Cryo-EM structures of two bovine adenovirus type 3 intermediates

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A B S T R A C T
Adenoviruses (Ads) infect hosts from all vertebrate species and have been investigated as vaccine vectors. We report here near-atomic structures of two bovine Ad type 3 (BAd3) intermediates obtained by cryo-electron microscopy. A comparison between the two intermediate structures reveals that the differences are localized in the fivefold vertex region, while their facet structures are identical. The overall facet structure of BAd3 exhibits a similar structure to human Ads; however, BAd3 protein IX has a unique conformation. Mass spectrometry and cryo-electron tomography analyses indicate that one intermediate structure represents the stage during DNA encapsidation, whilst the other intermediate structure represents a later stage. These results also suggest that cleavage of precursor protein VI occurs during, rather than after, the DNA encapsidation process. Overall, our results provide insights into the mechanism of Ad assembly, and allow the first structural comparison between human and nonhuman Ads at backbone level.

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Introduction

Adenoviruses (Ads) are nonenveloped, double-stranded DNA (dsDNA) viruses that can cause mild respiratory, gastrointestinal and eye infections in a variety of vertebrates, including humans, mammals, reptiles, amphibians, and fish (Davison et al., 2003; San Martin, 2012). In addition, Ads have a great potential as vectors for vaccination (Lasaro and Ertl, 2009). In addition, Ads have a great potential as vectors for vaccination (Lasaro and Ertl, 2009). The Ad dsDNA genome, together with four core proteins (V, VII, mu, and terminal protein) and multiple copies of Ad protease, is packaged in an icosahedral capsid of ~920 Å diameter, not including 12 flexible, trimeric fibers on icosahedral pentameric vertices. The Ad capsid consists of three major capsid proteins (hexon, penton base and fiber), as well as four minor capsid proteins (IIIa, VI, VIII and IX) (Liu et al., 2010; Reddy et al., 2010; Saban et al., 2006). All Ads with known structures share a similar capsid architecture, in which 240 trimeric hexons form 20 facets of the icosahedral capsid and 12 penton bases and 12 trimeric fibers form the 12 icosahedral vertices (San Martin, 2012). The fibers are of various lengths depending on the serotype, and are associated with different Ad receptors (Cao et al., 2012). The minor capsid proteins are located on the 20 capsid facets and form protein-protein networks to enhance the stability of the Ad capsid (Liu et al., 2010). The assembly of the Ad virion is believed to begin with a preassembled empty capsid formed by capsid proteins and precursor capsid proteins, followed by the insertion of the viral genomic DNA and core proteins (Ostapchuk and Hearing, 2005). It has been shown that interactions between the N-terminal domain of minor capsid protein IIIa and an Ad non-structural protein 52/55k are required to promote correct genome packaging (Ma and Hearing, 2011). The precursor capsid proteins are thought to be cleaved into the mature forms by an Ad protease following the DNA encapsidation process, an important step for the formation of infectious Ad particles (Mangel et al., 1997).

Bovine adenovirus type 3 (BAd3) is a nonhuman adenovirus and has a pathogenic effect on the respiratory tract of cattle (Darbyshire et al., 1968, 1965). Like all known human Ads, BAd3 belongs to the genus Mastadenovirus (Davison et al., 2003). The protein sequence identities between BAd3 and other human Ads (HAds) range from 23% to 70% (Reddy et al., 1998). Among HAds, human Ad type 5 (HAd5) is the most frequently used one in Ad-based vaccine studies. However, clinical use of HAd5 vectors is severely hampered by the widely prevalent, pre-existing immunity against HAd5 in the...
majority of the human population. To overcome pre-existing immunity against HAd5 vector vaccines in human beings (Barouch et al., 2004; Catanzaro et al., 2006), BAd3 and other nonhuman Ads have recently been developed and investigated as vaccine vectors (Bangari and Mittal, 2006; Singh et al., 2008).

Near-atomic resolution structures of human Ads have been reported recently (Cao et al., 2012; Liu et al., 2010; Reddy et al., 2010). Earlier studies also have resolved the structures of canine and ovine Ads at approximately 12 Å resolution (Pantelic et al., 2008; Schoehn et al., 2008). However, it is not possible to resolve α-helices and β-stands at such resolution. Because of this discrepancy in resolution, a direct structural comparison between human and nonhuman Ads at a comparable high resolution is not feasible. Here, we report two 3D structures of two BAd3 intermediates at near-atomic resolution determined by cryo-electron microscopy (cryo-EM) and single-particle reconstruction. The 3D reconstructions permit traces of the main chains of the hexon, penton base, IX, and VIII proteins. Our structural comparison between the two intermediates reveals that the differences are localized within the fivefold vertex region. In contrast, the facet structures of the two intermediates are shown to be identical. Based on mass spectrometry and cryo-electron tomography (cryo-ET) analysis, we propose that one intermediate structure represents the stage during DNA encapsidation, whilst the other intermediate structure represents a later stage in the development of BAd3 or partially degraded mature virions during sample preparation. These results also suggest that the cleavage of precursor protein VI occurs during, rather than after, the DNA encapsidation process. Structural comparison between BAd3 and human Ads with known structures (Cao et al., 2012; Lindert et al., 2009; Liu et al., 2010; Reddy et al., 2010) reveals that, except for loops in the outer surface of the capsid, the hexon, penton base, and VIII proteins of BAd3 exhibit nearly identical structures to those of human Ads. The most distinct structural difference between BAd3 and human Ads is that BAd3 protein IX exhibits a unique conformation, suggesting a different structural mechanism for capsid stability.

Results

Structure determination

BAd3-infected Madin–Darby Bovine Kidney (MDBK) cells were lysed 48 h after initiation of infection, and subjected to CsCl density gradient centrifugation. Two bands of BAd3 particles were observed in the gradient (Supplementary Fig. 1A). Cryo-EM images of the two bands of particles were collected using an FEI Titan Krios electron microscope. The 3D structure of particles isolated from the lighter band (LBPs) was reconstructed from 11,910 particle images and the 3D structure of particles from the heavier band (HBPs) was reconstructed from 28,505 particle images (Fig. 1A, B and Supplementary Fig. 1B). Icosahedral symmetry was enforced in the two reconstructions by using the Symmetry Adapted Spherical Harmonics method (Liu et al., 2008). The resolution of both structures was estimated to be approximately 4.5–5.0 Å based on the observation that all β-stands were separated or partially separated and that some distinctive side chains on loops and helices could be identified in both structures (Fig. 1A and B). Density maps of LBPs and HBPs allow us to build backbone models of the hexon, penton base, IX and VIII proteins. A structural comparison of LBPs and HBPs reveals that the differences are localized in the fivefold vertex region, while the facet structures of LBPs and HBPs are shown to be identical at the resolution of the reconstruction (Fig. 1A and B).

Overall structure of LBPs

The overall capsid structure of LBPs appears to be intact (Fig. 1A). The fibers present on the fivefold vertex are flexible, so only the bottom part, which interacts with the penton base, could be resolved (Fig. 1A). The 240 trimeric hexons and 12 pentameric penton bases dominate the capsid and exhibit an organization similar to that of HAd5 (Liu et al., 2010; Reddy et al., 2010; Saban et al., 2006). The icosahedral asymmetric unit of BAd3 contains four trimeric hexons, referred to as hexons 1–4 according to their locations in the asymmetric unit (Burnett, 1985). Hexon 1 is peripental, and hexons 2, 3, and 4 are referred to as group of nine (GON) hexons because each icosahedral facet contains 3 each of hexons 2, 3, and 4 (Figs. 1 and 2A). The 4 hexons in the asymmetric unit are shown to be identical at the resolution of the reconstruction, permitting us to average them to improve the signal-to-noise ratio. A structural comparison between the BAd3 and HAd5 hexons reveals that, except for loops in the outer surface of the capsid, they are almost identical (Fig. 1C). These loops belong to the hypervariable regions of the hexon protein from different Ad serotypes (Crawford-Miksza and Schnurr, 1996) and may reflect different cell tropisms. Among the GON hexons on the icosahedral facet of the capsid, there are three helix coiled-coils located at an icosahedral threefold axis and three local threefold axes (Figs. 1A and 2A, B). These helix bundles can be attributed to C-terminal domains of three copies of protein IX (described below).

The penton base protein structure in LBPs was not as well-resolved as that of the hexon protein, but the backbone model could still be traced (Supplementary Fig. 2A). Consistent with the 55% amino acid sequence identity between the BAd3 and HAd5 penton base proteins, fitting the atomic model of the HAd5 penton base protein (Liu et al., 2010) into its counterpart in the LB structure shows that they are almost identical except for a small number of loops.

Overall structure of HBPs

The structures of the icosahedral facets are shown to be identical at the resolution of the reconstruction for both LBPs and HBPs. However, in stark contrast to the structure of LBPs, the penton base and fiber are not visible in the structure of HBPs when the display contour is set to the normal level of 2.0σ, where σ is the standard deviation of the density map (Fig. 1B). The structure of HBPs appears to be “penton-less”; however, when it is filtered to 7 Å resolution with the display contour set to 1.2σ, a turret-like structure is visible on each fivefold vertex (Fig. 1D). The turret-like penton base consists of some rod-like structures and is smaller than the penton base in LBPs. No fiber was observed on the turret. The length of the longest rod on the fivefold vertex of the intermediate particle is almost identical to that of the longest α-helix (residues 166–186) in the penton base protein (Fig. 1E), implying that the turret-like structure is the conformationally changed penton base proteins. It is also possible that the turret-like structure is an internal capsid protein that takes the place of the penton base in the HBPs.

Interaction between proteins IX and hexon protein

Except for the fivefold vertex region, the capsid facets of LBPs and HBPs are identical (Fig. 1A and B). We observed some helix bundles located at the icosahedral threefold axis and three local threefold axes in the capsid facet (Figs. 1A, and 2A, B). These helix bundles were not observed at the corresponding locations in the capsid of HAd5 (Liu et al., 2010; Reddy et al., 2010). We assigned these helix bundles to the C-terminal segments of the three copies...
of protein IX. These trimers of protein IX that are located at each icosahedral threefold axis, and the three local threefold axes interact with GON hexons in the icosahedral facet (Fig. 2A).

In support of this C-terminal segment assignment, predictions by both the MULTICOILS and PAIRCOIL2 web servers (McDonnell et al., 2006; Wolf et al., 1997) reveal a strong propensity for coiled-coil formation by the C-terminal domain (residues 76–110) of the BAd3 protein IX. Earlier cryo-EM structures of HAd5 revealed that the C-terminal segments of the HAd5 protein IX present as a four-helix coiled-coil on the outer capsid surface of HAd5 (Liu et al., 2010; Saban et al., 2006). In contrast, no four-helix coiled-coil structure was observed in the corresponding locations in our BAd3 capsid. We also observed some densities attached to the distal ends of the coiled-coil in the capsid facet (Fig. 2B and Movie S1). These densities can be assigned to three C-terminal loops (after residue 110) of three protein IX monomers. Each of the three loops bridges a helix of the coiled-coil with a loop (residues 170–172) of hexon protein (Fig. 2B). The interactions between the protein IX at the icosahedral threefold axis and three copies of hexon 3 are essentially identical to the interactions of protein IX that are located at each icosahedral threefold axis, and the three local threefold axes interact with GON hexons in the icosahedral facet (Fig. 2A).

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Fig. 1. Structures of BAd3 LBP s and HBP s. The scale bar and color radial density scale apply to both panel A and panel B. (A) Radially-colored shaded-surface representation of LBP s (the display contour is 2.0σ) viewed along the twofold axis filtered at 7 Å resolution, in which four hexons in an asymmetric unit are labeled 1–4 and four coiled-coils are circled (left). A zoomed-in view of a pentameric region in LBP structure (middle). Density map (mesh) from LBP hexon superimposed on its atomic model (right). Some distinctive residues are labeled. (B) Radially-colored shaded-surface representation of HBP s (display contour 2.0σ) viewed along the twofold axis filtered at 7 Å resolution (left) and a zoomed-in view of a pentameric region in HBP structure (middle). Density map (mesh) from HBP hexon superimposed on its atomic model (right). (C) Density map (transparent density map) of a BAd3 hexon superimposed on an atomic model of HAd5 hexon. The three hexon protein monomers (ribbon) of HAd5 are in red, blue, and green. (D) Zoomed-in view of the fivelfold vertex region in HBP s (display contour 1.2σ). (E) Transparent view of the turret-like structure, in which five longest α-helices (residues 166–186 in the penton base protein) can be fitted.
between the protein IX at the local threefold axis and hexons 2, 3, and 4 (Fig. 2A). This structural organization of protein IX serves to tightly rivet the GON hexons together and forms a network of interactions in the icosahedral capsid (Fig. 2A), which may be essential to capsid stability.

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The N-terminal domain of BAd3 protein IX exhibits a nearly identical conformation to its counterparts in HAd5. Most side chains in the N-terminal segment of protein IX were clearly resolved while the side chains in the C-terminal helix were not resolved (Fig. 2C).

**Protein VIII located on the inner capsid of LBPs and HBPs**

There are two copies of protein VIII in the asymmetric unit, which serve as cementing proteins on the inner surface of the Ad capsid. One is located around the fivefold axis bridging the peripentonal hexon and the GON, and the other is located around the threefold axis and interacts with hexons 2, 3, and 4 to stabilize the GON hexons (Liu et al., 2010). On the inner surface of the HBP capsid, protein VIII is absent around the fivefold axis, while the protein VIII around the threefold axis is resolved clearly. On the inner surface of the LBP capsid, protein VIII around the fivefold axis is absent. Only the head domain of protein VIII (residues 4–61 and 170–214, according to the domain nomenclature of HAd5 protein VIII) around the threefold axis in both LBPs and HBPs was resolved (Supplementary Fig. 2C and D). The presence or absence of protein VIII around the fivefold axis may be related to the association or disassociation of the peripentonal hexons in the Ad capsid.

**Protein VI under the hexons in LBPs and HBPs**

Two loop-like structures are visible at the rim of the cavity entrance of all four hexons in the structures of both LBPs and HBPs (Fig. 3A). We assigned this loop to a segment of protein VI based on an established model of the HAd5 capsid (Saban et al., 2006). The backbones of the loops are resolved so that the zigzag backbone is visible, and the distance between two adjacent Cα was measured as 3.8 Å (Fig. 3B).

**Protein IIIa on the inner capsid of LBPs and HBPs**

BAd3 protein IIIa shares 52% sequence identity with its counterpart in HAd5. Just as in HAd5, BAd3 protein IIIa is located under the penton bases and peripentonal hexons around the fivefold axis. Protein IIIa has four domains, named the GOS-glue, connecting-helix, VIII-binding and core proximal domains according to HAd5 nomenclature (Liu et al., 2010). In both the LBP and HBP structures, only four helices in the GOS-glue and connecting-helix domains were resolved, while the VIII-binding and core proximal domains of protein IIIa were missing, probably due to their high structural flexibility. The four resolved helices in the LBP capsid fit well with those of HAd5 protein IIa (Fig. 3C and Movie S2). In contrast, the helix in the connecting-helix domain of the HBP capsid appeared to be slightly rotated compared to its counterpart in HAd5 (Fig. 3D and Movie S2). We propose that this structural difference is associated with the removal of the penton base, because this helix interacts with the N-terminal loop of the penton base protein in the LBP structure.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.virol.2013.12.012.

**Characterization of the infectiousness of LBPs and HBPs**

To characterize the biochemical properties of the two types of BAd3 particles, plaque assays were performed using MDBK cells, which were incubated with either LBPs or HBPs, and processed as described in the Methods section. Partially packed or empty virus particles were observed in the endosomes of cells incubated with LBPs. In contrast, virus particles were absent in cells incubated with HBPs (Supplementary Fig. 3). Importantly, neither LBP nor HBP-incubated samples formed viral plaques even after an extended incubation time of 7 days, indicating that LBPs can be internalized into endosomes but fail to replicate, while HBPs cannot internalize into endosomes. Taking into consideration the roles of the penton base and fibers, these results are consistent with our structural analysis. The LBPs, whose genomic DNA are...
partially packed, contain fibers and penton bases, and the HBPs have their penton bases and fibers missing. The plaque assays performed also indicated that no virion particles were mixed in with LBPs and HBPs. In addition, we observed a very weak band in our CsCl gradients. This weak band, when added to MDBK cells, was able to generate plaques, suggesting that these particles represent fully assembled BAd3 virions.

Cryo-ET analysis of LBPs and HBPs

The results of the plaque assays and the ultrathin sectioning observations of cells incubated with LBPs and HBPs suggest that neither LBPs nor HBPs contain virions. These results motivated us to perform cryo-ET analysis of LBPs and HBPs in an attempt to observe the structures in the absence of icosahedral symmetry. The central sections of the cryo-ET reconstructions show that there are 1 or 2 breaches in some capsids of LBPs, indicating that there are some channels of approximately 170–210 Å in diameter in the capsid (Fig. 4A). Moreover, pentagonal channels can also be observed in the central sections of some LBP capsids (Fig. 4B and Supplementary Fig. 4), indicating that these channels are located at the fivefold vertex. The size of the channels indicates that they arise from the missing penton bases and their peripentonal hexons at the fivefold vertex. The averaged structure from 31 cryo-ET reconstructions of LBPs (no symmetry was imposed) further confirmed that the channels are pentagonal and that peripentonal hexons are not present around the channel (Fig. 4C). The cryo-ET reconstruction of LBPs indicated that the single-particle reconstruction of LBPs, whose 12 fivefold vertices all appear to be intact (Fig. 1A), was caused by enforcing icosahedral symmetry during single-particle reconstruction. The fivefold vertex structure of LBPs by single-particle reconstruction is an averaged structure of all 12 fivefold vertices. As a result, the reconstruction does not reflect these fivefold channels. The central section of the cryo-ET reconstruction shows that the core of HBPs, which consists of genomic DNA and core proteins, appears to be more condensed than that in the central section of LBPs, indicating that HBPs contain more DNA or core proteins than LBPs (Fig. 4A and D).

SDS-PAGE and mass spectrometry analyses of LBPs and HBPs

We identified the precursor protein/proteins VII, 52k, and VI in LBPs and HBPs using mass spectrometry and SDS-PAGE (Fig. 5). The results showed that HBPs contain more core protein VII than LBPs. The results are in agreement with our cryo-ET analysis, which shows that the core of HBPs is more condensed than that of LBPs (Fig. 4A and D). In contrast, LBPs contain more 52k protein than HBPs (Fig. 5). The 52k protein of BAd3 is a homolog of the 52/55k protein in human Ads (Reddy et al., 1998). Previous studies of human Ads indicated that the 52/55k protein plays a role in encapsidating genomic DNA and is gradually lost as the Ads mature into virions (Gustin and Imperiale, 1998; Hasson et al., 1992). In addition, three of the protein bands in LBPs were identified as protein VI-associated polypeptides, while only one protein band in HBPs was identified as a protein VI-associated polypeptide (Fig. 5). Protein VI is produced in a precursor form and is cleaved at both the N-terminal and C-terminal sites by an Ad protease during Ad maturation. Cleavage at the two sites does not
proteins according to their molecular masses. bands in the two lanes were identified using mass spectrometry. The remaining protein bands in the two lanes were identified as the fiber, hexon, IIIa, penton base, and VIII proteins according to their molecular masses.

Discussion

Genomic DNA and core proteins of Ad are thought to be packed into a preassembled capsid through a fivefold channel (Christensen et al., 2008). The presence of the protein VI-associated polypeptide and 52k protein, and the amounts of protein VII and DNA in LBPs suggest that LBPs are assembly intermediates undergoing DNA encapsidation. It is intriguing that our cryo-ET analysis of LBPs reveals some channels at the fivefold vertex in which not only the penton base, but also the peripentonal hexons are absent. Because we did not observe the same channels in the cryo-ET reconstructions of HBPs, and given that both LBPs and HBPs were obtained using the same purification protocol, it is unlikely that these channels were introduced during the purification process. We suggest that at least one of the channels is for DNA and core proteins encapsidation (Fig. 4C). Given that protein IIIa is attached to the peripentonal hexons on the inner capsid surface (Liu et al., 2010), we reasoned that the protein IIIa molecules surrounding the channels of LBPs are also likely to be absent. Our structure excludes the possibility that the protein IIIa around the channel is available for interaction with core proteins outside the capsid during the DNA encapsidation (Ma and Hearing, 2011), but the flexible domains of protein IIIa located around the other fivefold vertices in our LBP structure imply that these protein IIIa molecules might play a role in DNA encapsidation.

Our plaque assays and ultrathin sections of cells have demonstrated that LBPs did not contain virions. Thus, the coexistence of precursor protein VI, pVIΔC, protein VI, and 52k protein in LBPs undergoing DNA encapsidation suggests that the cleavage of precursor protein VI occurs during, rather than after, the DNA encapsidation process. The identification of three protein VI-associated polypeptides with different molecular masses is consistent with previous biochemical results that the cleavage of precursor protein VI at the two sites does not occur simultaneously (Diouri et al., 1996). It also implies that the Ad protease is packaged within the preassembled capsid at the early stage of DNA encapsidation.

The cryo-ET and mass spectrometry analyses indicate that HBPs contain more DNA and protein VII than LBPs. The missing penton base and fiber might suggest that it is a disassembly intermediate, but on the other hand, the presence of a low level of the 52k protein in HBPs suggests that this might be a later assembly intermediate. Moreover, because we did not obtain a clear band of virions from the CsCl gradient, it is also possible that they are partially degraded mature virions during sample preparation. The structure of HBPs indicates that components of the BAd3 shell conserve the icosahedral order when the capsid is partially disrupted or incomplete.

Although neither the RGD nor the LDV motif, which are involved in binding of integrins at the host cell membrane, were observed in the sequence of the BAd3 penton base, the LDV motif seems to be replaced by an MDV motif (Reddy et al., 1998). Earlier structural studies of human Ad types 2, 3, and 5 showed that the fiber tail interacts with the grooves between adjoining penton base proteins (Cao et al., 2012; Fuschietti et al., 2006; Liu et al., 2011; Zubieta et al., 2005). Similar fiber-penton base interactions were also observed in our LBP structure (Supplementary Fig. 2B). This conservation is also reflected by the conserved amino acid residues 9–15 (FNLVYPP) in the BAd3 fiber tail involved in the interaction. The high structural similarity of penton base and penton base-fiber interaction between the BAd3 and HAd5 suggest that BAd3 has an internalization mechanism similar to HAd5.

The C-terminal domains of BAd3 protein IX form four three-helix coiled-coils located at an icosahedral threefold axis and three

Fig. 4. Cryo-ET analysis of LBPs and HBPs. (A) Breaches in some particles can be observed in a central section from the cryo-ET reconstruction of LBPs. (B) Pentagonal channels along the fivefold axis can be observed in a central section from the cryo-ET reconstruction of LBPs. (C) Averaged channel structure from cryo-ET reconstructions further confirms that the channels are pentagonal. (D) A central section from the cryo-ET reconstruction of HBPs.

Fig. 5. SDS-PAGE of the LBPs (left) and HBPs (right). The 52k, VI, VII, and IX proteins in LBPs and HBPs were identified using mass spectrometry. The remaining protein bands in the two lanes were identified as the fiber, hexon, IIIa, penton base, and VIII proteins according to their molecular masses.
local threefold axes in contrast to those of human Ads. Amino acid sequence alignment between protein IX homologs of HAd5 and BAd3 shows that the protein IX of BAd3 is shorter than its homolog in HAd5. The reduction in length renders the C-terminal segment of BAd3 protein IX unable to traverse two hexagon edges before forming the four-helix coiled-coils, as observed for the HAd5 homologue (Liu et al., 2010). Similar structural organization of protein IX homologs was also observed in the structures of canines Ad (Schoehn et al., 2008) and ovine Ad (Pantelic et al., 2008). However, due to the limited resolution in those reconstructions, the C-terminal helix and loop of protein IX in these structures could not be resolved with the required detail. The structural organization of protein IX may represent a major difference between human and nonhuman Ads. Consistent with our structure of the exposed C-terminal helix and loop of protein IX, incorporation of the RGD motif into the C-terminus results in significant augmentation of BAd3 fiber knob-independent infection of integrin-positive cells (Zakhartchouk et al., 2004), offering an opportunity to insert specific ligands to improve or alter transduction by BAd3 vectors. Our backbone model of BAd3 capsid proteins provides structural basis for designing better vectors based on this nonhuman virus.

Materials and methods

\textbf{BAd3 culture and purification}

MDBK cells were cultured to 80% confluence in 100-mm petri-dishes, and then infected with BAd3 at a MOI of 0.05. Cells were harvested 48 h after infection and freeze-thawed three times. Cellular organelles and debris were removed from the freeze-thawed lysates by centrifugation at 10,000 g for 1 h at 4°C, and the virus particles were concentrated from the supernatant by centrifugation at 50,000 g for 90 min at 4°C. The pellet was resuspended in 1 ml PBS and applied to a linear CsCl density gradient (0.454 g/ml). Ultracentrifugation was performed at 180,000 g for 12 h at 4°C. Two bands of virus particles were visible in the CsCl gradient and were collected for cryo-EM analysis.

\textbf{Cryo-EM, cryo-ET, 3D reconstruction, and atomic model building}

A 3.5 μl aliquot of the LBP or HBP sample was applied to a holey grid and blotted for 4 s in a chamber at 80% humidity using an FEI Vitrobot Mark IV. Viruses were imaged with an FEI Titan Krios cryo-electron microscope equipped with a Gatan UltraScan4000 (model 895) 16-megapixel CCD. Viruses were imaged at 300 kV at an absolute magnification of 125,390×, corresponding to a pixel size of 1.196 Å. The dose for each micrograph was approximately 20–25 e−/Å². The microscope was carefully aligned before image collection so that a maximum visible contrast transfer function ring of a carbon film image (20–25 e−/Å² dose) at ~1/3 Å⁻² spatial frequency was observed. The defocus value was set approximately 1.0–3.0 μm for all micrographs. The contrast transfer function correction for each micrograph was determined using the CTIFIT program from the EMAN package (Ludtke et al., 1999) based on incoherently averaged Fourier transforms of each image. The orientations and centers of all virus particles were determined using the IMIRs package (Liang et al., 2002) based on the common line strategy (Fuller et al., 1996). Selected particle images were combined and reconstructed using a reconstruction program based on icosahedral symmetry-adapted functions (Liu et al., 2008). The structure of the LBPs was reconstructed by cryo-EM and single-particle reconstruction using 11,910 particle images selected from 1275 cryo-EM images, and the structure of the HBPs was reconstructed using 28,505 particle images selected from 3585 cryo-EM micrographs.

A cryo-ET tilt series of the viral sample was taken using an FEI Titan Krios 300 kV electron microscope (FEI) with the Gatan UltraScan4000 CCD at 2k × 2k pixels mode. The magnification was set to an absolute magnification of 61,815×, corresponding to a pixel size of 4.8 Å, and the defocus was set to 5 μm. The angular range of the tilt series was from −70° to +70°, at tilt increments of 2° and a cumulative dose of ~90 e−/Å². The tilt series was aligned and reconstructed using the Protoomo package (Winkler, 2007). Cryo-ET reconstructions of LBPs were aligned using UCSF Chimera (Petterson et al., 2004) and averaged using program AVG3D in the EMAN package (Ludtke et al., 1999).

Protein subunit densities were segmented from the maps and visualized using UCSF Chimera (Petterson et al., 2004). Backbone models of hexon, penton base, VIII, and IX proteins were built based on the density maps of LBPs and HBPs using Coot (Emsley and Cowtan, 2004). Models were then refined in a pseudocrystallographic manner using the Crystallography and NMR System (Brunger et al., 1998) through a process that included simulated annealing refinement, crystallographic conjugate gradient minimization, and REFMAC5 idealization (Murshudov et al., 2011).

\textbf{Ultrathin sectioning}

After incubating with either LBPs or HBPs for 10 min, MDBK cells were washed twice, then fixed with 2% glutaraldehyde for 2 h, post-fixed with 1% OsO₄ for 2 h, dehydrated and embedded in Epon812. Samples were then ultrathin-sectioned at approximately 70 nm using a Leica EMUC6. Sections were stained with 2% uranyl acetate and 0.3% lead citrate and observed using an FEI Tecnai 20 electron microscope.

\textbf{Plaque assays}

MDBK monolayer cells were cultured in a 12-well plate. Each well was inoculated with 100 μl of diluted LBPs or HBPs (from 1 × 10⁻¹ to 1 × 10⁻⁶ dilution for each sample) and incubated for 1 h at room temperature to allow the viruses to fully adsorb on the cell monolayer. The inoculation in each well was then pipetted out and cells in each well were washed twice with PBS. Each well was overlaid with 2 ml of 0.4% agar, prepared by mixing melted 4% agar with a 10 fold volume of DMEM containing 2% FBS (fetal bovine serum). After the agar solidified at room temperature for approximately 10 min, the plate was incubated at 37°C for 7 days.

\textbf{Mass spectrometry analyses}

Samples of the LBPs and HBPs were run on a 15% SDS-PAGE gel. Protein bands were excised and digested individually. Bands were first cut into small gel plugs and washed twice in 200 μl distilled water for 10 min each time. Gel plugs were destained ultrasonically in 50 mM ammonium bicarbonate containing 40% acetonitrile for 5 min, then dehydrated in 200 μl 100% acetonitrile for 10 min and dried in a Speedvac (Labconco) for approximately 15 min. Reduction in 10 mM DTT (1 h, 56°C) and alkylation in 55 mM iodoacetamide (45 min, room temperature, in darkness) were then performed. The gel plugs were then washed twice with 50% acetonitrile in 25 mM ammonium bicarbonate, dried in a Speedvac, and digested overnight (37°C) with 10 μl of 0.1 μg sequence-grade modified trypsin (Promega, v5113) in 25 mM ammonium bicarbonate. Five microliters of 10% formic acid were added to terminate the enzymatic reaction, and the peptide mixture was then transferred to a sample vial for LC-MS/MS analysis using a ThermoFisher Finnigan LTQ linear ion trap mass spectrometer in line with a ThermoFisher Finnigan Surveyor MS.
Pump Plus HPLC system. The tryptic peptides generated were loaded onto a trap column (300SB-C18, 5 × 0.3 mm, 5 μm particles, Agilent Technologies), which was connected through a zero dead volume union to a self-packed analytical column (C18, 100 μm i.d. × 100 mm, 3 μm particles, SunChrom). The peptides were eluted over a gradient of acetonitrile and introduced online into the linear ion trap mass spectrometer (ThermoFisher Corporation) using nano electrospray ionization (ESI). MS data were analyzed using SEQUEST, searching against the NCBI protein database. Results were filtered, sorted, and displayed using BioWorksBrowser 3.3.1.

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Appendix A. Supplementary material

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References


