JOURNAL OF VIROLOGY, May 2006, p. 5082–5085 Vol. 80, No. 10
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.10.5082–5085.2006

Identification and Characterization of Novel Adeno-Associated Virus Isolates in ATCC Virus Stocks

Michael Schmidt,[1] Emmanuelle Grot,[1] Peter Cervenka,[1] Sandra Wainer,[1] Charles Buck,[2] and John A. Chiorini[1]*

Gene Therapy and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892,[1] and Department of Virology, American Type Culture Collection (ATCC), Manassas, Virginia 20108[2]

Received 19 October 2005/Accepted 20 February 2006

Adeno-associated viruses (AAVs) depend on a helper virus for efficient replication. To identify novel AAV isolates, we screened a diverse set of virus isolates for the presence of AAV DNA. AAVs found in 10 simian adenovirus isolates showed greater than 96% homology to AAV1 and AAV6 but had distinct biological properties. Two representatives of this group, AAV(VR-195) and AAV(VR-355), were studied in more detail. While the novel AAVs had high sequence homologies and required sialic acid for cell binding and transduction, differences were observed in lectin competition, resulting in distinct tropisms in human cancer cell lines.

Adeno-associated virus (AAV) is a member of the *Parvoviridae*, a virus family characterized by a single-stranded linear DNA genome enclosed by a capsid with icosahedral symmetry. A hallmark of the AAV life cycle is its dependency on a helper virus for efficient productive replication. Adenovirus was originally identified as the AAV helper virus, but other viruses, such as herpesvirus and cytomegalovirus (7), can provide helper functions for AAV replication. Virus stocks have therefore served as a rich source for the discovery of AAV isolates; AAV1, AAV2, AAV3, AAV4, AAV6, and bovine AAV were all found as contaminants of adenovirus preparations (1–3, 8, 10–12, 15).

In this article, we describe the analysis of virus stocks from the American Type Culture Collection (ATCC) for the presence of AAV DNA. Our goals were to identify ATCC virus isolates that contain AAV contaminations and, if novel, to characterize the isolates.

Virus stocks supplied by the ATCC were analyzed for the presence of AAV DNA by a PCR-based assay, as described previously (5). AAV DNA was detected in 13 of 137 samples analyzed. AAV contaminations were frequently detected in adenovirus isolates (26%) but not in herpesvirus, retrovirus, coronavirus, orthomyxovirus, poxvirus, or reovirus stocks. The entire coding regions for the AAV Rep and Cap open reading frames were PCR amplified and subcloned, and several clones from each isolate were sequenced and analyzed. Adenovirus-free stocks of the novel recombinant AAVs were produced by standard cotransfection protocols (14).

Ten of the AAVs detected in simian adenovirus stocks displayed at least 96% homology on the DNA level and 98% identity in the capsid amino acid sequence either to AAV1, to AAV6, or to each other. To determine whether these minor sequence changes could affect the biological activity of the isolates, 2 of the 10 isolates, AAV(VR-195), and AAV(VR-355), isolated from ATCC VR-195 and VR-355, respectively (GenBank accession numbers DQ180604 and DQ180605), were studied in greater detail. The VP1 capsid proteins of AAV(VR-195) and AAV(VR-355) differ by 7 and 6 amino acids, respectively, from that of AAV6. The locations of the divergent amino acids within the capsid were identified by superimposing the AAV(VR-195) and AAV(VR-355) VP1 sequences onto a pseudoatomic structure for AAV6 (Fig. 1). Divergent amino acids were found to cluster on the capsid surface around the threefold axis of symmetry, an area of the capsid that has been associated with receptor binding (6, 9). Since the amino acid changes among AAV(VR-195), AAV(VR-355), and AAV6 are surface exposed, we hypothesized that they may affect the biological properties of the capsid and cell tropism.

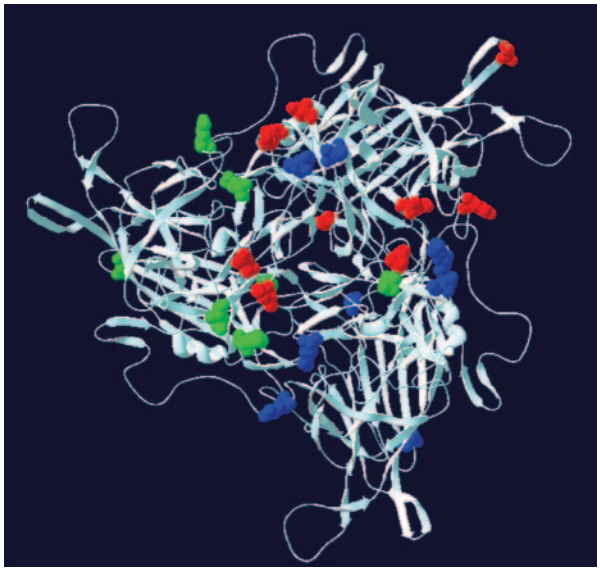

FIG. 1. Structure of AAV6 capsid subunits and comparison with rAAV(VR-195). Shown is a ribbon model of the capsid threefold axis of symmetry viewed from the top. The AAV6 VP sequence is shown in gray. Colored dots indicate AAV(VR-195)-specific amino acid changes compared to AAV6; changes in each VP3 subunit are displayed in a different color: red, green, or blue.

* Corresponding author. Mailing address: NIH 10/1N113, 10 Center Dr., MSC1190, Bethesda, MD 20892. Phone: (301) 496-4279. Fax: (301) 402-1228. E-mail: jchiorini@dir.nidcr.nih.gov.

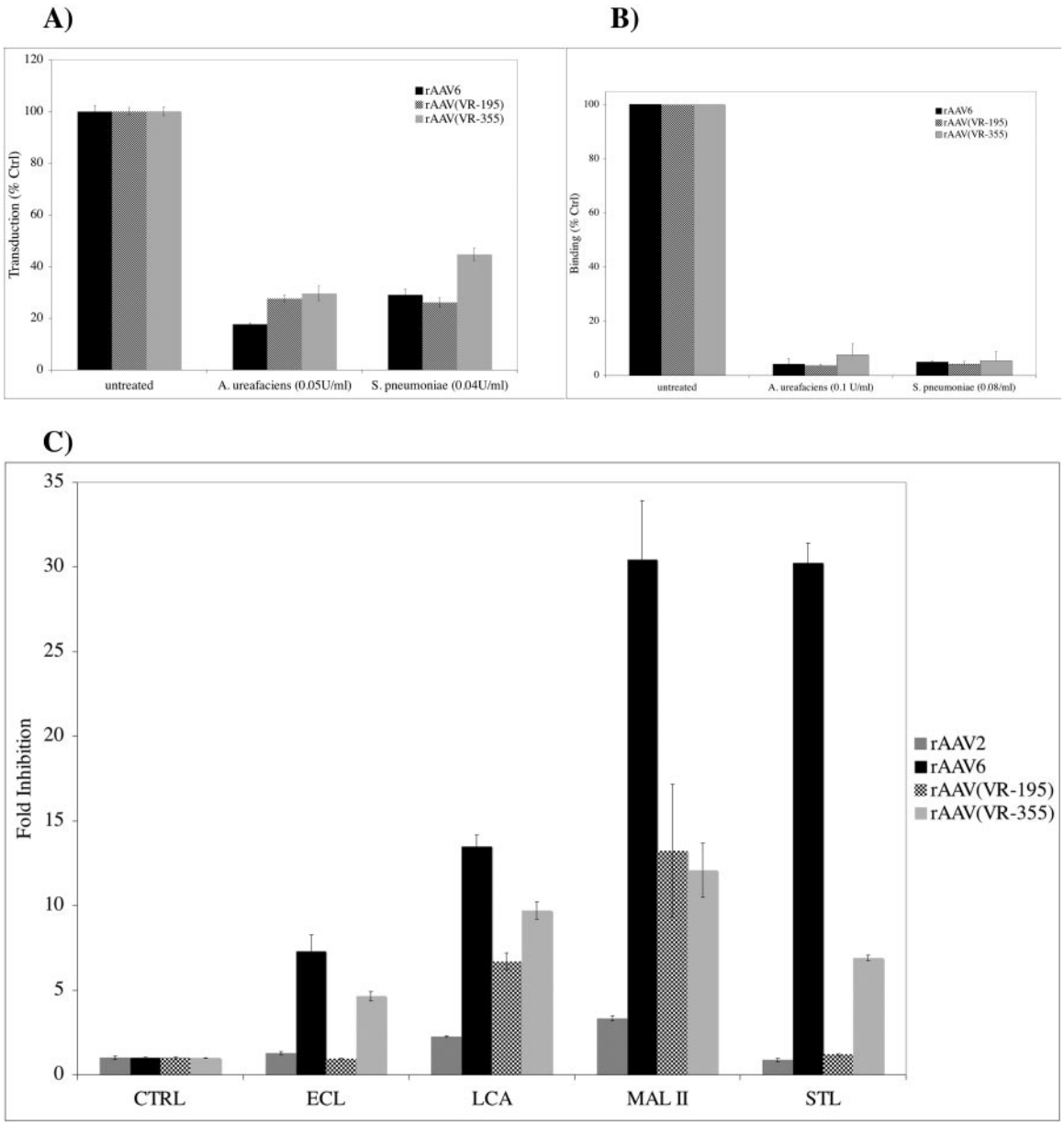

FIG. 2. rAAV6, rAAV(VR-195), and rAAV(VR-355) require sialic acid for transduction and cell binding. (A) Gene transfer mediated by rAAV6, rAAV(VR-195), and rAAV(VR-355) encoding GFP in COS cells, following neuraminidase pretreatment. Transduction was determined by flow cytometry. (B) Binding assayed by quantitative, real-time PCR. (C) Effects of lectins on transduction of COS cells. Values are means from three experiments; error bars represent standard deviations.

Sialic acid serves as the coreceptor for AAV4, AAV5, and AAV6 cell binding and transduction (4, 13, 16). To analyze whether sialic acid is required for transduction with AAV(VR-195) and AAV(VR-355) vectors, we studied the effects of the removal of cell surface sialic acids by neuraminidase treatment on transduction (Fig. 2A) and virus binding (Fig. 2B). Treatment of COS cells with a broad-spectrum neuraminidase from *Arthrobacter ureafaciens*, as well as neuraminidase specific for α-2,3-linked sialic acid from *Streptococcus pneumoniae*, inhibited transduction and binding of recombinant AAV6 (rAAV6), rAAV(VR-195), and rAAV(VR-355), suggesting that these viruses utilize α-2,3-linked sialic acid as a cell attachment factor. This result was confirmed in lectin competition assays (16). MalII, a lectin that recognizes α-2,3-linked sialic acid, inhibited transduction of rAAV-6, rAAV(VR-195), and rAAV(VR-355) (Fig. 2C). However, differences in lectin competition were observed with other lectins, suggesting distinct cell tropisms for each isolate. When we transduced COS cells in the presence of the mannose-specific lectins from *Lens culinaris* (LCA) and *Erythrina cristagalli* (ECL), we observed differences in their inhibitory potentials. While ECL, which recognizes α-mannose in conjugation with galactosyl (β-1,4)-*N*-acetylglucosamine,

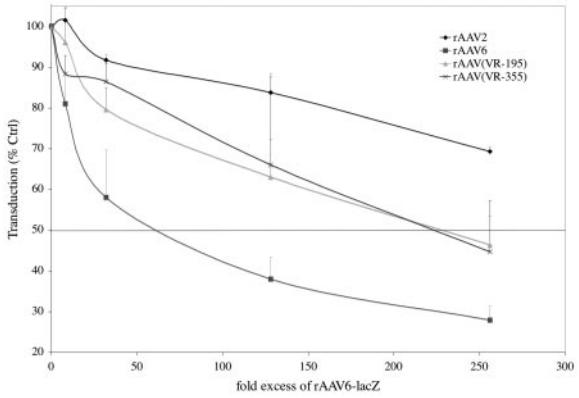

FIG. 3. AAV6 competition. COS cells were transduced with a constant amount of rAAV6, rAAV(VR-195), or rAAV(VR-355) expressing GFP after 60 min of preincubation with increasing titers of rAAV6-LacZ. At 48 h postinoculation, transduction was analyzed by flow cytometry. Values are means from three experiments; error bars represent standard deviations.

did not inhibit rAAV(VR-195), it reduced rAAV6 and rAAV(VR-355) transduction by seven- and fivefold, respectively. LCA inhibited all recombinant viruses tested and had an approximately twofold-higher inhibitory effect on rAAV6 than on rAAV(VR-195) or rAAV(VR355). *Solanum tuberosum* lectin (STL), which recognizes *N*-acetylglucosamine, did not inhibit rAAV(VR-195) but reduced rAAV6 and rAAV(VR-355) transduction by 30- and 12-fold, respectively. These results suggest that while all three viruses bind terminal α-2,3-linked sialic acid, the amino acid changes on the capsid surface appear to affect the cell binding activity of each isolate.

As a result of the few sequence changes on the surface of the rAAV(VR-195), rAAV(VR-355), and rAAV6 capsid, the viruses appear to exhibit biological characteristics that are different from each other. To investigate whether these viruses use distinct receptors, we assayed for changes in transduction during competition experiments between AAV6 and the other isolates. COS cells were preincubated with increasing doses of rAAV6-LacZ, followed by transduction with identical particle titers of either rAAV2, rAAV6, rAAV(VR-195), or rAAV(VR-355) expressing green fluorescent protein (GFP), and were counted by flow cytometry (Fig. 3). rAAV6-LacZ competition had the greatest effect on rAAV6-NLS-GFP transduction, with 50% inhibition of transduction at a 60-fold excess of the competitor, whereas a 220-fold excess was required for the same level of inhibition of rAAV(VR-195) or rAAV(VR-355). The stronger inhibition of rAAV6-NLS-GFP by rAAV6-LacZ than of rAAV(VR-195) or rAAV(VR-355) suggests that while rAAV6, rAAV(VR-195), and rAAV(VR-355) have a common attachment factor and potentially common receptors, differences in the attachment factor and receptor interaction exist. To analyze whether this difference also results in a change in tropism or transduction activity, six human cancer cell lines and African green monkey kidney cells, COS, were transduced with rAAV6, rAAV(VR-195), and rAAV(VR-355) (Fig. 4). AAV6 and AAV(VR-195) transduced COS, EKVX, IGROV1, and CAKI cells with similar efficiencies but were fourfold different in transduction of Ovcar5 cells and the central nervous system-derived SF295 cell

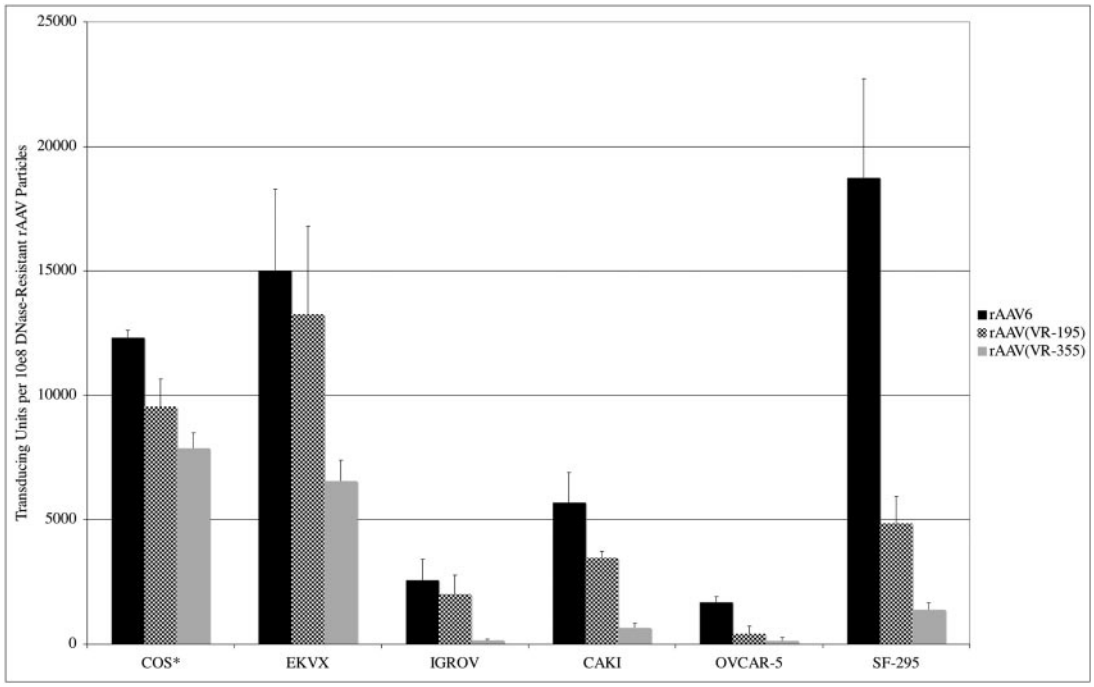

FIG. 4. Transduction efficiency in human cancer cell lines. The indicated cell lines were transduced with 1×10^8 particles of rAAV6, rAAV(VR-195), and rAAV(VR-355) expressing a nucleus-localized GFP. At 24 h postinoculation, transduction was analyzed by flow cytometry. Values are means from three experiments; error bars represent standard deviations. *, COS cell transduction is given as transducing units/2×10^7 particles.

line. AAV(VR-355) demonstrated efficient gene transfer in COS and EKVX cells, but transduction of Igrov1, CAKI, Ovcar5, and SF295 was 10- to 17-fold lower than for AAV6. The different transduction efficiencies of rAAV6, rAAV(VR-195), and rAAV(VR-355) suggest that each isolate may have a distinct cell tropism.

While it is accepted that different serotypes of AAV have unique tropisms, it has not been recognized that only a few amino acid changes can have a profound impact on the biological activity of an AAV particle and dramatically alter its tropism to a new cell type. In this study, we demonstrated that rAAV6, rAAV(VR-195), and rAAV(VR-355) require α-2,3-linked sialic acid for cell attachment and transduction but differ in sensitivities to lectin and in cross-competition and transduction activities on a panel of cells.

The amino acid changes among these three isolates are clustered on the surface of the particles, suggesting the identification of an important functional domain. Further characterization of the effects of these changes on the tropism and transduction activity of the viral particle and of their activity in vivo will be useful in understanding the AAV capsid and host cell interactions and in developing new vectors for gene transfer applications.

We thank Kathleen Bolland and the NIDCR sequencing core for excellent support, the NCI Fellows Editorial Board for editorial assistance, and David Russell for providing the AAV6 packaging plasmid used in this study.

This research was supported by the Intramural Research Program of the NIH and NIDCR.

REFERENCES

1. **Atchison, R. W., B. C. Casto, and W. M. Hammon.** 1965. Adenovirus-associated defective virus particles. Science **149:**754–756.
2. **Chiorini, J. A., L. Yang, Y. Liu, B. Safer, and R. M. Kotin.** 1997. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. J. Virol. **71:**6823–6833.
3. **Hoggan, M. D., N. R. Blacklow, and W. P. Rowe.** 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. Proc. Natl. Acad. Sci. USA **55:**1467–1474.
4. **Kaludov, N., K. E. Brown, R. W. Walters, J. Zabner, and J. A. Chiorini.** 2001. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. J. Virol. **75:**6884–6893.
5. **Katano, H., S. Afione, M. Schmidt, and J. A. Chiorini.** 2004. Identification of adeno-associated virus contamination in cell and virus stocks by PCR. BioTechniques **36:**676–680.
6. **Kern, A., K. Schmidt, C. Leder, O. J. Muller, C. E. Wobus, K. Bettinger, C. W. Von der Lieth, J. A. King, and J. A. Kleinschmidt.** 2003. Identification of a heparin-binding motif on adeno-associated virus type 2 capsids. J. Virol. **77:**11072–11081.
7. **McPherson, R. A., L. J. Rosenthal, and J. A. Rose.** 1985. Human cytomegalovirus completely helps adeno-associated virus replication. Virology **147:**217–222.
8. **Muramatsu, S., H. Mizukami, N. S. Young, and K. E. Brown.** 1996. Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. Virology **221:**208–217.
9. **Opie, S. R., K. H. Warrington, Jr., M. Agbandje-McKenna, S. Zolotukhin, and N. Muzyczka.** 2003. Identification of amino acid residues in the capsid proteins of adeno-associated virus type 2 that contribute to heparan sulfate proteoglycan binding. J. Virol. **77:**6995–7006.
10. **Rutledge, E. A., C. L. Halbert, and D. W. Russell.** 1998. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J. Virol. **72:**309–319.
11. **Samulski, R. J., K. I. Berns, M. Tan, and N. Muzyczka.** 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. Proc. Natl. Acad. Sci. USA **79:**2077–2081.
12. **Schmidt, M., H. Katano, I. Bossis, and J. A. Chiorini.** 2004. Cloning and characterization of a bovine adeno-associated virus. J. Virol. **78:**6509–6516.
13. **Seiler, M. P., A. D. Miller, J. Zabner, and C. L. Halbert.** 2006. Adeno-associated virus types 5 and 6 use distinct receptors for cell entry. Hum. Gene Ther. **17:**10–19.
14. **Smith, R. H., S. A. Afione, and R. M. Kotin.** 2002. Transposase-mediated construction of an integrated adeno-associated virus type 5 helper plasmid. BioTechniques **33:**204–206, 208, 210–211.
15. **Srivastava, A., E. W. Lusby, and K. I. Berns.** 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. J. Virol. **45:**555–564.
16. **Walters, R. W., S. M. Yi, S. Keshavjee, K. E. Brown, M. J. Welsh, J. A. Chiorini, and J. Zabner.** 2001. Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. J. Biol. Chem. **276:**20610–20616.