

Characterization of the Capsid Protein Glycosylation of Adeno-Associated Virus Type 2 by High-Resolution Mass Spectrometry†

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Received 16 November 2005/Accepted 3 March 2006

Adeno-associated virus type 2 (AAV-2) capsid proteins have eight sequence motifs that are potential sites for O- or N-linked glycosylation. Three are in prominent surface locations, close to the sites of cellular receptor attachment and to neutralizing epitopes on or near protrusions surrounding the three-fold axes, raising the possibility that AAV-2 might use glycosylation as a means of immune escape or for preventing reattachment on release of progeny virus. Peptide mapping and structural analysis by Fourier transform ion cyclotron resonance mass spectrometry demonstrates, however, no glycosylation of the capsid protein for virus prepared in cultured HeLa cells.

Adeno-associated viruses (AAVs) are small single-stranded DNA parvoviruses (36). There may be more than the eight serotypes originally identified, with humans or other primates as their primary hosts (13, 16). Although ~80% of the population is seropositive due to natural exposure to AAV (11, 34), AAV has not been associated with disease (3). This is one of the reasons that it is being developed as a vector for in vivo gene therapy (5, 33).

Parvoviruses are small (250 Å) unenveloped viruses in which a single-stranded DNA (ssDNA) genome is surrounded by a T=1 icosahedrally symmetric capsid containing 60 copies of the capsid protein. Parvoviruses contain three or four variants of the capsid protein, differing in length at the N terminus (36). In AAV type 2 (AAV-2), viral protein 3 (VP3; 533 amino acids) constitutes 80% (by mass) of the capsid (60). Alternative mRNA splicing gives variants VP2 and VP1, which are extended at the N terminus by 65 and 202 residues, respectively. Although present in the crystals, the VP1 and VP2 unique regions were not seen in the atomic structure (58)—it appears that the 533 residues common to VP1, VP2, and VP3 occupy symmetry-equivalent positions in the capsid. The VP1 unique addition encodes nuclear localization signals (22, 56) and a phospholipase A2 domain (15) that is likely needed for the virus to escape into the cytoplasm from the endosome (12a). The location of the VP1 unique region has been debated, but

it looks as if it can move from the inner to outer surfaces (12, 26, 59, 60).

The primary cellular receptor for AAV serotypes 1 to 3 is heparan sulfate (HS) proteoglycan (50). Coreceptors are likely also involved, possibly fibroblast growth factor receptor and integrin $\alpha_v\beta_5$ (39, 40, 42, 49). AAV enters cells through endosomes (1) and is transported quickly into the nucleus (1, 44). Newly synthesized capsid proteins must be transported to the nucleolus for assembly of the DNA-containing virions (41), and specific binding of a nucleolus-targeting protein to VP2, and of nucleolin to AAV-2 virions has been reported (22, 41). Thus, the natural life cycle requires interactions with a number of other macromolecules in which glycosylation of the capsid proteins, if present, might be important.

Indeed, glycoproteins figure prominently in a number of other viruses, especially enveloped viruses, where they are often exposed on the outer surface. Mutations at the glycosylation sites often interfere with viral entry, infectivity, tissue specificity, or host range, implying roles in cell recognition, membrane fusion, and cell entry (8, 27, 43, 46, 53). Among nonenveloped viruses, glycosylation of structural proteins is less common. However, the fiber proteins of adenovirus, specifically types 2 and 5, are O glycosylated (6). The presence of the carbohydrate modulates the antigenicity in adenovirus (6). In rotavirus, it has been possible to select monoclonal antibody neutralization escape mutants (29) that have new sites of glycosylation in the epitope. Indeed, it has been suggested for human immunodeficiency virus type 1 (57) that glycosylation sites provide variability that allows the virus to escape immune detection of nearby conserved amino acids at the cellular-receptor binding site.

Our characterization of potential AAV-2 glycosylation sites started with a search through the sequence for likely motifs. It is possible to search for putative sites for N-linked glycosylation, for which there are several motifs (2), but for O-linked glycosylation, searching for motifs is more challenging (18, 24). The sequences of the capsid proteins were screened by use of

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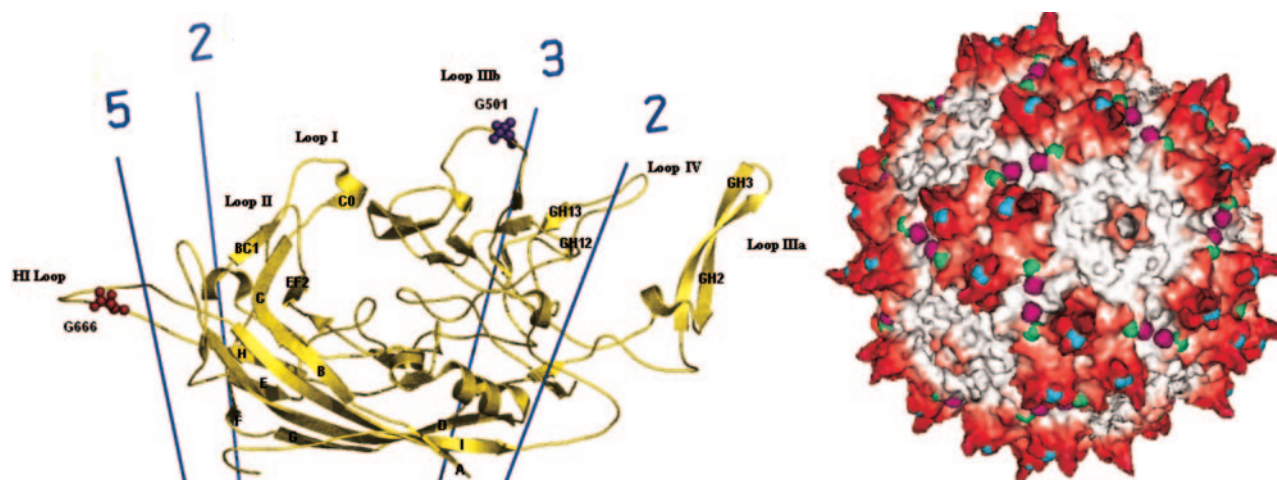


FIG. 1. (Left) Sequence motifs characteristic of glycosaminoglycan modification are shown as spheres on the part of the capsid subunit structure that is common to VP1, VP2, and VP3 (58). Asn₄₉₆ to Ser₄₉₈ and Asn₅₁₉ to Ser₅₂₁ are on outer loops near the three- and five-fold axes respectively. (Right) In the assembled capsid, these sequences are in prominent positions on the outer surface. Asn₄₉₆ to Ser₄₉₈ are shown in blue near the tips of protrusions that surround each three-fold axis. Asn₅₁₉ to Ser₅₂₁ are shown in green near the five-fold axes.

MOTIFS of the Wisconsin Package (Accelrys, Inc.) and the PROSITE database (23). Within the common region of VP1 and VP2, there were two SGXG motifs at positions 157 (SGTG) and 195 (SGLG) (VP1 numbering) (4). These motifs are associated with glycosaminoglycan modification, but only the first at position 157 is preceded by two acidic amino acids in the -5 to -3 positions (EPDSSSGTG) closely resembling the consensus that requires two acidic residues between -4 and -2 of the SGXG conserved sequence (4). Searches for NXS/T motifs that can be associated with N-linked glycosylation (14) led to six AAV-2 sequences all within the part common to VP1, VP2, and VP3. Two (VP1_{223–225} and VP1_{408–410}) are near the inner surface of the capsid (Fig. 1), but the four on the outer surface are easier to imagine having relevance to virus-host interactions. NPS_{656–658} (VP1 numbering) can be eliminated from consideration because the presence of a proline strongly reduces the likelihood of glycosylation (14). NGS_{382–384}, NNS_{496–498}, and NKS_{705–707} remain as candidate sites.

Examination of the location of the candidate sites on the atomic structure of AAV-2 (58) shows that all are in prominent surface locations (Fig. 1). Of particular interest was the location of NNS_{496–498} at the tip of the protrusions that surround each three-fold axis. It is in a location where glycosylation could impact both cell receptor binding and antigenicity. Heparan sulfates are usually bound by basic residues (35). Electrostatic calculations from the atomic structure showed that residues RGNR_{585–588} were at the center of a positively charged surface patch that was predicted to be the core of the receptor binding site (58), a prediction that was subsequently confirmed through mutagenesis (25, 38). Although far apart in the structure of an individual subunit, NNS_{496–498} and RGNR_{585–588} come together in the assembled capsid, as adjacent loops from neighboring three-fold related subunits (58). The candidate glycosylation sequence is at the tip of the protrusion whereas the receptor-binding site is on the inner side of the protrusion (58), but separated by only 3.1 Å, i.e., in immediate van der Waals contact and well within a likely footprint of the heparan sulfate receptor. Furthermore, NNS_{496–498} is

within the major linear epitope of neutralizing antibody C37B, a monoclonal antibody that inhibits receptor attachment (55). The remaining two candidate glycosylation sites (NGS_{382–384}, and NKS_{705–707}) are well separated within a subunit but come together on surface when contributed by adjacent subunits. They are on the opposite side of the RGNR_{585–588} receptor-site, but only 21 to 25 Å away, still conceivably within a possible receptor footprint. Neither of these candidate glycosylation sites is within a neutralizing epitope yet mapped, but they are close. NGS_{382–384} is immediately adjacent in primary structure to epitope 2 of monoclonal antibody A20, and His₁₃₄, which is a surface neighbor of both NGS_{382–384} and NKS_{705–707}, is at the start of A20 epitope 1 (55, 58). With these candidate glycosylation sites having potentially significant impact upon both antigenicity and receptor binding, it became imperative to characterize glycosylation experimentally.

For experimental characterization, AAV-2 was prepared according to Xie et al. (60). The methods involve propagation of AAV-2 in cultured human (HeLa) cells, so it is not unreasonable to expect that glycosylation patterns should resemble those in a human host. There might be some differences based on cell type and with the higher in vitro propagation levels that could saturate posttranslational modification pathways. To reduce the potential for such heterogeneity, the virus was harvested 48 h postinfection (cf. 72 h). It is reasonable to expect that at least some of the virus produced would have glycosylation mimicking that occurring naturally. Virus was purified by cesium chloride density gradient ultracentrifugation repeated three times.

Various enzymatic, chemical, and immunochemical analyses can reveal the presence of glycans (21, 28, 51). For preliminary screening, methods were chosen that had broad specificity—chemical oxidations detected with either fluorescence or immunoassay. For both assays, capsid proteins from denatured AAV-2 particles were electrophoretically separated on a 4 to 12% Bis-Tris gel (NuPage, Inc.). The Glycoprofile III fluorescent glycoprotein detection kit (Sigma, Inc.) was applied to a fixed gel according to the manufacturer's instructions. Briefly,

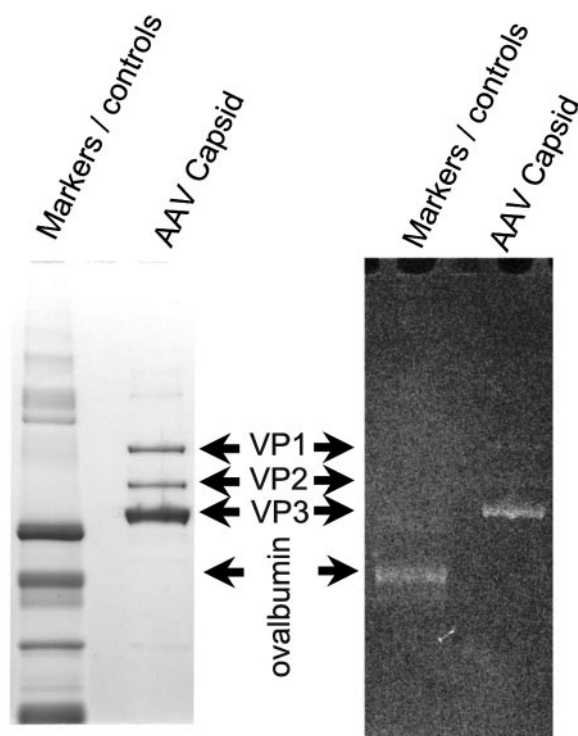


FIG. 2. Glycosylation detection through fluorescence labeling. The left panel shows an EZBlue-stained gel with denatured AAV-2 capsids run alongside the ProteoProfile PTM markers (Sigma-Aldrich, Inc.). The right panel shows the gel stained with Glycoprofile III (Sigma-Aldrich, Inc.) with fluorescent imaging. VP3 fluorescent staining suggests glycosylation commensurate with ovalbumin. The weak fluorescent staining of VP1 and VP2 is not inconsistent with their ~ 10 -fold-lower concentration and glycosylation of a region common to VP1, VP2, and VP3.

potential glycoconjugates were oxidized with a periodic acid solution, stained with dansyl hydrazide, destained, and imaged using a UV transilluminator with a yellow filter. EZBlue (Sigma, Inc) was then used to stain all proteins. The band for VP3 fluoresced with an intensity similar to that of ovalbumin (Fig. 2). This band could correspond to approximately an 8-mer attached at a single site or smaller modifications at several sites. The weak fluorescent staining of VP1 and VP2 is not inconsistent with the 10-fold-lower levels of VP1 and VP2, and with modification of a site or sites common to VP1, VP2, and VP3.

The second assay used the digoxigenin (DIG) glycan detection kit (Roche, Inc.) according to the manufacturer's instructions. Potential carbohydrates were oxidized on the intact capsid with periodate caproic acid hydrazide and then labeled with digoxigenin. The particles were then denatured and separated electrophoretically. The proteins were transferred to a nitrocellulose membrane, then hybridized with an antidigoxigenin antibody conjugated to alkaline phosphatase so that activity could be measured colorimetrically. VP3 stained positively, but so did several unglycosylated proteins tested as negative controls. Thus, both chemical assays were giving inconclusive results. These types of tests are known to be difficult, with the real possibility of false positives. A more definitive method was

sought, and the positive, if equivocal, chemical detection results motivated a mass spectrometric analysis.

Our choice would be liquid chromatography (LC)-Fourier transform ion cyclotron mass spectrometry (FT-ICR MS), taking advantage of the resources of the National High Magnetic Field Laboratory (NHMFL). There are important differences relative to the more familiar matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The pre-separation of the samples by LC circumvents the problem of selective desorption/ionization, while the high resolution and mass accuracy provided by ICR permit the analysis of large glycopeptides (48). Also, the analysis of intact glycopeptides permits the assignment of specific sugar structures to a specific amino acid residue (19). Thus, it is preferable with FT-ICR MS to characterize directly the glycopeptides, rather than cleave off the carbohydrate as might be needed with MALDI-TOF MS, a particular challenge with O-linked glycosylation due to the lack of suitable enzymes.

One disadvantage of FT-ICR MS is the relatively large amount of sample needed. For the liquid chromatography mass spectrometry-mass spectrometry (LCMS-MS) methods used to confirm the identity of potentially glycosylated peptides, larger quantities (up to 200 μg) would be needed than for MALDI-TOF MS. Such quantities are orders of magnitude greater than those normally prepared in culture for virological research. Replication of AAV-2 in HeLa cells is high yield relative to other cell types (37), and we took advantage of extensive past efforts to maximize production for crystallographic structure determination (60)—no further development of AAV-2 production in HeLa cells was required for the FT-ICR MS characterization.

Samples for mass spectrometry were extracted from denatured gels and proteolytically fragmented. Stained VP3 and VP1 bands from eight lanes loaded with 8 μg denatured virus were excised from the gel and destained in 50% acetonitrile–25 mM ammonium bicarbonate until no color remained. Gel pieces were vacuum dried and then incubated overnight at 37°C with 15 μl of 10 $\mu\text{g}/\mu\text{l}$ with trypsin, Asp-N, or Glu-C (Sigma-Aldrich, Inc.). Peptides were eluted from the gel pieces with 75% acetonitrile and 0.25% formic acid. VP1 required additional deionization with a ZipTip (Millipore, Inc). Samples were stored at -20°C . VP3, available in larger amounts, was analyzed first, followed by VP1.

Protein digests were dissolved in acetonitrile–0.5% formic acid (aqueous) at a concentration of approximately 10 μM and ionized by microelectrospray (9, 10). In order to obtain improved signal/noise ratio, some digests were purified with a miniaturized system (ZipTips, Millipore, Bedford, MA) according to the manufacturer's instructions. Accurate mass analysis was performed with data obtained with a custom-built 7T or 9.4T Fourier transform ion cyclotron mass spectrometer (31, 45, 54). A one-dimensional nano-liquid chromatography (nano-LC) system (Eksigent, Livermore, CA) was interfaced with microelectrospray to the 7T FT-ICR MS and used for separation of protein digests over a 125-nl C_{18} cartridge (Opti-Pak, Optimize Technologies, Inc., Oregon City, OR). The nano-LC setup permitted efficient "peak parking" of proteolytic peptide ions. Peak-parked ions were stored waveform inverse Fourier transform (SWIFT) (17, 32) isolated in the

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