and associated core proteins, these capsids are sealed, and the staining agent cannot penetrate. Cryo-EM images (Fig. 4E) showed that, in agreement with the negative-staining observations, some L2 particles seemed to lack capsid fragments (Fig. 4E, black arrows), while L3 capsids in general looked intact. To our knowledge, the Ad5/FC31 L3 genomeless, structurally complete capsids would be the first authentic HAdV-C5 virus-like particles (VLPs) described and, as such, an ideal candidate for the development of epitope display-based vaccines with low biosafety hurdles.

Comparing the structures of Ad5/FC31 light particles with the two high-resolution models for mature HAdV-C5. One of the main differences between the two alternative models for the structure of HAdV-C5 currently available, cryo-EM (9) and X-ray (11), is the identity of the proteins forming the pinwheel feature beneath the vertices. In the X-ray model, but not in the cryo-EM one, core protein V forms part of the pinwheel (Fig. 1 and 5A).

Since neither the L2 nor the L3 particles produced by Ad5/FC31 contain this protein (Fig. 4B), we fitted the complete X-ray model into our cryo-EM maps to assess whether these were lacking density at the proposed location for V. The X-ray model for V (11) contains 72 of the 368 residues in the protein in two fragments (208 to 219 and 236 to 295). A short helix formed by the smaller fragment, together with residues 236 to 273, forms a more or less compact, globular domain, while the rest of the traced residues adopt an extended structure. As shown in Fig. 5B, in both the L2 and L3 cryo-EM maps only part of the extended arm (residues 273 to 285) protrudes from the density, while the globular domain is completely covered by it. That is, more than 80% of the residues traced for V in the crystallographic structure are well covered by density in maps of particles lacking this protein. Furthermore, the density coverage for the traced polypeptide V fragment is practically the same in the Ad5/FC31 light-particle maps and in a cryo-EM map of the HAdV-C2 *ts1* mutant, which does contain the full genome and accompanying set of proteins (Fig. 5B) (63). Therefore, our results disagree with the assignment of polypeptide V to the pinwheel region, providing independent evidence to support the cryo-EM high-resolution model (9). Accordingly, we use this model for interpretation of our maps in the rest of this report.

Differences between mature HAdV-C5 and Ad5/FC31 incomplete particles. Despite the absence of genome and core proteins, both L2 and L3 particles contained some heterogeneous material inside, which in L2 tended to be close to the inner capsid

FIG 4 Molecular characterization of Ad5/FC31 light particles. (A) Silver-stained denaturing electrophoresis showing the proteins present in L2, L3, and heavy (H, mature virions) Ad5/FC31 particles purified at 56 hpi. (B) Purified viral particles analyzed by Western blotting against selected AdV proteins. Particles from the light and heavy CsCl gradient bands of HAdV-C5 wild-type (wt), Ad5/FC31 (FC31), and the thermosensitive HAdV-C2 mutant *ts1*, which does not undergo maturation and contains the precursor version of all AVP targets, were probed with the indicated antibodies. Similar amounts of particles ($\sim 6 \times 10^9$ virus particles) were loaded in each well. In the Western blot for L1 52/55k, the Ad5/FC31 L3 lane is shown with two different exposures (L3 and L3*) for better appreciation of the different bands. Signal for a previously described short isoform of the putative packaging ATPase IVa2 (73) is also observed in some cases, most notably in the Ad5/FC31 L2 particles. (C) Analysis of DNA extracted from purified Ad5/FC31 heavy (H) and light (L2 and L3) particles, as indicated. A nondenaturing electrophoresis in a 0.8% agarose gel (left) and its corresponding Southern blot probed with digested viral genome (right) are shown. A star (*) indicates the position of the full Ad5/FC31 genome; arrows indicate a DNA fragment between 2.5 and 2 kbp observed in L3. SphI, HAdV-C2 *ts1* genome digested with SphI enzyme; M, DNA size markers (bp). (D) Negative-staining EM images showing the overall structure of Ad5/FC31 L2, L3, and heavy particles, as indicated. (E) Cryo-electron micrographs showing Ad5/FC31 L2, L3, and heavy particles. In L2, a particle with a continuous layer of internal density is indicated with a white arrow, and disrupted particles are indicated with black arrows. Scale bar, 100 nm.

surface and occasionally appeared as a continuous layer (Fig. 4E, white arrow). Both the L2 and L3 3D maps presented a weak (but stronger than noise) internal layer of density, consistent with contents that were heterogeneous and disordered (Fig. 6A). Radial average profiles of the 3D maps (Fig. 6C) indicated that in L2 this layer was stronger directly beneath the capsid (radii of 220 to 320 Å). Conversely, in L3 the shell adjacent to the capsid presented a density minimum, while the strongest gray values for the disordered contents were found at lower radii (170 to 270 Å). The results described indicate that, although disordered, the contents in L2 particles are preferentially located close to the inner capsid

surface, suggesting their interaction with coat proteins. These interactions would be disrupted in L3, and the internal material falls from the outer shell to the center of the cavity inside the genome-less particle.

Difference maps calculated by subtracting a map created from the HAdV-C5 cryo-EM model (filtered at 12.3 Å) from our L2 and L3 maps revealed density present in Ad5/FC31 light particles but not in the EM model for the HAdV-C5 virion (Fig. 7). On the outer part of the capsid, these densities were limited to the fiber and some hexon loops, as expected, since these were not traced in the high-resolution model (9). Inside the particle, extra densities

FIG 5 Comparison of Ad5/FC31 L2 and L3 particles lacking polypeptide V with the two available structural models for the HAdV-C5 full virion. (A) Ribbon representation of the proteins assigned to the pinwheel feature located beneath the vertex region in the cryo-EM (EM) (PDB accession number 3IYN) and X-ray (XR) (PDB 4CWU) models of full, mature HAdV-C5. Polypeptide IIIa is depicted in yellow, V is in green, VI is in red, and VIII is in orange. (B) The pinwheel proteins from the cryo-EM or X-ray HAdV-C5 model are shown fitted into the Ad5/FC31 L2 and L3 cryo-EM maps, as indicated. For comparison, atomic models were also fitted to a previously published cryo-EM map of the HAdV-C2 *ts1* mutant (63), filtered to the same resolution as the Ad5/FC31 light-particle maps. This mutant, stalled at the immature state, contains the fully packaged genome and core proteins. The view is from inside the capsid, along a 5-fold symmetry axis. Most of the polypeptide V residues modeled in the X-ray study (except those in the stretch limited by two arrowheads, residues 273 to 285) are well covered by density in all three cryo-EM maps, including those of particles lacking this protein. The cryo-EM maps (in semitransparent gray) are contoured at 1.4σ above the average density (1.46σ for *ts1*). The position of the 5-fold axis is indicated by a black pentagon. Scale bar, 50 Å.

corresponding to the weak layers directly observable in the maps (Fig. 6) were detected. In L2 particles, the low-density shell located directly beneath the capsid surface appeared stronger close to the vertex region, as shown by isosurface representations of the difference map at a threshold of 1.5σ (Fig. 7A and B, thin arrow). An isosurface is generated by depicting voxels where the density values (gray level) cross a certain threshold. A threshold of 1.5σ means that all voxels enclosed by the isosurface have density values at least 1.5 times the standard deviation above the mean density value of the map. These are the strongest differences between the Ad5/FC31 L2 particle and the mature virion. This point was further corroborated by calculation of the L2 particle radial average profile after two complementary sets of conical masks were applied: one that excludes the vertices and another one that conserves only the vertex regions and excludes the rest of the particle (Fig. 6D). The density in the shell adjacent to the inner capsid surface (radii of 220 to 320 Å) is higher in the masked map con-

taining only the vertices than in the rest, with density values similar to those of the fiber (radii of 410 to 440 Å). This observation indicates that the disordered material, although not following icosahedral symmetry, binds preferentially to internal vertex components. Additionally, at the same threshold (1.5σ above the map average density), small differences were found within all internal hexon cavities and lying directly on the inner capsid surface (Fig. 7B, thick arrow and arrowhead, respectively). At a low threshold (0.75σ) the difference density completely filled the hexon cavities, and all the observed features connected, giving rise to an 80-nm-thick inner shell. Conversely, in L3 the vertex region is devoid of difference density (Fig. 7C, circle, and E), while other features observed in L2 are present. These include the differences within the hexon cavity and on the inner capsid surface (Fig. 7D, thick arrow and arrowhead, respectively). As in L2, at a low threshold the L3 difference density fills all hexon cavities and connects with the small features on the inner capsid surface, forming a network

FIG 6 Cryo-EM reconstructions of Ad5/FC31 L2 and L3 particles. (A) Central sections of the Ad5/FC31 L2 and L3 maps, as indicated. The view is along a 2-fold symmetry axis. A central section of an HAdV-C2 *ts1* mutant map is also shown for comparison, with the positions of one 5-fold and one 2-fold icosahedral symmetry axis indicated by a white pentagon and a white oval, respectively. All maps are low-pass filtered to 12.3-Å resolution, and higher density is shown in white. Scale bar, 200 Å. (B) Resolution curves for all maps used in this study. The FSC threshold of 0.143 is indicated by a dotted line. (C) Radial average profiles of the Ad5/FC31 L2 and L3 maps, compared to the profile of the full HAdV-C2 *ts1* map. (D) Radial average profile of the complete Ad5/FC31 L2 particle (L2_ALL; the curve is the same as that in panel C) compared to radial average profiles calculated from the same map after masks are applied that either exclude the vertex regions (L2_NO_VERTICES) or conserve only the vertex regions and exclude the rest of the particle (L2_VERTICES).

that contacts hexons at multiple points but not the pinwheel proteins beneath the vertex (Fig. 7D, E, and G). A thicker layer of weak density appears at more internal radii, disconnected from the icosahedral shell and the difference densities on the inner capsid surface (Fig. 7D and F).

The main difference in molecular composition between L2 and L3 is the maturation state of proteins L1 52/55k and VI (Fig. 4A and B). The packaging protein L1 52/55k can form homo-oligomers and links shell (IIIa) to core (VII, dsDNA, and AVP) components (17, 37, 40–42). Polypeptide VI (copy number, 360 [66]) binds to hexon and to dsDNA (67, 68), and its C-terminal peptide is a cofactor for the virus protease (65). The HAdV-C5 *ts147* mutant is defective for hexon nuclear import, probably due to lack of

proper interaction between hexon and polypeptide VI. Modeling of hexon structural changes in *ts147* suggested that VI binds to the internal cavity of the hexon trimer (69). Polypeptide VI is not icosahedrally ordered, and only weak density for small fragments of VI has been observed in previous structural studies (9, 11). Of these two proteins, L1 52/55k suffers the most drastic changes upon maturation, with up to 14 potential cleavages which disrupt its links with capsid and core, leading to its eventual removal from the mature particle (40). Polypeptide VI (250 residues) is cleaved at its N and C termini (33 and 11 residues, respectively) but remains in the mature particle, and its only structural change resulting from maturation detected so far is a lower degree of icosahedral ordering in the hexon-interacting region (8, 63, 70).

FIG 7 Differences between Ad5/FC31 light particles and the cryo-EM HAdV-C5 high-resolution model. (A and B) Differences showing additional density in L2. (C and D) Additional density in L3. The HAdV-C5 map (gray) is contoured at 1 standard deviation above the average map density (1σ), and the difference maps (blue) are contoured at the indicated thresholds, chosen to show similar features in L2 and L3. The highest contour levels reveal the strongest differences. Panels A and C show the back half of the viral particle cut open for visualization of the internal difference density. In panels B and D only a slab is shown for further clarity. The view is along a 2-fold symmetry axis. Pentagons, triangles, and ovals indicate the positions of the 5-fold, 3-fold, and 2-fold icosahedral symmetry axes. The thick and thin arrows, circle, and arrowhead point to different details of the difference map discussed in the main text. f, fiber; hl, hexon loops. (E, F, and G) Details of the L2 and L3 difference maps, with the various proteins in the HAdV-C5 cryo-EM model in different colors: IIIa in yellow, VIII in orange, penton base in pale pink, peripentonal hexons in pale cyan, and other hexons in pale tan. The view in panel E is from inside the particle along a 5-fold axis; panel F shows a section across the vertex, and panel G shows a complete facet (delineated with a white triangle) as seen from inside the L3 particle. Difference maps in (E to G) are contoured at 1.6σ except for the map of L3 in panel F, which is at 1.2σ . Scale bars, 100 Å (A to D) and 50 Å (E to G).

FIG 8 Schematics depicting the interpretation of the difference maps. Cartoons showing a cross-section of the L2 (A) and L3 (B) particles with symbols for pVI/VI in red and L1 52/55k in blue, using a darker shade for copies bound to the vertex region (IIIa, yellow).

Based on these considerations, we attribute the densities in common in the L2 and L3 difference maps, that is, differences within hexon cavities connected to a network of weak density on the inner hexon surfaces, to polypeptide VI (Fig. 7G and 8, red symbols). This assignment is consistent with previous identifications of short VI fragments within or near the hexon cavity (9, 11, 63, 70). The weak network in contact with hexon inner surfaces, presumably formed by the rest of the polypeptide VI chain, would have been difficult to visualize in studies of genome-containing particles where the noisy core would have obscured its signal.

The rest of the differences, making up the thick shell connected to the capsid in L2 but disconnected in L3, would correspond to L1 52/55k (Fig. 8, blue symbols). The molecular mass of HAdV-C5 L1 52/55k is 47 kDa. It has been reported that approximately 100 copies of L1 52/55k are present in HAdV-C5 wild-type light-density particles (39). Considering an average protein specific volume of $0.73 \text{ cm}^3/\text{g}$, this copy number would give a volume of $5.7 \times 10^6 \text{ \AA}^3$. The volume occupied by the L2 difference density within a 350-Å-radius sphere, when rendered at 1.5σ above the map average value (such as in Fig. 7A) is $5.0 \times 10^6 \text{ \AA}^3$, in good agreement with the volume expected for 100 copies of full-length L1 52/55k. When the rendering threshold is lowered to include weaker density (as in the rightmost panel in Fig. 7B), the volume occupied by the difference density is $74.4 \times 10^6 \text{ \AA}^3$. This does not mean, however, that there would be 1,000 instead of 100 copies of L1 52/55k per particle because the calculation does not take into account the density level of the voxels included. This density is weak, reflecting variable occupancy and lack of symmetry in L1 52/55k. This kind of estimation contains a great deal of uncertainty, given the difficulty to establish the exact copy number and volumetric limits of a protein not following icosahedral symmetry and most likely having a different copy number in each virus particle in a preparation containing particles with different disruption degrees (Fig. 4). We conclude that the size of the difference regions assigned to full-length L1 52/55k in L2 particles is within the range expected for the protein mass, with all the caveats explained above. The strongest difference density beneath the vertices in L2, spanning the pinwheel feature (Fig. 7E and F), is consistent with previously observed interactions between L1 52/55k and polypeptide IIIa (17) and with polypeptide IVa2, reported to be present at a single vertex (24, 41). However, our results do not indicate binding of L1 52/55k at a singular vertex but at a variable number of them. The formation of a thick shell starting from this preferential interaction with the vertices is consistent with the L1 52/55k homo-ol-

gomerization properties (40) (Fig. 8A). Lack of L1 52/55k density beneath the vertices in L3 suggests that the full-length protein is required to establish interactions with IIIa. Cleaved L1 52/55k fragments are no longer able to interact with capsid proteins or with each other (40) and fall to the center of the empty particle, where they remain trapped (Fig. 8B).

Implications for the role of L1 52/55k in adenovirus assembly. Based on our results and on previous research, we propose the following model for the role of L1 52/55k in adenovirus assembly. First, full-length L1 52/55k would bind to IIIa/IVa2 (17, 41) at one or more capsid vertices, growing from there a disordered shell made up by more L1 52/55k molecules (40). L1 52/55k at the vertices would be poised to engage incoming genomes, located close to other packaging proteins (IVa2) (24) that would help complete packaging. The thick L1 52/55k shell would act like Velcro, tethering the incoming genome to the capsid. As packaging proceeds, proteolytic processing would release L1 52/55k from the capsid shell, the genome, and other L1 52/55k molecules (40). In this way, L1 52/55k is removed from the virus particle, and links between capsid and core are eliminated so that the genome is free to be released at the nuclear pore in the last stages of uncoating. In the HAdV-C2 *ts1* mutant, however, where maturation does not occur, fully packaged immature particles contain a considerable amount of unprocessed L1 52/55k (40), and the core remains attached to capsid fragments even under highly stressful conditions at which the genome is completely lost from wild-type virions (71, 72).

Conclusions. We present a molecular and structural study of two different kinds of human AdV light-density particles presenting different maturation states of the packaging protein L1 52/55k and polypeptide VI. Lack of core protein V in these particles allowed assessment of the two alternative models for the location of minor coat proteins in the mature capsid, providing evidence to support the cryo-EM model. We show for the first time the organization of a component of the adenovirus packaging machinery in the viral particle and its changes upon partial maturation.

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