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Electron microscopy analysis of a disaccharide analog complex reveals receptor interactions of adeno-associated virus

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ABSTRACT

Mechanistic studies of macromolecular complexes often feature X-ray structures of complexes with bound ligands. The attachment of adeno-associated virus (AAV) to cell surface glycosaminoglycans (GAGs) is an example that has not proven amenable to crystallography, because the binding of GAG analogs disrupts lattice contacts. The interactions of AAV with GAGs are of interest in mediating the cell specificity of AAV-based gene therapy vectors. Previous electron microscopy led to differing conclusions on the exact binding site and the existence of large ligand-induced conformational changes in the virus. Conformational changes are expected during cell entry, but it has remained unclear whether the electron microscopy provided evidence of their induction by GAG-binding. Taking advantage of automated data collection, careful processing and new methods of structure refinement, the structure of AAV-DJ complexed with sucrose octasulfate is determined by electron microscopy difference map analysis to 4.8 Å resolution. At this higher resolution, individual sulfate groups are discernible, providing a stereochemical validation of map interpretation, and highlighting interactions with two surface arginines that have been implicated in genetic studies. Conformational changes induced by the SOS are modest and limited to the loop most directly interacting with the ligand. While the resolution attainable will depend on sample order and other factors, there are an increasing number of macromolecular complexes that can be studied by *cryo*-electron microscopy at resolutions beyond 5 Å, for which the approaches used here could be used to characterize the binding of inhibitors and other small molecule effectors when crystallography is not tractable.

1. Introduction

Adeno-associated virus (AAV) is a small human single-stranded DNA virus that is enjoying laboratory and clinical successes as a recombinant vector in experimental gene therapies (Carter et al., 2008; Nathwani et al., 2011). The first step in endosomal-mediated viral entry is attachment to one of several extracellular glycans used by different AAVs (Kaludov et al., 2001; Shen et al., 2011), heparan sulfate proteoglycan (HSPG) for type species AAV-2 (Summerford and Samulski, 1998). These interactions have been shown to be important *in vivo* to the tissue tropism of vectors (Shen et al., 2012), and are of intense interest in efforts to modulate the specificity of cell transduction and limit off-target transgene expression (DiPrimio et al., 2010).

This study focuses on AAV-DJ, a recombinant variant that was selected for resistance to pooled human neutralizing sera and liver-tropism out of a library created by gene shuffling among natural AAV serotypes (Grimm et al., 2008). AAV-DJ is among the first of a growing number of AAV gene delivery vehicles showing varying tissue tropisms in pre-clinical studies and produced through the application of combinatorial technologies (Asokan et al., 2012; Maheshri et al., 2006). AAV-DJ is a chimeric mix of serotypes 2, 8 and 9. AAV-DJ was chosen to check for possible changes in primary receptor binding in the selection of this retargeted recombinant variant. It was also chosen, because the *cryo*-electron microscopy (*cryo*-EM) of native AAV-DJ at 4.5 Å resolution (Lerch et al., 2012) suggested that it might be possible to visualize a receptor complex at sufficient resolution to address questions about AAV cell

Abbreviations: AAV, adeno-associated virus; CTF, contrast transfer function; EM, electron microscopy; FSC, Fourier shell correlation; HBD, heparin-binding domain; HSPG, heparan sulfate proteoglycan; VLP, virus-like particle.

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attachment. The *cryo*-EM of native AAV-DJ virus-like particles (VLPs) had showed a structure that was highly homologous to AAV-2 (Lerch et al., 2012), differing mostly at the binding site of a neutralizing AAV-2 antibody (McCraw et al., 2012), and differing less at the hypothesized site of heparin-binding.

The general area for glycan attachment in AAV-2 was predicted from surface charge seen in the AAV-2 crystal structure (Xie et al., 2002). The region contains five basic amino acids implicated by mutagenesis in heparin-binding and/or cell attachment (Kern et al., 2003). Neither co-crystallization nor soaking yielded high resolution diffraction, so structural studies proceeded through *cryo*-EM analysis of AAV-2 complexed with heparin fragments (Levy et al., 2009; O'Donnell et al., 2009). In the Levy et al. (2009) study at 18 Å resolution, difference map features at 0.5–1.0σ were interpreted as a bound heparin oligosaccharide, and virus conformational changes that would be induced in making the expected interactions with AAV-2 Arg_{585} and Arg_{588}. In the 8 Å study of O'Donnell et al. (2009), stronger difference density (6σ) showed the heparin oligosaccharide in immediate contact with Arg_{585} and Arg_{588} and there was no evidence of large-scale conformational change. Nevertheless, the Levy et al. (2009) conclusions are widely cited because they paint an appealing picture of how conformational changes expected at some point during endosomal entry might be triggered (Levy et al., 2009), whereas the O'Donnell et al. (2009) implies that any triggering occurs at some later undetermined post-attachment step. Neither study produced sufficiently detailed maps for recognition of chemical features of the ligand that would have provided decisive internal validation of the map interpretation. This is the goal of the current research. It required not only higher experimental resolution, but use of a different receptor analog. The earlier studies had used chromatographic fractions of heparin whose heterogeneity would limit our ability to identify detailed chemical features of the ligand even if the virus could be imaged at high enough resolution.

Sucrose octasulfate (SOS) is a commonly used glycosamino glycan (GAG) analog that is chemically homogeneous. With 55 non-hydrogen atoms, a 1 nm dimension and a mass at 1% of the target, the small size of the ligand would present challenges in resolution and sensitivity, which would be compounded by partial occupancy and perhaps by flexibility in ligand binding. Structure–function studies of complexes with small ligands (substrates, inhibitors, effectors and other ligands) have a storied history in protein crystallography (Johnson and Phillips, 1965; Sigler et al., 1966; Stryer et al., 1964). Early in this history, it was recognized that difference map analysis provided a sensitive means of imaging molecular interactions (Henderson and Moffat, 1971). Difference imaging has been used with EM to localize subunits in large complexes (Stewart et al., 1993), but small ligands have largely remained in the exclusive purview of X-ray crystallography due to the aforementioned challenges. AAV would provide a case study, exploring the current capacity of EM in the analysis of small ligand binding to macromolecular complexes in cases where ligand binding is incompatible with well-ordered crystal lattices.

2. Materials and methods

Virus-like particles (VLPs) of AAV-DJ were expressed in insect cells from a baculovirus construct as previously described (Lerch et al., 2012). Empty capsids were purified by three rounds of CsCl density gradient ultracentrifugation, as before, followed by additional chromatography using a heparin affinity column. Following elution with an NaCl gradient, AAV-DJ was dialyzed into 10 mM Tris buffer pH 6.8 with 125 mM NaCl and 1 mM $MgCl_2$. SOS (Sigma–Aldrich) was added in 400-fold molar excess relative to capsid subunit at 3 mg/mL and incubated for 45 min at room temperature. Immediately before flash-freezing the AAV-DJ was diluted to 1.5 mg/mL.

Sample aliquots of 3 μL were placed on 400 mesh carbon-coated grids (C-flat) that had been glow discharged for 5 s on a Gatan Model 950. Samples were flash-frozen by plunging into liquid ethane from an environment of 100% humidity and 4 °C, using a Vitrobot Mark IV (FEI Inc.), blotting both sides of the grid for 2.5 s. Data were collected on an FEI Titan Krios at 120 keV using an electron dose of 15 $e^-/Å^2$. Nominal defocus on the microscope was randomly chosen between −0.8 μm and −2.0 μm. The resulting images had a mean estimated defocus of −1.58 μm with a standard deviation of 0.49 μm. Images were recorded at a magnification of 120,000× on a 4 k × 4 k pixel Gatan Ultrascan CCD camera, which resulted in a pixel size of 0.65 Å at the specimen level. 8155 high magnification images were automatically acquired over the course of 4 days using Leginon (Suloway et al., 2005), and 5207 of the images were selected for further processing.

Particles were processed semi-automatically using Appion (Lander et al., 2009) for picking, contrast transfer function (CTF) estimation, and stack making. Particles were selected automatically using template matching. CTFs were estimated with the ACE (Automated CTF Estimation) software package (Mallick et al., 2005), rejecting images with confidence value <0.7, and flipping phases for individual particles according to their ACE-estimated defocus. These analyses resulted in 94,123 particles that were used for alignment and classification. FREALIGN (Grigorieff, 2007) was used to refine Euler angles and generate the reconstruction, starting from Euler angles estimated by EMAN using an initial model generated directly from the data using the starticos sub-program. Euler angle refinement in FREALIGN converged in 12 iterations, and 70,725 particles contributed to the final EM map. Resolution was estimated from the EM data at 4.8 Å by $FSC_{0.143}$ ($FSC_{0.5}$ = 5.6 Å) (Böttcher et al., 1997; Grigorieff, 2000; Harauz and van Heel, 1986; Rosenthal and Henderson, 2003; Sousa and Grigorieff, 2007). Signal attenuation was corrected using EM-Bfactor (Fernandez et al., 2008) using a cut-off of 4.8 Å.

A difference map was calculated between the newly acquired reconstruction of the AAVDJ–SOS complex and the previously published native AAV-DJ (Lerch et al., 2012). A magnification correction was first refined for each reconstruction using RSRef (Chapman et al., 2013) by least-squares minimizing the differences between density levels of the reconstruction and those calculated from the crystal structure of homolog AAV-2 that had been superimposed rigidly according to the icosahedral symmetry elements. The relative magnification corrections are remarkably consistent, considering that they were refined independently against different reconstructions, and were small, 1.011 for native and 1.010 for complex. Nevertheless, at the outside of a 275 Å diameter virus a 1.01 correction corresponds to a 1.4 Å positional error, and its application appreciably affects the superimposition of the AAV structure on the SOS difference peak.

After a preliminary atomic refinement (see below), the difference map calculation was improved modestly by first bringing the density levels of each reconstruction to a common scale using the atomic models as a reference. An additive and multiplicative constant were applied to all voxels in each reconstruction that least squares minimized differences from the calculated density within an envelope drawn 4.9 Å around the respective atomic models (Chapman et al., 2013). Following subtraction of the native reconstruction from the complex, the difference map was low-pass filtered to 5.0 Å using EMAN (Ludtke et al., 1999), minimizing possible artifacts arising from the slightly different resolution limits (4.8 and 4.5 Å, respectively).

An atomic model for SOS was placed approximately in the difference map using Coot (Emsley et al., 2010). RSRef (Chapman et al., 2013) was used for the atomic refinements described below.